1995

Whirling Disease

F-W Knapp #87093

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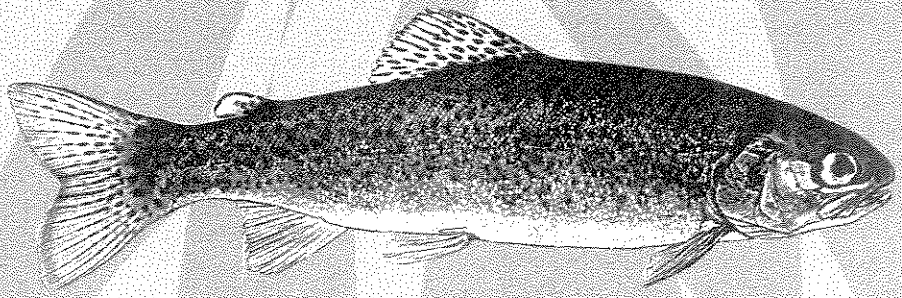

Whirling Disease in Salmonid Fishes

Prepared as a Scientific Resource by:

Stuart E. Knapp, Karl M. Johnson,
Shawna M. Button and Matthew C. Rognlie

Veterinary Molecular Biology Laboratory
Montana State University-Bozeman

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PREFACE

The recent discovery of whirling disease in rainbow trout in Montana has caused a sudden growth of interest in whirling disease research. We recognize that some scientists may have limited accessibility to libraries and the literature. We therefore desired to give various individuals interested in whirling disease research immediate access to key papers. As a result, we have prepared this bibliography on whirling disease in salmonid fishes. The majority of the papers in this bibliography are research articles published in peer-reviewed journals, although we have also included some reports, abstracts, leaflets and book chapters that deal directly with *Myxosoma cerebralis*. In addition to the bibliographical listing of articles, we have attached reprints of each article when possible. Reprinting the articles would not have been possible without the generosity of publishers who, almost without exception, have given copyright permission and allowed the papers to be recopied at no charge. Because a goal of this project was to provide only courtesy copies of this bibliography, any requests for royalty charges were noted and only the abstract was printed for the articles in question.

Articles included in this bibliography were selected with essentially one criterium: they deal specifically with whirling disease or *M. cerebralis*, including any intermediate life stages. We have put together as many of these key papers as we could find. Since some papers may have been omitted, we apologize in advance, and request from the user the favor of correspondence if you have identified such papers. It is our intent to continue to collect articles on this subject and create an addendum in a year or two.

The first part of this document is numbered in lowercase Roman numerals, and contains a Table of Contents, a Subject Index and the bibliographic listing of papers. The second part of the document contains the copied articles and is numbered with Arabic numerals. The listing of articles is alphabetized and numbered, and paper's number is also found in the upper right hand corner of odd-numbered pages. We hope this will aid in finding each article of interest. The Subject Index was made so the reader might have a starting point for finding articles dealing with a specific area of whirling disease. The Index is not comprehensive; rather we have extracted key words from the titles, index descriptors, abstracts or introduction when appropriate. We hope the Index will accelerate the usefulness of the bibliography for the reader.

The authors gratefully acknowledge the support by Montana State University-Bozeman and the Montana Department of Fish, Wildlife and Parks.

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AN ENDEMIC DISEASE OF SALMONIDS IN SAKHALIN ISLAND

E. A. Bogdanov

State Research Institute for Marine and Fresh Water Fish Culture

(Presented by E. N. Pavlovskii, April 16, 1960)
Translated from Doklady Akademii Nauk SSSR, Vol. 134, No. 6, pp. 1501-1503, October, 1960
Original article submitted April 4, 1960

One of the best documented diseases of young Salmonid fish is "vertigo," a disease caused by the Myxosporidian parasite Myxosoma cerebralis (Hofer, 1903) Plehn, 1905.

According to the literature, this disease is widespread in Salmonid hosts and occurs in many European countries. M. cerebralis was first discovered in Germany [1-4] and during the subsequent decade in France [5], Italy [6], Czechoslovakia [7] and Poland [8]. There are some accounts of its occurrence in Denmark.

Vertigo in Salmonids was not recorded in the Soviet Union until comparatively recently. Symptoms of the disease were first noted in rainbow trout from the "Ropsha" fish farms, Leningrad Region, in 1952. Later, this disease was observed in young Black Sea trout at the Chernorechensk fish farm, Abkhazia, and spores of M. cerebralis were found in wild Black Sea trout (Salmo trutta labrax) and rainbow trout (S. irideus), inhabiting the Black Sea rivers [9].

So far, it would appear that this parasite has been recorded only from Eastern and Western Europe. However, in 1959, the author found and studied vertigo in Pacific salmon of the genus Oncorhynchus, at the fish hatcheries in Southern Sakhalin Island.

The infection was found in young ketas (O. keta), gorbuschas (O. gorbuscha) and simes (O. masu) and detailed parasitological dissection revealed its presence in the following wild species: Salvelinus leucomaenis, S. malma morpha and young O. masu. Specimens of these fishes were taken from the Kalininka, Lesna, Serakavo and Taranaika rivers. The first of these rivers flows into the Terpenia Strait, the second into the Sea of Okhotsk near the township of Okhotsk, the third into the Gulf of Terpenia and the last into Anivskii Bay. In addition to the above-mentioned localities, numerous fish were examined from hatcheries, fish farms and reservoirs in the Kalininskii, Lesnoi, Sokolovskii and Taranaiskii catchment areas.

Spores of M. cerebralis were discovered in cartilaginous tissue from the skulls of O. masu (64%), Salvelinus leucomaenis (44%) and S. malma morpha (in all five specimens examined). The extent of the infection among the different species of fish varied from one river to another. Thus, spores of M. cerebralis were found in all the specimens of O. masu examined from the Kalininka and Serakavo rivers, whereas only one individual from the Taranaika River was found to be infected. Salvelinus leucomaenis from the Lesna and Serakavo rivers were found to be less heavily infected than those from the Kalininka River. All the individuals of S. malma morpha from the Kalininka and Lesna rivers were found, on dissection, to be infected with this parasite. At a magnification of 40 × 15 diameters, a single field of vision usually yielded one M. cerebralis spore when preparations of S. malma morpha were examined. Preparations of O. masu tissue showed higher infestation by the parasite; at a magnification of 40 × 15 diameters up to 15-16 spores were visible in a single field of vision.

These observations suggest that in the Far East, particularly in the Sakhalin Islands, there is an endemic focus of parasitism associated with M. cerebralis. Spores of this parasite have been observed in fish of the genera Oncorhyncus and Salvelinus from the rivers of the western, eastern and southern coasts of Southern Sakhalin Island. M. cerebralis may, therefore, be described as having a wide distribution in this area. The fact that this pathogen occurs in a region of intensive fish cultivation has very considerable practical importance. Salmonids between five and six months old are known to suffer considerable damage from the activities of this myxosporidian; the infected fish show obvious symptoms of disease and many die.

Young salmonids suffering from vertigo may be distinguished from healthy individuals by several external signs, e. g., the blackening of the posterior half of the body, the curvature of the spine, the rudimentary nature of the operculum, the uncoordinated movements which they exhibit, etc.

The author's researches into the biology of this parasite have shown that the fish become infected from the moment when they commence active feeding. The plasmodial stage in the life cycle is the stage which is most harmful to the young Salmonids, for it is the plas-

modium which infiltrates into the cartilaginous tissue of the host's skull and destroys it. The young fish begin to die from vertigo as early as July. By then the diseased fish have all acquired their abnormal pigmentation. Depending on the time of hatching of the fish fry and on climatic conditions, sporulation of M. cerebralis may be observed during the month of August. Spores of the myxosporidian are found in the external medium after the death of the diseased fish and the dissolution of their tissues. They are also passed through the alimentary tract of living fish suffering from vertigo. Consequent upon this latter state of affairs, wild fish entering hatcheries and fish farms, where young Salmonids are raised, prove a constant source of M. cerebralis infection.

Under the conditions prevailing in the Sakhalin Islands three species of wild Salmonid fish, namely Salvelinus leucomaenis, S. malma and Oncorhynchus masu, are the main disseminators of M. cerebralis spores.

Observations, carried out in 1959 on young ketas, gorbuschas and on their hybrids, also on young simes, have shown that from May to the beginning of June these species are free from any externally apparent

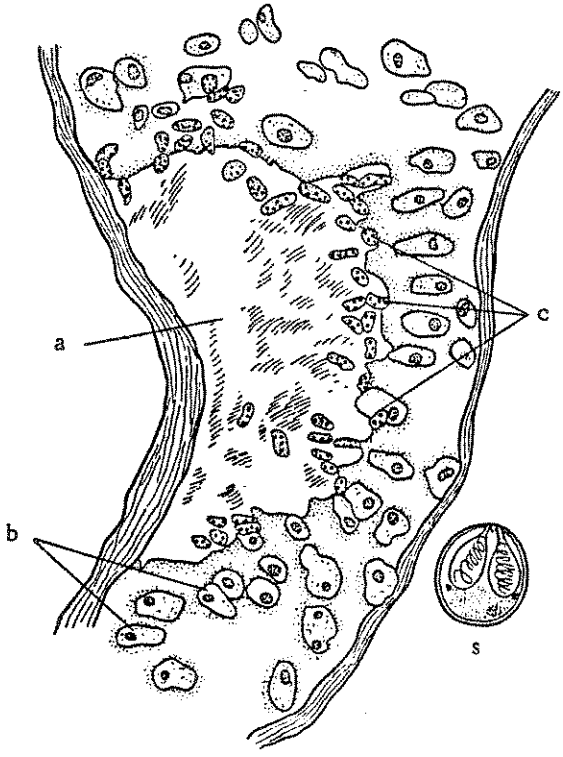

Fig. 1. Transverse section through the skull of a young autumn O. keta smolt infected with Myxosoma cerebralis (stained Ehrlich's hematoxylin): a) cavity formed as a result of the parasite destroying the cartilage; b) chondrocytes with nuclei; c) plasmodium of parasite penetrating cartilage, together with chondrocytes; s) spore of M. cerebralis (with polar capsules).

symptoms of vertigo. This period of time coincides with the seaward migration of the Salmonid smolts in natural waters. However it must be admitted that young fish in the early stages of vertigo infection reveal no external signs of the disease, and that it is not possible to detect the plasmodium of M. cerebralis in any general parasitological examination. Consequently, it was decided to make histological sections of the cartilaginous skulls of young fishes at this time of the year. For this purpose six month old keta smolts were taken from the Kalinskii fish farm* on June 2, 1959.

As a result of the investigation it was established that plasmodia of M. cerebralis were present in the cartilage of the skull and the branchial arches of these fish at the time. Characteristic of this phase of the disease was the destruction of the cartilaginous tissue of the skull (Fig. 1). The fact that the fish were not shedding spores at the time, can be explained in terms of the conditions under which the young fish are reared. The water in the rearing tanks is at a low temperature (usually 3-4°, very occasionally reaching 7°), consequently the development of the parasite is retarded and sporulation does not occur during the period when the salmon smolts would normally descend the Kalininka River for the sea. Lengthening the time of sojourn in the hatcheries until July and August may result in the smolts showing symptoms of vertigo and shedding ripe spores of M. cerebralis.

M. cerebralis is, therefore, not only a parasite of the wild Salmonids, Oncorhynchus and Salvelinus in the Sakhalin Islands but also occurs in young ketas reared under artificial conditions. These young fish are released into rivers at a time when the parasite is beginning to sporulate, and further development takes place when the fish reaches the sea.

The discovery of a new center of distribution for M. cerebralis demonstrates the need for carrying out additional investigations, with a view to determining levels and dynamics of infection in young fish belonging to various species of the genus Oncorhynchus. In addition there is need to study the biology of this parasite, particularly in relation to the conditions prevailing in Sakhalin Island, together with other allied problems.

Artificial rearing of Pacific salmon is widely practiced in Japan [10], in the United States [11] and also in Canada. In these countries, there are considerable numbers of fish hatcheries and fish farms where millions of young salmon of the genus Oncorhynchus are reared annually. For this reason the determination of the distributional range of M. cerebralis and its occurrence among

*S. leucomaenis, O. masu, and S. malma were all constant visitors to this particular fish farm, as well as inhabiting the reservoirs from which the water in the fish farm was derived. These species, therefore, furnished the M. cerebralis spores, which increased the incidence of infection among the resident Salmonid smolts.

Oncorhynchus species and those of other salmon genera, both in fish farms and in natural waters, is of great scientific and practical importance.

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AN EFFICIENT CONCENTRATION AND PURIFICATION METHOD FOR SPORES OF *MYXOSOMA CEREBRALIS*

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THE PRIMARY NEED FOR SPORE CONCENTRATION techniques has been for the purpose of increasing the reliability of diagnosing whirling disease in trout. In present diagnostic procedures, skulls from suspect fish are minced and the watery suspension examined microscopically for spores [*1*]. Such suspensions are too dilute and debris-laden to be efficient for use in the diagnosis of early or mild infections of whirling disease or for immunological studies requiring spores. Large amounts of clean and highly concentrated spores are necessary for practical studies dealing with spore physiology, tissue culture, and immunologic studies.

This paper describes a simple, efficient method for production of clean, concentrated spore suspensions by mechanical homogenation and chemical digestion of infected trout tissues.

MATERIALS AND METHODS

Trout from 4 to 6 inches in length and exhibiting whirling disease signs were processed in lots of 5 to 6 fish. The fish were skinned and defleshed leaving only head and spine intact. The heads and spines were cut into small pieces with scissors and placed in a Waring blendor chamber. The chamber was filled with ice-cold distilled water and the tissues homogenized for 5 to 10 minutes.

The homogenate was mixed 1:1 with a digestive solution consisting of 2 percent dried pepsin (Merck and Co., Inc. Rahway, N.J.) and 2 percent HCl, and placed on a magnetic stirrer for 3 to 6 hours. The 2 percent digestive solution was prepared by dissolving 20 grams of dried pepsin and adding 20 milliliters of concentrated HCl per liter of distilled water. The digested homogenate was passed through sieves having 72 micron openings to remove any large debris.

The filtrate was placed in 250 milliliter centrifuge tubes and spun at 1,000 xg for 15 minutes. The supernatant was drawn off, and the pellets resuspended in distilled water and pooled into one large centrifuge tube. Twenty milliliters of ethyl ether was added to the suspended spore-debris mixture which was shaken well and spun at 200 xg for 10 minutes. The ethyl ether layer and the water-ether interface were drawn off leaving only the spores in the water fraction below.

The spores were washed twice in distilled water by alternate resuspension and centrifugation at 1,000 xg. Finally, the spores were resuspended in a known volume of water, counted in a hemacytometer chamber, treated with penicillin and streptomycin (200 μ penicillin/ml, 200 μg streptomycin/ml), and either stored at 4°C or frozen.

RESULTS

Several lots of 5 to 6 trout 4 to 6 inches in length were processed. Each lot yielded a white pellet of pure spores containing 10 to 12 million intact spores. No debris could be observed in samples of resuspended spores examined microscopically.

DISCUSSION

The modified concentration technique for *Myxosoma cerebralis* spores described above

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has been shown to be an efficient, simple method to produce spore suspensions sufficiently pure and concentrated for diagnosis as well as experimental applications. Rydlo [2] described a cruder and more time-consuming method of spore concentration employing chemical digestion only. He cut the infected fish into chunks and placed them in an acid pepsin solution for several hours. The filtrate of the digestion was again digested in acid pepsin for an additional 10 to 20 hours. Rydlo's process was only intended for diagnosis and was unsuitable for producing a clean suspension. His aim was only to qualitatively determine the presence of spores in various organs of the trout.

The initial defleshing and mechanical homogenation of the skulls and spines of the trout allowed rapid and thorough digestion of the tissues including bones and cartilage by the acid pepsin. A trace of undigested residue was collected when the digested homogenate was passed through the 72-micron-opening sieve. Apparently, only spores, very small suspended particles, and dissolved components are left in the homogenate after screening with the sieve. Mixing with ether removes fatty substances and suspended particles into the ether layer or the water-ether interface. The use of ether in a *M. cerebralis* spore concentration method was first described by workers of the Eastern Fish Disease Laboratory at the Whirling Disease Workshop, March 21-22, 1973, Leetown, W. Va. After centrifugation at low speeds (with the ether), the water phase contains only spores—most of which have collected in a visible white pellet allowing the ether phase to be drawn off with little or no loss of spores. This method of spore concentration will produce consistent results when followed closely. The critical steps probably include thorough defleshing of skull and spine, using fresh and sufficient acid pepsin (at least 1:1 with the homogenate), and centrifugation at the proper speeds.

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Parasitology Research

Experimental transmission of two *Myxobolus* spp. developing bisporogeny via tubificid worms

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Abstract. Spores of *Myxobolus cotti* El-Matbouli and Hoffmann 1987 and *M. cerebralis* Hofer 1903 (fresh or after 5 months in mud) are taken in by tubifex worms and develop in their gut epithelium cells into actinosporea of the genus *Triactinomyxon*. *Triactinomyxon* deriving from *M. cotti* differ distinctly from those derived from *M. cerebralis* in morphology and the number of sporozoites in the epispore. It could be shown that *Triactinomyxon* spores infect fish either via the water (bullhead, rainbow trout) or by feeding of infected tubifex (rainbow trout), developing into *Myxobolus* spores in central nervous tissue (bullhead) or cartilage (rainbow trout). Fresh or ripened spores of *M. cotti* and *M. cerebralis* were not infectious for bullheads or rainbow trout, respectively. The results of our experiments confirm the hypothesis that the life cycle of *M. cotti* and *M. cerebralis* includes an intermediate host and a metamorphosis into actinosporea of the genus *Triactinomyxon*.

All published transmission experiments with members of the class Myxosporea have been done with *Myxobolus cerebralis* (syn. *Myxosoma cerebralis*), the agent of whirling disease in salmonid fish. All transmission trials were ineffective until a new aspect to this unsolved problem was revealed by Markiw and Wolf (1983), Wolf and Markiw (1984) and Wolf et al. (1986), who presented the theory not only that tubificid worms are necessary as intermediate hosts, but also that a transformation of *M. cerebralis* to an actinosporean (*Triactinomyxon gyrosalmo*) occurs in tubifex, and only this *Triactinomyxon* can infect salmonids. These results have not generally been accepted; thus, Hamilton and Canning (1987) reported that *M. cerebralis* spores do not induce a higher prevalence of *Triactinomyxon* in tubifex.

The present paper gives further evidence for the involvement of tubifex or actinosporean in the life cycle of *Myxobolus* spp. using *M. cotti*, which infects the CNS of bullhead (*Cottus gobio*) (El-Matbouli and Hoffmann 1987), and *M. cerebralis*, which invades the cartilage of salmonids, especially rainbow trout (*Salmo gairdneri*).

Reprint requests to: M. El-Matbouli

Materials and methods

Two series of experiments were carried out in 1986 and 1987. For the first series (experiment 1), four bullheads were fed homogenated spinal cord of bullheads infected with *M. cotti*. The bullheads were kept in a 20-l glass aquarium to obtain feces for microscopic examination. In experiment 2, spores of *M. cotti* deriving from CNS were transferred to a 20-l glass aquarium with a 5-cm-thick layer of sterilized sand on the bottom. After filling the aquarium with water (temperature, 16°–17° C), 50 g tubifex worms (90% *Tubifex tubifex* Müller and 10% *T. ignotus* Stolc and *Limnodrillus hoffmeisteri* Claparède) was added (tank 1). As a control, 50 g tubifex was put in another 20-l aquarium with a 5-cm-thick layer of sterilized sand on the bottom (tank 2).

Every 2 days, samples of tubifex from both tanks were taken for squash preparations, and other samples were fixed in 5% buffered formalin and embedded in Parablast. Sections 5 µm thick were stained with hemotoxylin and eosin and Giemsa's solutions. After the appearance of *Triactinomyxon* spores in the gut of worms or the water in tank 1, the tubifex were washed with distilled water. These *Triactinomyxon*-containing tubifex were put into capsules of a meshwork with 200-µm width and transferred to a 20-l aquarium containing bullheads from an environment without Myxosporea. The bullheads were transferred 20 days later to a new aquarium without tubifex at 12°–15° C.

In the second series (experiment 3), 20 rainbow trout fry (4–6 cm long) were put in a 100-l tank containing spores of *M. cerebralis* ripened in tubifex-free mud for 4 months. In experiment 4, the conditions were identical to those in experiment 2, except that *M. cerebralis* spores were used instead of those of *M. cotti*.

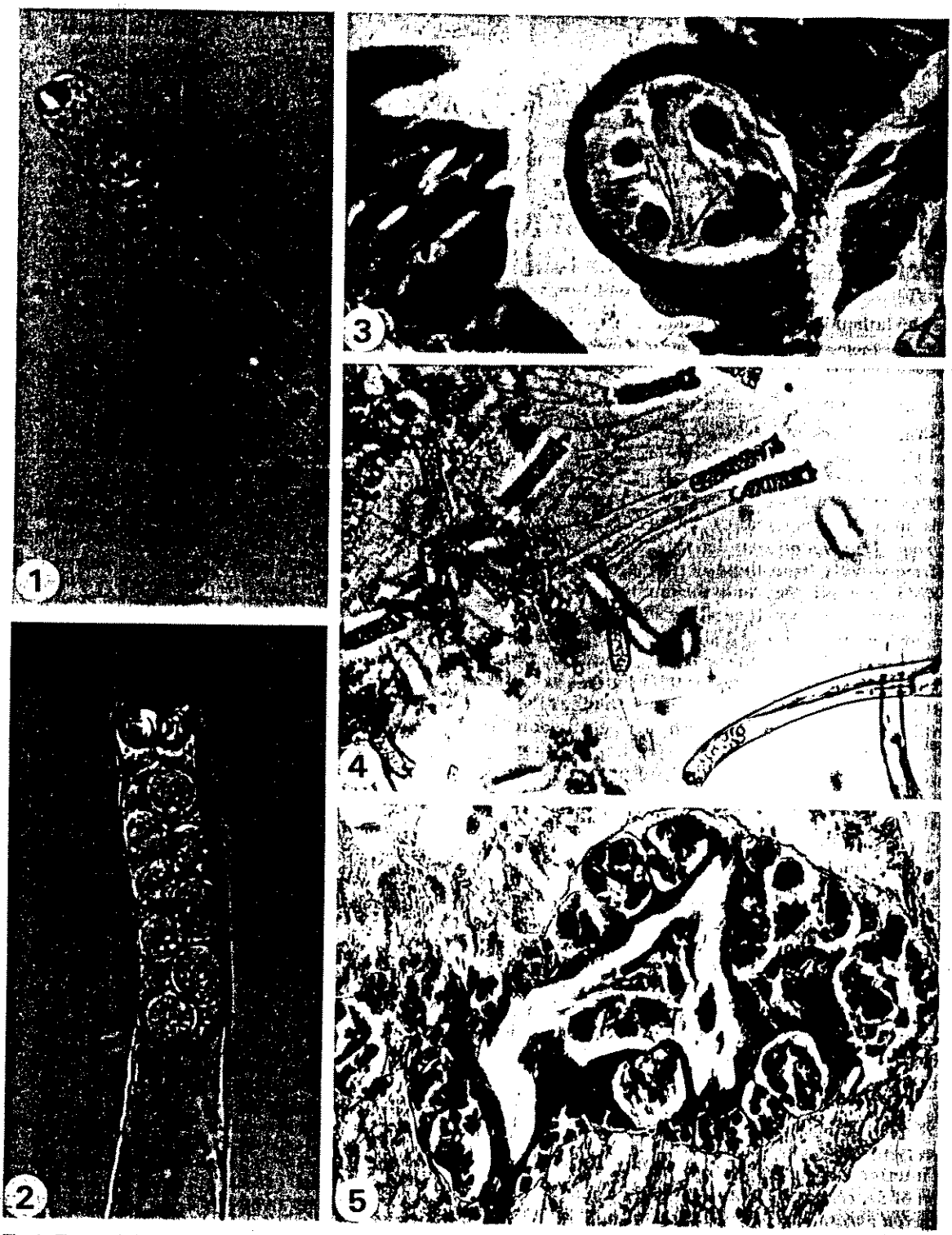

Fig. 1. *T. myxoboli cotti* from the homogenate of infected tubifex: epispore with 16 sporozoites. ×925. **Fig. 2.** *T. myxoboli cotti* isolated from water: epispore with 8 sporozoites. ×1200. **Fig. 3.** Pansporocyst of *T. myxobolus cotti* in the gut epithelium of an infected tubifex. Giemsa; ×825. **Fig. 4.** *T. gyrosalmo* from the homogenate of infected tubifex: epispore with 32–50 sporozoites. ×350. **Fig. 5.** Spores of *T. gyrosalmo* in the gut epithelium of an infected tubifex. Giemsa; ×350

Results

Series I – transmission of Myxobolus cotti

Experiment 1: direct transmission. Feeding of healthy bullheads with material from the CNS of infected bullheads containing spores of *M. cotti* did not result in any changes in the recipients, but unchanged spores could regularly be found in their feces.

Experiment 2: transmission via tubifex. In samples of tubifex taken every 2 days for squash and histologic examination, developing stages and spores of a member of the genus *Triactinomyxon* were detected beginning on day 80. This *Triactinomyxon* was anchor-shaped and had three polar capsules; the anterior cavity (epispore) contained 16 spherical sporozoites (Fig. 1). On day 125, living and dead spores could be seen attached to the glass wall and swimming freely in the water; the free stages contained only eight spherical sporozoites (Fig. 2) whose total length (epispore and styl) was 88.64 µm on average. Histologically, masses of pansporocysts could be found in the gut epithelium (Fig. 3) and free *Triactinomyxon* spores were detected in the gut lumen of tubifex. Tubifex from control aquaria without contact to material from the CNS of infected bullheads did not show evidence of *Triactinomyxon* spores or developing stages. In one of three bullheads in the tank with infected tubifex, spores of *M. cotti* could be detected in the spinal cord on day 130 after the first contact with tubifex.

Series II – transmission of M. cerebralis

Experiment 3: direct transmission. Rainbow trout fry in the tank containing spores of *M. cerebralis* ripened in tubifex-free mud did not show clinical signs of whirling disease. All postmortem examinations gave negative results during the 5 months of observation.

Experiment 4: transmission via tubifex. On day 82, pansporocysts and spores of an actinosporean of the genus *Triactinomyxon* were found in squash preparations of tubifex. As in experiment 2, the spores were anchor-shaped, with three polar capsules, but the epispores contained 32–50 spherical sporozoites (Fig. 4). Histologically, developing stages could be detected in the gut epithelium on day 82 (Fig. 5). Beginning on day 94, freely swimming spores could be observed. There was no difference between spores in the squash preparation and those in the water; their arm length was 135.62 µm on average. Control tubifex did not contain any stage of *Triactinomyxon*. The continuation of this experiment was modified: infected tubifex were divided into two groups.

Table 1. Infection of young rainbow trout fry with *M. cerebralis* via the water

Fish number	Time between infection and clinical signs (in days)	Time between infection and necropsy (in days)	Occurrence of *M. cerebralis*
1	86	120	+
2	96	116	+
3	–	140	+
4	96	172	+

Table 2. Results of oral infection of rainbow trout fry with *M. cerebralis*

Fish number	Time between infection and clinical signs (in days)	Time between infection and necropsy (in days)	Occurrence of *M. cerebralis*
1	–	60	–
2	–	95	+
3	95	102	+
4	–	115	+
5	–	118	+
6	–	121	–
7	–	131	+

Experiment 4a: transmission via water. As in experiment 2, infected worms were encapsulated in meshwork cages and transferred to aquaria with rainbow trout fry (4–5 cm long). Beginning on day 86 after indirect contact with tubificids, typical signs of whirling disease (black tail, whirling) were observed. Both the squash preparation and histologic slides showed evidence of *M. cerebralis* in all trout (Table 1).

Experiment 4b. Seven rainbow trout were fed with the remaining tubifex. Whirling disease could be proven in five trout between days 95 and 131 after feeding (Table 2).

Discussion

The results of the present study support the theory that tubificid worms are obligate intermediate hosts for Myxobolidae. Not only *M. cerebralis* but also *M. cotti* are transmitted by tubifex. Whereas the actinosporea spp. deriving from *M. cerebralis*

was identical to that described by Wolf and Markiw (1984), *T. gyrosalmo*, the *Triactinomyxon* deriving from *M. cotti* strongly differs morphologically. Therefore, we suggest that the latter be named *Triactinomyxon myxoboli cotti*; this name should also document that *Myxobolus* and *Triactinomyxon* are only different stages of the same parasite. Current systematics (Levine et al. 1980) should therefore be revised, since they present results also favor the theory that the use of an intermediate host is commonly necessary in Myxosporea.

It is very surprising that sporogony takes place twice during the life cycle of these parasites; there are several possible explanations for this. The first could be that the original hosts were only tubificids and that after the phylogenetic appearance of vertebrates, an additional host was involved. In terms of the distribution of the parasites, the inclusion of freely swimming *Triactinomyxon* spores favors positive contact with the next host. Especially in trout, contact with *Myxobolus* spores is improbable except by cannibalism; in fact, only fry and fingerlings of salmonids can become clinically infected, whereas spore development takes place in larger fish (Hoffman 1962). The spores are found in body tissue that does not come into contact with the environment, which enables release only after the death of the fish, after which most of the spores will appear at the bottom of the water. Direct contact with freely swimming trout is improbable, but tubificid worms can easily come into contact with the spores. The multiplication of these parasites in tubificid worms in combination with the development of freely swimming stages favors effective contact with the final host.

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Effects of Freezing, Aging, and Passage through the Alimentary Canal of Predatory Animals on the Viability of *Myxobolus cerebralis* Spores[1]

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Abstract.—In transmission experiments with tubificids *Tubifex tubifex* as primary hosts and fry of rainbow trout *Oncorhynchus mykiss* as secondary hosts, it was shown that *Myxobolus cerebralis* spores can tolerate freezing at −20°C for at least 3 months, aging in mud at 13°C for at least 5 months, and passage through the guts of northern pike *Esox lucius* or mallards *Anas platyrhynchos* without loss of infectivity.

The failure of many experiments designed to infect fish directly with freshly isolated spores of *Myxobolus cerebralis* has led to the general opinion that spores need to ripen by aging 4–5 months in mud (Hoffman 1962; Hoffman and Putz 1969; Putz 1970; Putz and Hoffman 1970; Schäperclaus 1979). Plehn (1904, 1924) suggested that spores were resistant to drying and freezing, and had a long survival time. Hoffman and Putz (1971) further suggested that aging of previously frozen spores for about 4 months was necessary for infectivity.

Meyers et al (1970) and Schäperclaus (1979) implicated avian vectors in the transmission of *M. cerebralis* by finding spores in the feces of great blue herons *Ardea herodias*. Hoffman and Putz (1970) were unable to infect fry with heron feces containing *M. cerebralis* spores aged 4 months; however, it was unknown as to whether the spores were viable. Taylor and Lott (1978) were able to infect trout in ponds in which feces from contaminated waterfowl were suspended.

Clarification of the life cycle of *M. cerebralis*, which includes a tubificid intermediate host (Wolf and Markiw 1984; El-Matbouli and Hoffman 1989), has allowed experimental transmission of whirling disease. The ability to complete the life cycle in the laboratory has enabled us to investigate factors that affect the viability of *M. cerebralis* spores.

Methods

Experiment 1: Transmission of M. cerebralis via Tubifex tubifex infected with fresh spores.—Spores of *M. cerebralis* were mechanically isolated and enriched. (Infected cartilage was disrupted, filtered, and centrifuged, and the resulting pellets were resuspended in phosphate-buffered saline [PBS]). A 20-L glass aquarium with a 5-cm-thick layer of sterilized sand was filled with tap water. Afterwards, 100 g of tubificids (90% *Tubifex tubifex*, 10% *Tubifex ignotus* and *Limnodrilus hoffmeisteri*) of our laboratory-reared specific-pathogen-free (SPF) stock were distributed on the sand layer. The tubificids were regularly tested and shown to be free of *Actinosporea*. Aquaria were undisturbed for 2 d to allow the tubificids to burrow into the sand. Water flow was then stopped, and *M. cerebralis* spores were added in a ratio of about 50 spores per tubificid worm. Water temperature was 16–17°C. At 90 d after initial exposure, waterborne triactinomyxon spores (considered the intermediate stage of *M. cerebralis*) were detected. The maximum numbers of triactinomyxon spores were found in the water 120–140 d after exposure.

At day 120 after exposure, infected tubificids were transferred to a capsule with a 200-μm-mesh screen allowing only triactinomyxon spores to leave. This capsule was placed in a 50-L tank with 30 fry of rainbow trout *Oncorhynchus mykiss* (3–5 cm in length) from our SPF stock. Aquaria were aerated, but water flow was stopped for 7 d. After 7 d, the capsule containing the infected tubificids was removed, and water flow was set to about 800 mL/min. Water temperature was 12–13°C. The fry were observed daily for clinical signs of whirling disease.

Experiment 2: Transmission of M. cerebralis via T. tubifex infected with aged spores.—Spores of *M. cerebralis* were added to a 20-L aquarium with a 5-cm-thick layer of sterilized sand and a water

[1] This study was supported by the Deutsche Forschungsgemeinschaft (grant DFG HO645/8-1).

TABLE 1.—Results of infections of rainbow trout fingerlings with treated spores of *Myxobolus cerebralis*.

Spore treatment	*N*	First detection of triactinomyxon in water (days after exposure)	Number of fish (%) with whirling disease 130 d after exposure to triactinomyxon spores	
			Clinical signs	*M. cerebralis* spores in head cartilages
None (fresh)	30	90	22 (73%)	28 (93%)
Aging in mud	30	80	20 (67%)	30 (100%)
Freezing				
2 months	30	78	18 (60%)	29 (97%)
3 months	30	81	16 (53%)	28 (93%)
Spores passed through the alimentary canal or piscivore				
Northern pike	15	75	11 (73%)	12 (80%)
Mallard	15	84	13 (87%)	13 (87%)

flow of about 400 mL/min. Tap water of 13°C was used. Aquaria were undisturbed for 5 months to allow the spores to age. After this time, 100 g of SPF tubificid worms were added in a ratio of one tubificid worm to 50 spores. As in experiment 1, 120 d after exposure to spores, tubificids were encapsulated in mesh cages and transferred to aquaria containing 30 rainbow trout fry.

A negative control consisted of placing 20 rainbow trout fry into a tank containing sand with spores aged for 5 months.

Experiment 3: Transmission of M. cerebralis via T. tubifex infected with frozen spores.—Ten spore-bearing rainbow trout heads were frozen for 2 months, and another 10 for 4 months. After freezing, *M. cerebralis* spores were isolated and enriched as in experiment 1. The two groups of frozen spores were added to separate aquaria, each containing 100 g of tubificid worms. The aquaria contained sterilized sand as described in experiment 1. At 120 d after exposure, the tubificids were isolated from the sand, encapsulated in mesh cages, and transferred to one of two aquaria containing 30 rainbow trout fry. Further procedures were as in experiment 1.

Experiment 4: Transmission of M. cerebralis via T. tubifex infected with M. cerebralis after passage of spores through fish.—Over a period of 2 weeks, 10 experimentally infected rainbow trout fingerlings (7–8 cm long) with strong clinical signs of whirling disease were fed to five northern pike *Esox lucius* (26–30 cm long). During 15 d, feces were collected, suspended in PBS and passed through a 200-μm-mesh sieve to remove gross particles. Feces were then passed through 100-, 50-, and 25-μm-mesh filters. The final filtrate with spores was centrifuged at 130 × gravity for 10 min. The pellets with *M. cerebralis* spores were resuspended in PBS. The suspension of harvested spores was added to a 20-L aquarium with sterile sand and 50 g of tubificids, as in experiment 1. At 120 d after exposure, tubificids were removed and exposed to 15 rainbow trout fry as described in experiment 1.

Over a 1-week period, 10 spore-bearing rainbow trout heads were fed to three mallards *Anas platyrhynchos*. Feces from the ducks were collected for 7 d, and spores of *M. cerebralis* were isolated as from fish feces and used to infect 15 rainbow trout fry.

Controls for each experiment consisted of one aquarium with a 5-cm-thick sand layer and 100 g tubificids from the same population but without addition of spores. The water and the tubificids were regularly examined for the presence of triactinomyxon spores.

Results

The timing of the first appearance of waterborne triactinomyxon spores was similar for all experiments in which *M. cerebralis* spores were in contact with tubificids. Triactinomyxon spores first appeared at an average of 81 d (range, 75–90 d) after exposure of tubificids to the different treated spores of *M. cerebralis*. These triactinomyxon spores were identical to those described in Wolf and Markiw (1984), and by El-Matbouli and Hoffmann (1989). In the tank containing only aged *M. cerebralis* spores but no tubificids, triactinomyxon spores could not be detected at any time.

In all groups of rainbow trout fingerlings exposed to triactinomyxon spores, a high percentage developed clinical (53–77%) or morphological (80–100%) signs of whirling disease (Table 1). In contrast, among fish having contact only with aged spores of *M. cerebralis*, neither clinical signs nor

spores in head cartilages were detected. Control fish from the breeding stock were free of signs of disease.

Discussion

This study demonstrated that spores of *M. cerebralis* survive for at least 3 months at −20°C. In addition, spores passed through the alimentary canals of northern pike and mallards remained viable. The ability of spores to survive passage through the intestines of piscivorous fishes, birds, or mammals is important in the natural distribution of whirling disease, because affected salmonids can be easily caught by predators. Therefore, the implications of transferring fish-eating animals to other regions or continents free of *M. cerebralis* must be considered. The results of this study support the hypotheses of Meyers et al. (1970), Taylor and Lott (1978), and Schäperclaus (1979) on the role of fish-eating birds in the distribution of whirling disease.

In contrast to the suggestion that *M. cerebralis* spores must ripen in mud for 6 months (Schäperclaus 1931; Hoffman et al. 1962; Putz and Hoffman 1970) or age for 4 months after freezing (Hoffman and Putz 1971) to become infective, our results show no difference between fresh and mud-ripened spores with regard to induction of triactinomyxon development in tubificids. There is a long period between release of *M. cerebralis* spores in the water and first signs of whirling disease because the parasite stages in the first host (*T. tubifex*) and the second host (salmonid fish) each take about 3 months to develop (Wolf and Markiw, 1984; El-Matbouli and Hoffmann 1989).

Freezing down to −20°C did not inhibit the infectivity of *M. cerebralis* spores. This is an important consideration in the shipment of frozen food fish. Resistance to freezing seems to be a common attribute of *Myxobolus* spores, because spores of *M. cotti* and *M. pavlovskii* have a comparable quality (our unpublished observations).

In conclusion, the treated spores of *M. cerebralis* (fresh, aged 5 months in mud, frozen at −20°C for 3 months, or passaged through the alimentary canals of northern pike and mallards) were ingested, along with mud particles, by oligochaetes of the genus *Tubifex*. Inside the tubificids, the spores differentiated into the fish-infective stage, the triactinomyxon stage. The resilient qualities of *Myxobolus* spores as well as their possible transmission through piscivorous predators must be considered in programs for prophylaxis and eradication of whirling disease.

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Light and electron microscopic observations on the route of the triactinomyxon-sporoplasm of *Myxobolus cerebralis* from epidermis into rainbow trout cartilage

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Penetration of triactinomyxon-sporoplasms of *Myxobolus cerebralis* through skin, fins, gills and buccal cavity have been demonstrated experimentally in rainbow trout. Furthermore the multiplication-stages of penetrated triactinomyxon-sporoplasms reach the cartilage via peripheral nerves and the central nervous system (CNS). This is in contrast to the assumption that the agent reaches the cartilage via blood, lymph, and/or coelomic fluid. During the first hour following penetration, the sporoplasm migrates between the epidermal cells. Then, it enters the epithelia and multiplies intracellularly. These stages migrate deeper into the subcutis, then through the peripheral nerves and CNS. After about 21 days the parasites reach the head cartilages. During their migration they also multiply to increase parasite numbers. The ultrastructure of the proliferative phase (presporogonic development) and the sporogonic phase of the life cycle are demonstrated and discussed.

Key words: *Triactinomyxon*; *Myxobolus cerebralis*; epidermis; CNS; cartilage.

INTRODUCTION

Whirling disease is mainly a chronic disease of trout fry and fingerlings caused by the myxosporean *Myxobolus cerebralis*. The increased resistance of older fish is due to ossification of cartilages as well as a result of an acquired immunity (Hoffmann *et al.*, unpub. obs.). The first confirmed outbreak occurred in Germany as early as 1903 among rainbow trout *Oncorhynchus mykiss* (Walbaum), a fish species which was not indigenous to Europe (Hofer, 1903). The transcontinental spread of whirling disease and its economic importance has increased with the worldwide development of salmonid farming. The disease has therefore been included in the International Office of Epizootics list of important infectious diseases of fresh water fish. *M. cerebralis* has been recorded in 18 species of salmonids (Halliday, 1976). Rainbow trout is the most seriously affected species, brook trout *Salvelinus fontinalis* Mitchill less severely, and brown trout *Salmo trutta* L., seems to be the most resistant (Hoffman *et al.*, 1962).

Wolf & Markiw (1984) and El-Matbouli & Hoffmann (1989) demonstrated that *M. cerebralis* has a two-host life cycle involving fish and a tubificid oligochaete. During the last 3 years a series of studies has proven a two-host-life cycle of other myxosporean species including an actinosporean developing in an oligochaete (Table I). Two different stages of sporogony occur, one in each host. *M. cerebralis* spores in fish cartilage are the infectious stage for *Tubifex tubifex* and transform in their gut epithelial cells into an actinosporean triactinomyxon.

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TABLE I. Myxosporean species requiring oligochaetes as intermediate hosts in their life cycle

Myxosporea species	Fish host	Oligochaete host	Actinosporea genus	Authors
Myxobolus cerebralis	Rainbow trout (*Oncorhynchus mykiss* Walbaum)	*Tubifex tubifex*	Triactinomyxon	Wolf & Markiw (1984); El-Matbouli & Hoffmann (1989)
Myxobolus cotti	Bullhead (*Cottus gobio* L.)	*Tubifex tubifex*	Triactinomyxon	El-Matbouli & Hoffmann (1989)
Myxobolus pavlovskii	Silver carp [*Hypophthalmichthys molitrix* (Valenciennes)]	*Tubifex tubifex*	Hexactinomyxon	El-Matbouli & Hoffmann (1991); Ruidisch *et al.* (1991)
Myxobolus sp.	Goldfish (*Carassius auratus* L.)	*Branchiura sowerbyi*	Raabia	Yokoyama *et al.* (1991)
Unknown Myxosporean	Channel catfish (*Ictalurus punctatus* Rafinesque)	*Dero digitata*	Aurantiactinomyxon	Styer *et al.* (1991)
Hoferellus carassii	Goldfish (*Carassius auratus*)	Not published	Aurantiactinomyxon	El-Matbouli *et al.* (1992*b*)
Hoferellus cyprini	Carp (*Cyprinus carpio* L.)	*Nais* sp.	Not published	Großheider & Körting (1992)
Ceratomyxa shasta	Rainbow trout (*Oncorhynchus mykiss*)	*Nais* sp.	Aurantiactinomyxon	Bartholomew *et al.* (unpublished)
Myxobolus carassii	Golden orfe (*Leuciscus idus*)	*Tubifex tubifex*	Triactinomyxon	El-Matbouli & Hoffmann (1993)
Myxidium giardi	Eel (*Anguilla anguilla* L.)	*Tubifex* sp.	Aurantiactinomyxon	Benajiba & Marques (1993)
Myxobolus arcticus	Sockeye salmon (*Oncorhynchus nerca* W.)	*Stylodrilus heringianus*	Triactinomyxon	Kent *et al.* (1993)

Only this is able to infect salmonid fish and induce whirling disease. Susceptible salmonids either ingest infected tubificids harbouring the specific triactinomyxon or contact waterborne triactinomyxon spores which penetrate the fish surface (Wolf & Markiw, 1984; El-Matbouli & Hoffmann, 1989).

The initial penetration of the triactinomyxon-sporoplasm into the fish host takes place in the epithelium of fins, skin, gills and intestine (Markiw, 1989; El-Matbouli *et al.*, 1992*a*). These authors demonstrated experimentally that within a very short time (5–10 min) after initial exposure, individual forms of aggregates of triactinomyxon-sporoplasms (12–18 μm) were found in the superficial epithelium. The peak of infection was evident in the fish epidermis 2 to 4 h after exposure. Aggregates of penetrated sporoplasms were no longer evident after 8 h and after 24 h only single sporoplasms were recognizable, scattered in deeper epithelial layers (Markiw, 1989; El-Matbouli *et al.*, 1992*a*). How, in what form and where in the fish the penetrated triactinomyxon-sporoplasms reach the cartilage has been a matter for speculation for a long time. The purpose of this study was to determine the route of the penetrated triactinomyxon-sporoplasm into the cartilage of rainbow trout fry.

MATERIAL AND METHODS

EXPERIMENTAL PRODUCTION AND ENRICHMENT OF TRIACTINOMYXON SPORES

Parts of the skulls of 60 rainbow trout clinically affected by whirling disease were minced in small pieces and homogenized in an Ultra Turax, suspended in phosphate buffered saline (PBS) and passed through screens with mesh size of 1000, 500, 250 and 100 μm. The final filtrate was centrifuged and the resulting pellets containing *Myxobolus cerebralis* spores were resuspended in PBS. Three trials were performed with the harvested spores. In each trial one-third of the harvested spore suspension was kept in a 12-l glass aquarium containing a 5-cm-thick layer of sterilized mud, 200 g actinosporea-free tubificid oligochaetes (90% *Tubifex tubifex*, 10% *T. ignotus* Stolc and *Limnodrilus hoffmeisteri* Claparede) and filled with tap water. In all three trials, each tubificid worm was exposed to about 200 *M. cerebralis* spores.

To harvest triactinomyxon spores we started the trials in 2-week intervals. As a control, tubificids from the same population were kept in another aquarium under identical conditions except for *M. cerebralis* spores. Water flow was stopped for 24 h then a slow flow of water was provided at a rate of 10 ml min^{-1}. Water temperature was 13–15° C. One hundred and twenty days after initial exposure of tubificid worms to *M. cerebralis* spores, the waterborne triactinomyxon spores were collected every 2–3 days by filtering 75% of the aquarium water through a 20-μm-mesh screen. This water was replaced by fresh tap water.

EXPERIMENTAL INFECTION OF RAINBOW TROUT FRY

After each filtration process, the harvested waterborne triactinomyxon spores were exposed to 14–21-day-old specific-pathogen-free (SPF) rainbow trout. In all infection trials, each fish was exposed to an average of 10 000 waterborne triactinomyxon spores. The trout were exposed for a maximum of 1 h and were then transferred to tanks supplied with tap water. Samples of water (100 ml) were also filtered through 20-μm-mesh screen 10 min after fish were in contact with triactinomyxon. Retained triactinomyxon spores were examined microscopically immediately. Eight fish were taken at 5, 10, 15, 20, 25, 30, 40, 50 min and thereafter one fish every hour for 18 h after contact with triactinomyxon spores; then one fish was examined daily for 130 days. Trout were anaesthetized with chlorobutanol (1.1.1-trichlor-methylpropanol 0·1 g l^{-1} water). Blood was collected and smeared from the caudal vessel after cutting off the caudal fin. Fish were then necropsied

and parts of fins, skin, muscles, gills, skull, brain, spinal cord and parenchymatous organs were fixed in 5% buffered formalin and embedded in Historesin. Sections were stained with H&E and Giemsa's solutions. Blood smears were air-dried, fixed in methanol and stained with Diff-Quick. Specimens of fins, skin, cartilage and central nervous system were also fixed in 6·25% Sorensen-phosphate buffered glutaraldehyde (pH 7·4) for 3 h and post-fixed with 1% OsO_4 in the same buffer for 2 h. After dehydration, they were embedded in Epon-812. Semithin sections were stained with toluidine blue and safranin, ultrathin sections were contrasted with uranyl acetate and lead citrate, and examined by light respectively transmission electron microscope (Zeiss EM 109).

RESULTS

In all three trials, triactinomyxon spores appeared in the water 94 days on average after tubificids had been exposed to *M. cerebralis* spores. The waterborne triactinomyxon spore, as described by Wolf & Markiw (1984) and El-Matbouli & Hoffmann (1989), is anchor-shaped with three polar capsules at the top (Fig. 1). The style with epispore measures about 140 µm and contains a minimum of 64 spherical sporoplasm cells (sporozoites). The tapering arms measure 175–200 µm. The maximum amount of waterborne triactinomyxon spores was obtained between 110 and 160 days post-exposure. Infected tubificids were shown to release triactinomyxon spores for more than 12 months after their first appearance. Spores or developmental stages of triactinomyxon could not be found in negative controls, and trout exposed to the control tank remained free of *M. cerebralis* spores.

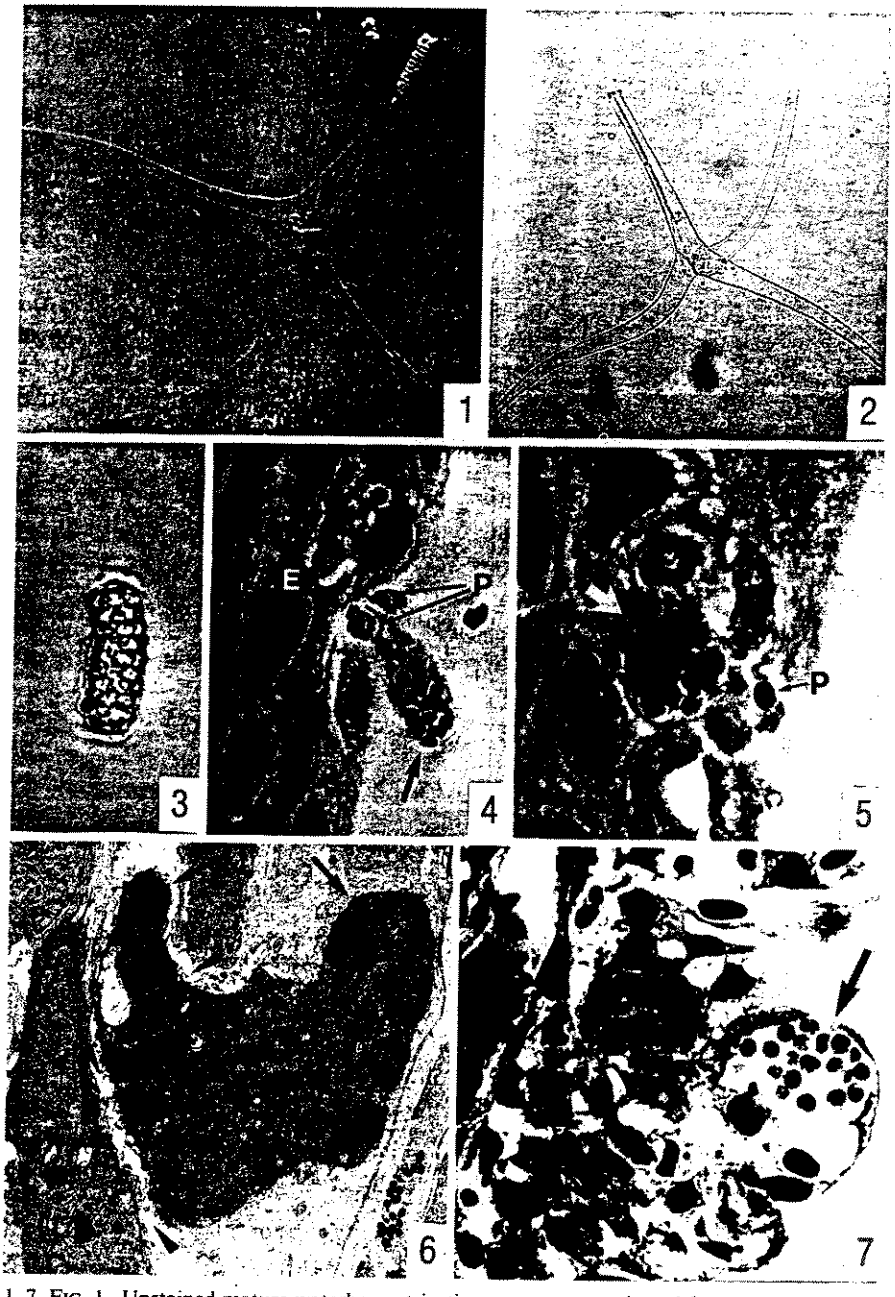
INFECTION OF TROUT FRY

Thirty minutes post-exposure of trout fry to waterborne triactinomyxon spores, filtered water contained between 40–60% of triactinomyxon spores which had released sporoplasms. These consisted of spore-shells without polar capsules and sporoplasm cells (Fig. 2). Occasionally, single, naked sporoplasms were found free in filtered water. These were membrane-bound, elongate and filled with sporoplasm cells (Fig. 3).

EXAMINATION OF SKIN AND FINS OF TROUT FRY

Within 5 min of exposure of trout fry to triactinomyxon spores, histological examination of skin and fins revealed triactinomyxon spores just penetrating the epidermis (Fig. 4). Moreover, the polar capsules of the penetrated triactinomyxon spore could be demonstrated in surface epithelia (Fig. 4). At 15 min post-exposure the penetrated triactinomyxon sporoplasm was recognizable deeper in the epidermis (Fig. 5). At 5 to 15 min post-exposure aggregates of sporoplasm cells were compact, intensively stained with Giemsa and their structure could not be differentiated by light microscope. Their cells measured 2·0 to 2·5 µm in diameter. The number of these membrane-bound cells ranged from 20 to 60, sometimes more. Their recognition was frequently facilitated by the occurrence of a surrounding lytic zone.

Electron microscopically, it could be shown that the sporoplasm aggregates found 1 h post-exposure were situated intercellularly in the epidermis (Fig. 6). Sporoplasm cells (about 20 cells) were electron-dense and in close contact with each other and seemed to be enveloped with a large cell. Each cell within these membrane-bounded aggregates possessed a large, distinct nucleus with a dense,



FIGS 1–7. FIG. 1. Unstained mature waterborne triactinomyxon spore released from *T. tubifex*, 120 days post-exposure, × 260. FIG. 2. Empty spore-shell of waterborne triactinomyxon spore, 30 min post-exposure to trout fry, unstained fresh preparation, × 260. FIG. 3. Free sporoplasm of triactinomyxon found in water containing triactinomyxon spores, 30 min post-exposure of trout fry, × 700. FIG. 4. Triactinomyxon-sporoplasm (arrow) penetrating the epidermis (E) of caudal fin, 5 min post-exposure. P, Polar capsules, × 700. FIG. 5. Compact aggregate of sporoplasm (arrow) between epithelial cells of the caudal fin, 15 min post-exposure. P, Polar capsule, × 1050. FIG. 6. Electronmicrograph of interepithelial localized aggregate of triactinomyxon-sporoplasm forming pseudopodia-like projections (arrows); lesions of the epidermis (arrowheads); 1 h post-exposure, × 2800. FIG. 7. Penetrated triactinomyxon-sporoplasm in the gill epithelial cells (arrow), 30 min post-exposure, Giemsa, × 700.

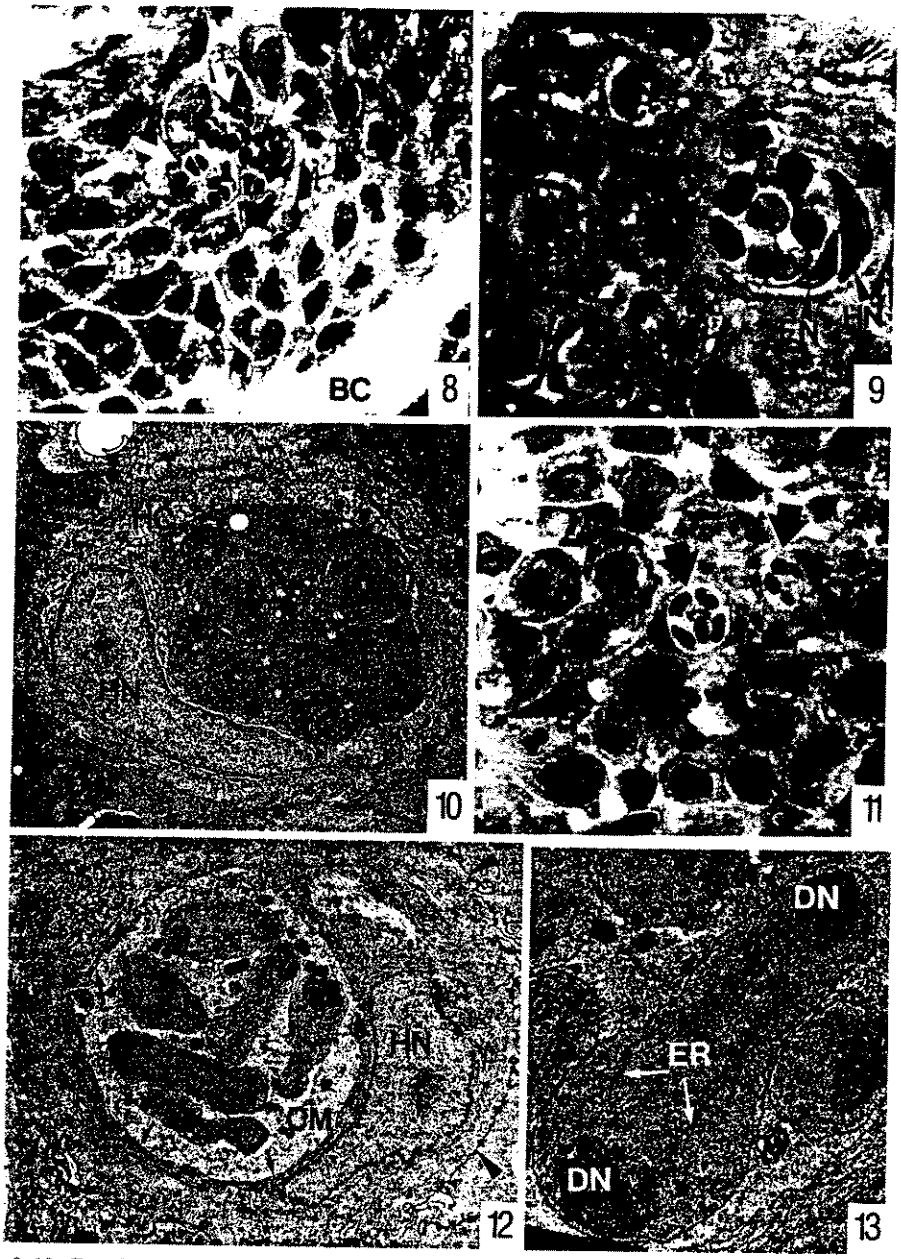
homogeneous nucleoplasm, and a cytoplasm which appeared much denser than that of the surrounding matrix. Moreover, osmiophilic bodies, endoplasmic reticulum and electron-dense mitochondria were observed within these cells. It was evident from serial sections that these intercellular, membrane-bounded aggregates formed pseudopodia-like projections between epidermal cells (Fig. 6).

At 30 min post-exposure, individual aggregates of triactinomyxon sporoplasms were also found in the epithelium of the gills (Fig. 7) and in the skin of all examined trout. However, the greatest number of penetrated triactinomyxon sporoplasms were observed in caudal fin sections, 1 h after initial exposure of trout to waterborne triactinomyxon spores. At this time the sporoplasm cells appeared not only as compact aggregates in the epidermis but also as many smaller clusters of cells, which occurred intracellularly in epithelia (Fig. 8). Each cluster was enveloped by one cell with an eccentric, large nucleus. Infected epithelial cells had a narrow cytoplasmic border and their nucleus was compressed to one side (Figs 9 and 10).

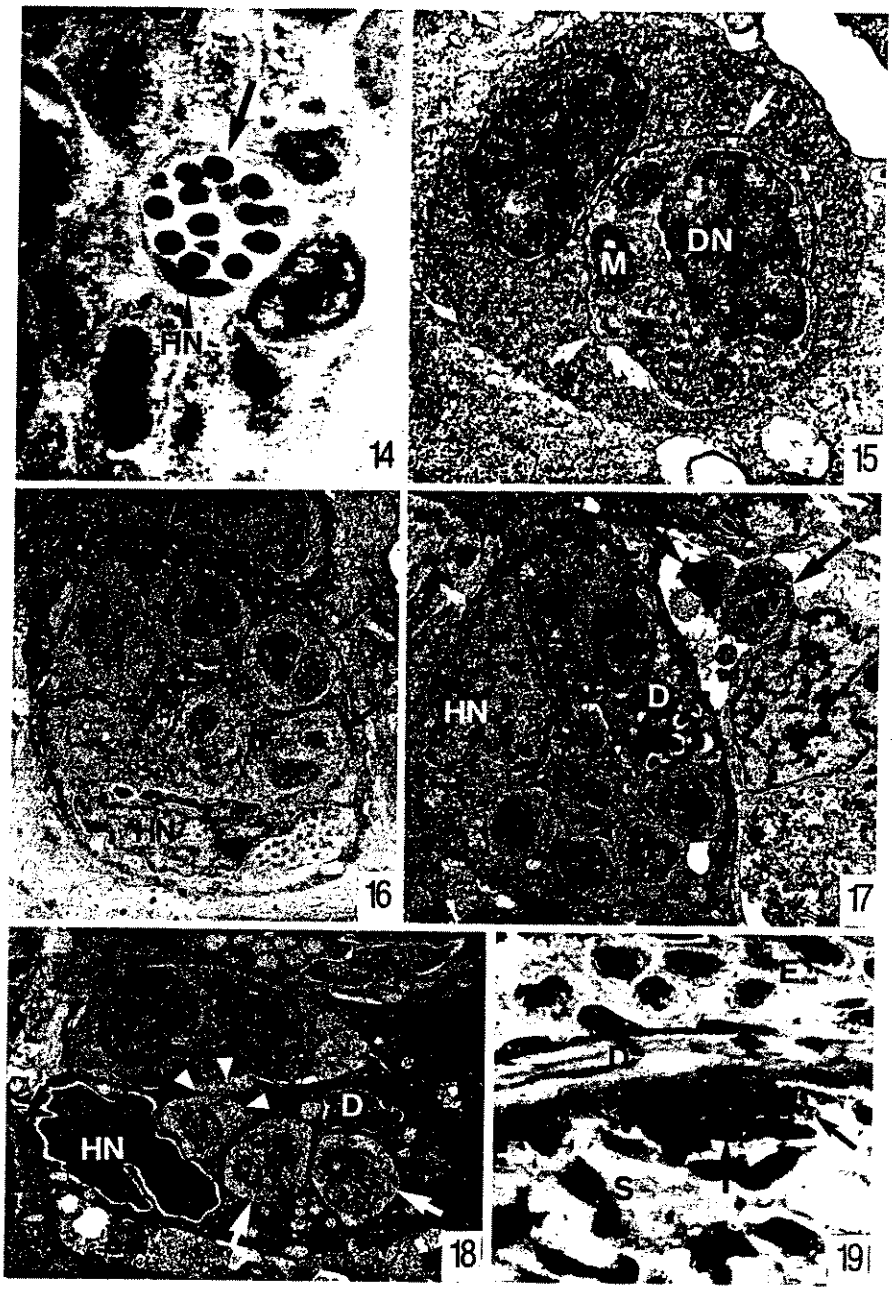
Within 2 h post-exposure some telophase stages of mitosis were observed in sporoplasm aggregates infecting the caudal fin of trout fry. The cells were elongated and contained two intensively stained spots situated one at each pole of the cell. At this time, all intra-epithelial cells appeared to be in a synchronous cell cycle (Fig. 11). Electron microscopic observations revealed that the dividing sporoplasm cells are enveloped by a large cell containing electron-lucent cytoplasmic matrix and are located intracellularly in the epidermis. The epithelial cell nucleus was pushed between the sporoplasm aggregate and the plasmalemma (Fig. 12). The two daughter nuclei of the dividing sporoplasm cells were electron-dense and occurred at opposite sides of the cells in telophase (Fig. 13). Their cytoplasm contained mitochondria and endoplasmic reticulum. Four hours post-exposure, membrane-bound intra-epithelial sporoplasm cells, each containing two nuclei, were observed (Fig. 14). In these cells, endoplasmic reticulum surrounded one of the daughter nuclei and produced the plasmalemma of the envelope cell (Fig. 15).

Fish necropsied 7 h post-exposure showed membrane-bound intraepithelial sporoplasm cells consisting of primary cells each enveloping one secondary cell (Fig. 16). These stages were found 2 h later free in the host cell cytoplasm without a surrounding membrane. Single cell-doublet of these stages was degenerate and did not follow a regular course of development. These degenerate stages showed dilatation of cell organelles and karyolysis (Fig. 17). Intra-epithelial developmental stages are released by rupture of the plasmalemma of the epithelial cell into the intercellular spaces at 8 h post-exposure (Fig. 18). These migrating parasitic stages could be found in trout epidermis until 12 h post-exposure. Between 12 h and 20 h only single, often degenerated stages, were found. Twenty-four hours post-exposure, no stages or aggregates were detectable in the epidermis.

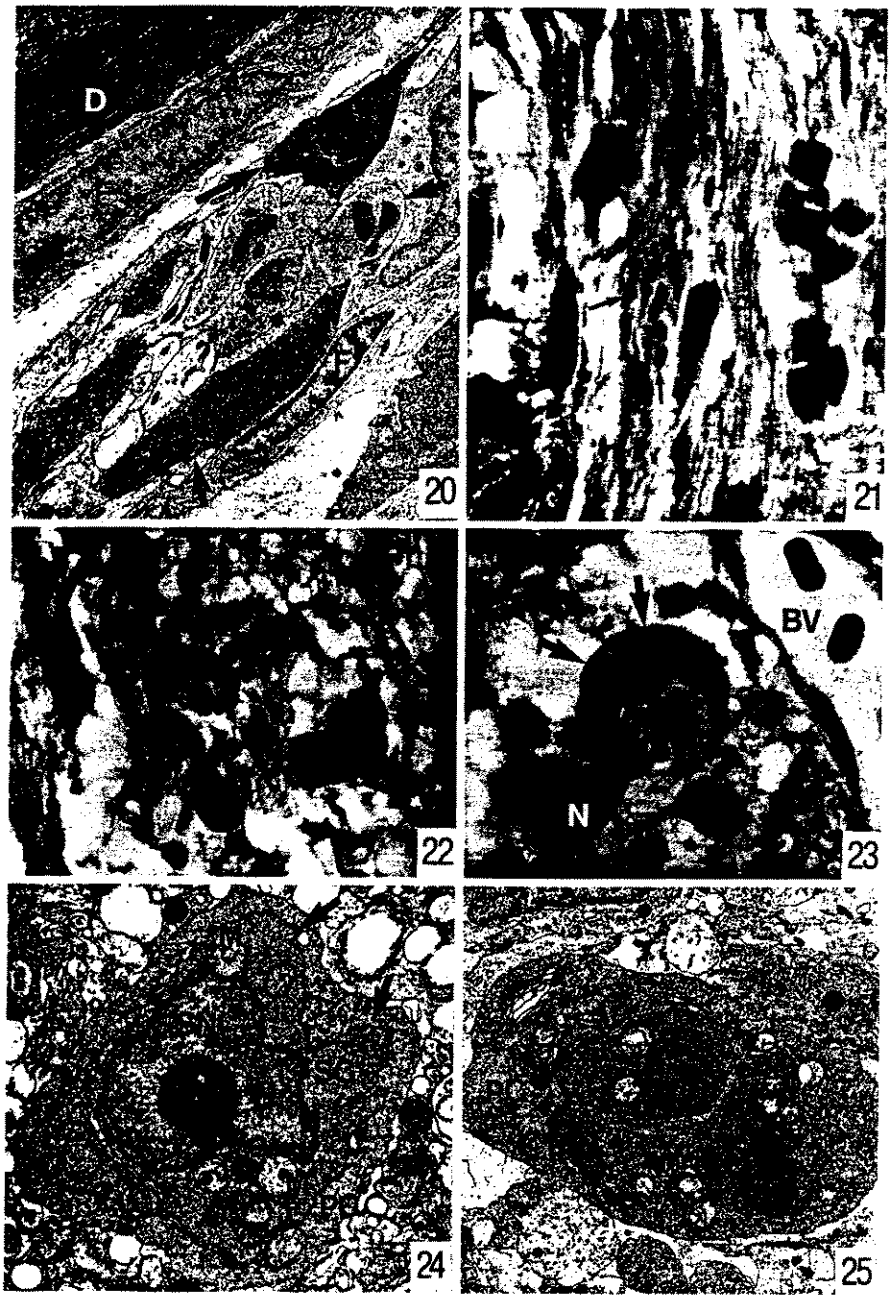
The first observable appearance of developmental stages in the subcutis was 2 days post-exposure. In sections of pectoral fins, compact parasitic aggregates of more than 20 cells were situated in the subcutis (Fig. 19). Each cell of these aggregates included one secondary cell (Fig. 20). The cytoplasm of both cells were electron-dense and contained free ribosomes and mitochondria. These stages in the subcutis were found until 4 days post-exposure. The search for the



FIGS 8–13. FIG. 8. Buccal cavity mucosa (BC) with numerous intracellular aggregates of dark stained sporoplasms (arrows), 1 h post-exposure. Giemsa. × 700. FIG. 9. Part of dorsal skin with one infected epithelial cell; the host cell nucleus (arrow) is compressed to one side of the cell; EN, enveloping cell nucleus; HN, host cell nucleus, 1 h post-exposure. Giemsa. × 1050. FIG. 10. Electronmicrograph of intracellular membrane-bounded sporoplasm in dorsal skin. Note the margination of the host cell cytoplasm (HC) and displacement of the host cell nucleus (HN), 1 h post-exposure. × 4400. FIG. 11. Part of caudal fin with two infected epidermal cells (arrows). All sporoplasm cells showing the telophase of mitosis, 2 h post-exposure. Giemsa. × 700. FIG. 12. Electronmicrograph of dividing sporoplasm cells. The cytoplasmic matrix (CM) of the aggregate contains numerous mitochondria and is less dense than that of the host cell. Note that the sporoplasm cells are surrounded with membrane (arrows) and also the displacement of the host cell nucleus (HN). Host cell plasmalemma (arrowheads) is also evident. × 4400. FIG. 13. Higher magnification of a dividing sporoplasm cell in late telophase. Two daughter nuclei (DN) have formed at each pole and nuclear membranes are reappearing. A narrow cytoplasmic bridge still connects the two daughter cells; ER, endoplasmic reticulum; M, mitochondrium. × 10 000.



FIGS 14–19. FIG. 14. Synchronous endogeneous divisions of intra-epithelial localized sporoplasm cells (arrow) in pectoral fin. Note that all parasitic cells are binucleate, HN, host cell nucleus; 4 h post-exposure. Giemsa, × 1050. FIG. 15. Electronmicrograph of a binucleate cell. Note that vesicles of the endoplasmic reticulum (arrows) envelop one of the daughter nuclei (DN) to produce the plasmalemma of the enveloped cell, M, mitochondrium. × 19 000. FIG. 16. Intra-epithelial membrane-bound sporoplasm cells consisting of cell-doublets (arrow). HN, Host cell nucleus, 7 h post-exposure, × 4400. FIG. 17. Cell-doublets free within the host cell cytoplasm. One showing degeneration process (D); HN, host cell nucleus; arrows indicates extracellular located cell-doublets; 9 h post-exposure, × 4400. FIG. 18. Infected epithelial cell in the dorsal fin containing cell-doublets within the host cell cytoplasm (arrows). Note one unit piercing the host cell plasmalemma to enter the extracellular space (arrowheads). D, Degenerated cell; HN, host cell nucleus; 8 h post-exposure, × 4400. FIG. 19. Aggregates of parasitic stages in the subcutis of the pectoral fin (arrows). E, Epidermis; D, dermis; S, subcutis; 2 days post-exposure, Giemsa, × 700.



FIGS 20–25. FIG. 20. Electronmicrograph of migrating cell-doublets (arrows) in the subcutis. D, Dermis; F, fibrocytes. × 4400. FIG. 21. Spindle-shaped and elongated developmental stages (arrows) between nerve fibres of spinal cord; 4 days post-exposure. Giemsa, × 1050. FIG. 22. Rounded developmental stages (arrows) in the medulla oblongata; 6 days post-exposure. Giemsa, × 1050. FIG. 23. Membrane-bound developmental stages (arrows) in a head ganglion near the auditory organ. N, Nerve cells; BV, blood vessel with erythrocytes; 20 days post-exposure. Giemsa, × 1050. FIG. 24. Cell-doublet consisting of primary cell (PC) and secondary cell (SC) between nerve fibres of spinal cord. Note that the enveloped secondary cell has pseudopodia-like cytoplasmic protrusions (arrows); SN, secondary cell nucleus; M, mitochondria; 14 days post-exposure. × 10 000. FIG. 25. Primary cell (PC) enveloping two secondary cells (SC) in the head ganglia. PN, Primary cell nucleus; M, mitochondria; 16 days post-exposure. × 6100.

presence of developmental stages in the muscles was only once achieved on histological section of head muscle, two cell doublets were detected between muscle bundles.

CENTRAL NERVOUS SYSTEM (CNS)

Four to 24 days post-exposure of trout fry to triactinomyxon spores, different developmental stages of the parasite were detected in nearly all parts of the CNS and in some peripheral nerves. However these stages were found most frequently in the dorsal part of the spinal cord, in the grey matter of the brain and in the head ganglia.

Stages found between nerve fibres of the spinal cord and along peripheral nerves were spindle-shaped and elongate (Fig. 21). They measured 1·5 µm wide and 6·5 µm long. At the transition of the spinal cord to the medulla oblongata the white matter was dispersed, the developmental stages were rounded with a diameter of about 2·6 µm (Fig. 22). Stages observed 14 days post-exposure reached a diameter of 2·8–3·8 µm. Compact aggregates of developmental stages containing numerous parasitic cells were surrounded by a thin membrane. They were detected 20 days post-exposure in the head ganglia near the auditory capsule (Fig. 23).

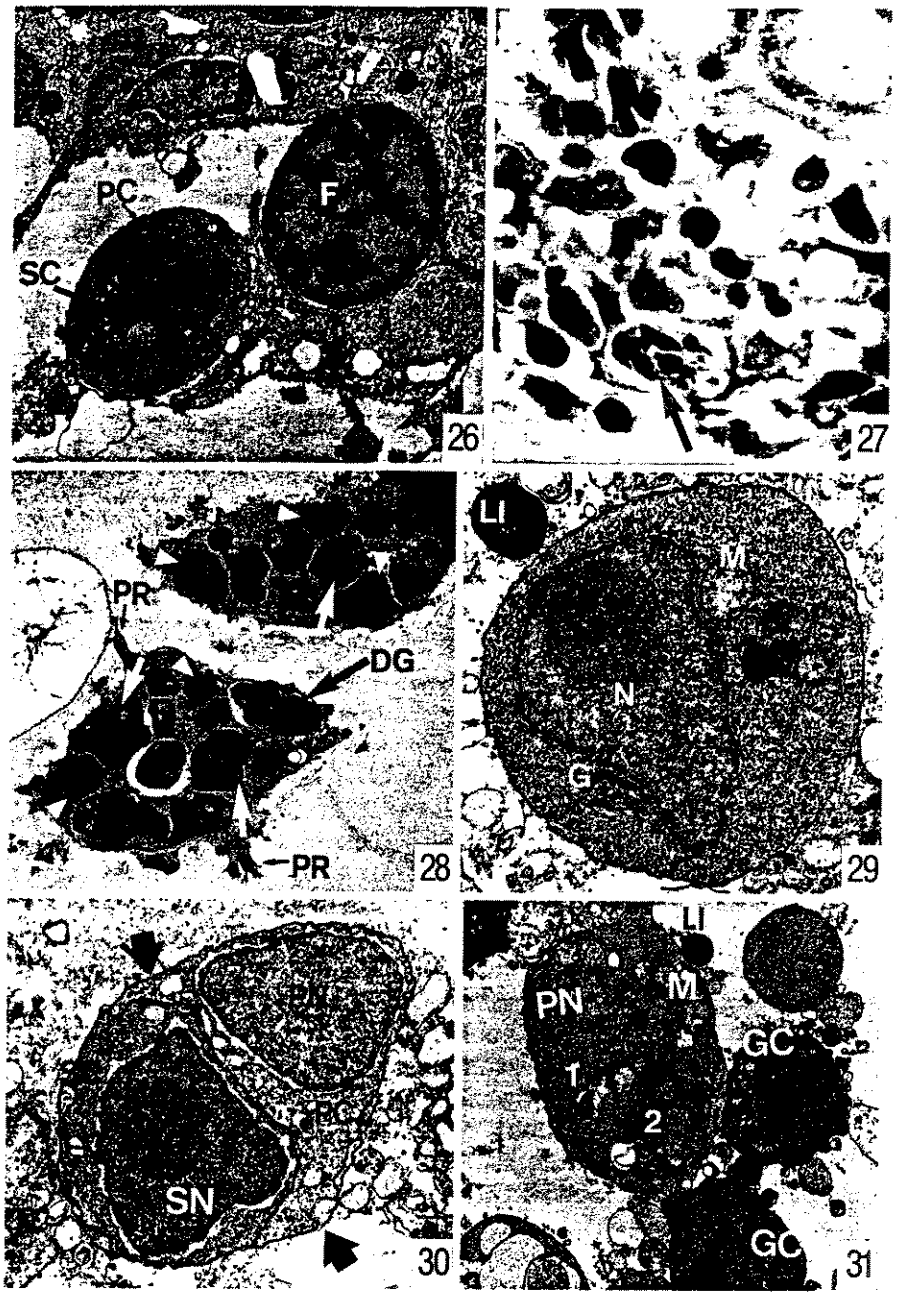
As far as could be concluded ultrastructurally, the earliest developmental stages in the nervous tissues were cell-doublets. The secondary cell of these stages had pseudopodia-like cytoplasmic protrusions (Fig. 24). The nucleus of the secondary cell was remarkably large and had a distinct nucleolus (Fig. 24). In more advanced neural stages, the primary cells contained two or numerous secondary cells inside (Fig. 25). Their cytoplasm included, in contrast to that of the primary cell, numerous free ribosomes so that it has a dense appearance. In addition to free ribosomes, there was rough endoplasmic reticulum and several mitochondria in the cytoplasm of both cell types. Neither tissue reactions nor degeneration of nerve tissues in areas around the parasite stages were recognized.

MENINGES

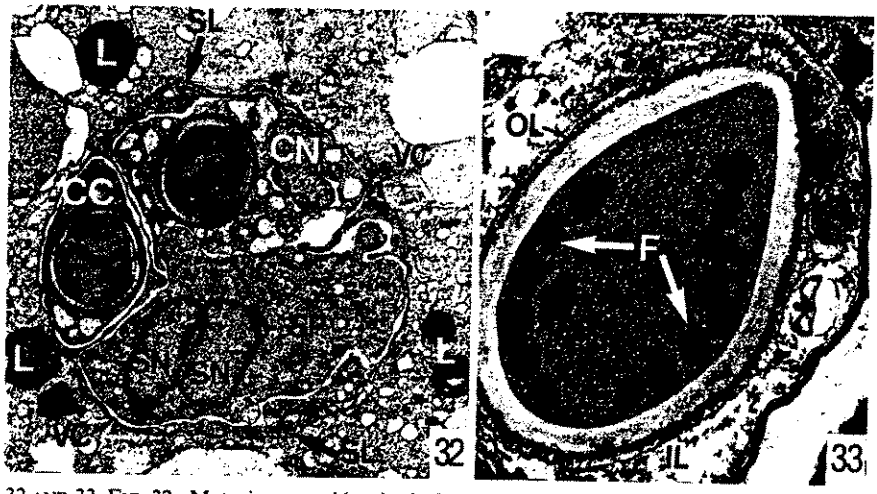
Early developmental stages composed of one secondary cell within the enveloping primary cell resembling those in the nervous tissues were also observed 14 days post-exposure in the connective tissues between the vertebrae of the spinal column (Fig. 26). Twenty-four days post-exposure of triactinomyxon spores to trout, aggregates of parasite stages were also detected in the brain meninges near the head cartilages (Fig. 27).

CARTILAGE

The penetration of parasitic stages in the form of cell-doublets, into head cartilages could be observed 20 days post-exposure. Ultrastructural studies showed that large plasmodia formed pseudopodia projecting into their surrounding cartilages (Fig. 28). The electron lucent cartilage matrix was lysed around the parasites. The plasmodia contained vegetative nuclei and generative cells inside. Some of the generative cells showed late telophase of mitosis. Forty days post-exposure, in addition to plasmodia, there were one-cell stages scattered in the cartilage (Fig. 29). These stages were remarkably similar to the generative cells inside the plasmodia. They had an eccentric nucleolus, and a hyperchromatic nuclear membrane. Adherence of two generative cells (80 days



FIGS 26–31. FIG. 26. Cell-doublet in the connective tissues between the vertebra of spinal column. PC, Primary cell; SC, secondary cell; F, fibrocyte; 14 days post-exposure. × 6100. FIG. 27. Aggregate of parasite stages (arrow) between the fibrocytes of brain meninges. 24 days post-exposure. Giemsa, × 1050. FIG. 28. Two plasmodia in the head cartilage containing generative cells (arrows) and vegetative nuclei (arrow heads). DG, Dividing generative cell. Note the pseudopodia of the plasmodia (PR) and the lysis of the surrounding cartilage; 40 days post-exposure. × 2800. FIG. 29. One-cell-stage in the head cartilage probably released from the plasmodia. N, Excentric nucleus; M, mitochondria; G, Golgi apparatus; LI, lipid inclusion; 64 days post-exposure. × 19 000. FIG. 30. Two generative cells, one electron lucent and just enveloping the other to become the pericyste (PC), the enveloped one is electron dense and becomes the sporogonic cell (SC). Note the cytoplasmic projections of the pericyste surrounding the sporogonic cell (arrows). PN, Pericyste nucleus; SN, sporogonic cell nucleus; 80 days post-exposure. × 19 000. FIG. 31. Early pansporoblast with two sporogonic cells (1 and 2). PN, Pansporoblast cell nucleus; M, mitochondria; GC, free generative cells in the head cartilage; LI, lipid droplet; 110 days post-exposure. × 6100.



FIGS 32 AND 33. FIG. 32. Maturing sporoblast in the head cartilage. SP, Sporoplasm with two nuclei (SN) and the characteristic sporoplasmosomes (arrows); CC, two capsulogenic cells with polar capsules; CN, capsulogenic cell nucleus; VC, two valvogenic cells; SL, sutural line; L, lipid droplet in the surrounding cartilage. × 6100. FIG. 33. Longitudinal section through a maturing polar capsule. Note the seven respectively eight helically arranged coils of the membrane-bound filament (F). OL, IL, Outer and inner layers of the capsular wall. × 19 000.

post-exposure), one just enveloping the other represented the first recognizable step of sporogenesis of *M. cerebralis* (Fig. 30). Subsequent developmental events involved division of the enveloped cell (sporogonic cell) resulting in pansporoblast stages which consisted of 2 to 12 sporogonic cells, all situated within a non-dividing envelope cell (mother cell). A three-cell pansporoblast, apparently resulting from division of the sporogonic cell within an envelope cell is shown in Fig. 31. The cytoplasm of all three cells contained several mitochondria with loose cristae, which were detached from the inner membranes forming circular rings in the matrix.

The sporogenesis of *M. cerebralis* was asynchronous and resulted in the formation of a disporic pansporoblast. In each spore, a pair of valvogenic cells enclosed capsulogenic cells and a sporoplasm and each forms half of the spore valve. Where the valvogenic cells meet, thickened crests from apposing cells are joined by a continuous septate junction forming the sutural ridge and suture line. In more mature spores, discontinuous, electron-dense substances appeared in the cytoplasm of the valvogenic cells (Fig. 32). During the spore maturation, the valvogenic cells form a narrow layer around the spore and their organelles degenerate and become incorporated into the valves. Two capsulogenic cells, each containing a large club-shaped capsular primordium are enclosed by the valvogenic cells. The capsular primordia were enclosed by a membrane which overlies an electron-dense zone followed by an electron lucent one. The base of the primordium is rounded and the narrow end of the apex grows as an elongated, external tube. Longitudinally, as well as transversely, sectioned tubes were filled with a moderately electron-dense, substance. In advanced stages of spore maturation, capsular primordia were transformed to mature polar capsules containing well organized polar filaments. In most sections the filament had seven to eight coils (Fig. 33). As soon as the polar

capsule primordium appeared, the sporoplasm could be recognized easily because of its characteristic formation of cell organelles. The sporoplasm was binucleate (Fig. 32). In mature spores, the sporoplasm filled all of the space beneath both polar capsules and partially extended between them. Several electron-dense bodies (sporoplasmosomes) were always present in the sporoplasm cell (Fig. 32). In all sections, where early and later sporogenesis was observed, round to oval lipid droplets were found in the surrounding cartilage tissue. At this time the chondrocytes were phagocytized by the plasmodia and the cartilage was destroyed.

Examination of blood smears from all necropsied fish did not reveal any myxosporean stages.

DISCUSSION

The ability to produce and enrich the triactinomyxon spores experimentally by tubificid worms made it possible to perform this study. For a period of 70 days about 30 000 to 60 000 triactinomyxon spores could be harvested per day which then were used to infect trout fry.

It has been demonstrated previously that the actinosporean triactinomyxon initiates whirling disease (Wolf & Markiw, 1984; El-Matbouli & Hoffmann, 1989). Regarding the portals of entry for salmonid whirling disease, Markiw (1989) and El-Matbouli *et al.* (1992*a*) showed that the triactinomyxon sporoplasm penetrated the epidermis of exposed trout less than 10 min after contact. However, the role of the polar capsule-filaments, the transfer mechanism and the further development remained unexplained. The present study corroborates these findings further. In addition, it describes the route and development of penetrated triactinomyxon sporoplasms into the cartilage.

Within 5 min post-exposure, the triactinomyxon spores begin to attach to the epidermis of the trout. At this point the triactinomyxon spores extrude their polar filaments into the epidermis. Figure 4 reveals that the purpose of the polar filaments is to anchor the spore during the penetration of the sporoplasm into the epidermis of trout. However the mechanism that causes the spores to extrude their polar filaments is still unknown. According to the host specificity, triactinomyxon spores of whirling disease recognize specifically their final host. In an unpublished study, we observed that the epidermis of cyprinid fish (*Cyprinus carpio* L. & *Carassius auratus* L.) exposed to triactinomyxon spores of *M. cerebralis* were free from sporoplasm aggregates and the triactinomyxon spores remained intact. How the initial stage is able to identify the fish host is enigmatic, though presumably through chemotaxis. The results of this study are summarized in Fig. 34. The developmental sequence of the agent of whirling disease in the fish host is as follows.

Infection of trout begins as the triactinomyxon-sporoplasms penetrate the epidermis. During the first 60 min following penetration, the sporoplasm aggregates remained compact and migrated intercellularly in the epidermis respectively gill epithelium. This is in contrast to Daniels *et al.* (1976) and Markiw (1989) who reported only intracellular occurrence of the parasite stages at this time. After 60 min, the sporoplasm-enveloping cell disintegrates and each sporoplasm cell penetrates an epidermal or gill epithelial cell. The first division

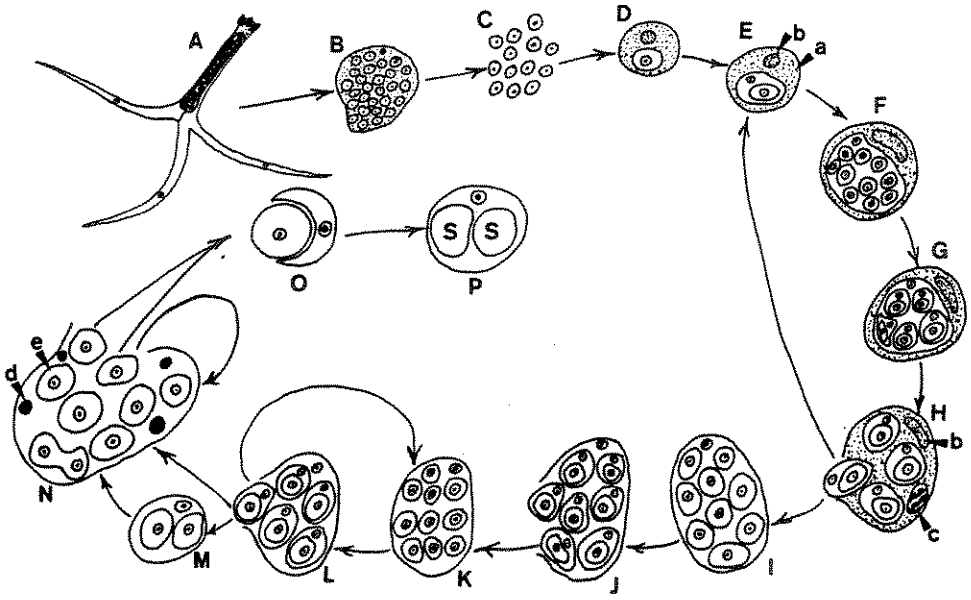


FIG. 34. Life cycle of *Myxobolus cerebralis* within the fish host (rainbow trout). B to M: Presporogonic phase of the life cycle. N to P: Sporogonic phase. B and C, Occurring inter-epithelial. D to H, Intra-epithelial. I and J, Intercellular in the subcutis. K to M, In nervous tissue. M to P, In the cartilage. A, Waterborne triactinomyxon spore produced in *Tubifex tubifex*. B, Compact triactinomyxon sporoplasm between epithelial cells. C, Interepithelial free sporoplasm cells, each penetrates an epithelial cell (D). E, Intracellular endogeneous cell division of the parasite producing primary and secondary cells. F, Numerous secondary cells resulting from mitotic divisions. G, Endogeneous division of the secondary cells producing cell-doublets. H, Cell-doublets rupture the aggregate membrane and pierce the host cell plasmalemma, then penetrate other epithelial cells or migrate deeper into the dermis and subcutis. I, Intercellular growth of the cell-doublets in the subcutis through mitosis. J, Endogeneous divisions in the subcutis. K and L, Similar to I and J but in nervous tissue. M, Triplet in CNS and cartilage. N, Plasmodium in head cartilage. O and P, Formation of pansporoblast arising from the unification of two cells, pericyste and sporogonic cell. S, Sporoblast. a, Host epithelial cell, b, host cell nucleus, c, degenerated cell-doublet, d, vegetative nucleus, e, generative cell.

of these now intracellular localized sporoplasm cells is an endogeneous cleavage producing within the enveloping cell (primary cell), an inner one (secondary cell). A series of rapid synchronous mitotic divisions occurs resulting in an increase in the number of secondary cells. At this time (2 h post-penetration) intracellularly localized enveloped groups of parasitic stages were recognizable in sections scattered in three to five neighbouring epithelial cells of skin and fins. A nuclear division of the enveloped secondary cells, in which vesicles of the endoplasmic reticulum surround one of the daughter nuclei, occurs to produce the plasmalemma of the tertiary cell. These intra-epithelial membrane-bound parasitic stages, composed of cell-doublets, are comparable with the blood stages of *Sphaerospora* species described by Lom *et al.* (1983, 1985), Molnar (1988), Baska & Molnar (1988), Dykova *et al.* (1990) and Suppamattaya *et al.* (1993). The presence of stages of enveloping (primary) cells containing enveloped (secondary) ones are considered as the most important characteristic of myxosporeans (Lom, 1987).

Following formation of cell-doublets within the membrane-bounded intra-epithelial aggregates, these cell-doublets rupture the aggregate membrane and

enter the host cell cytoplasm. At this time, single cell-doublets seemed to be destroyed within the cytoplasm of the host epithelial cell. Daniels *et al.* (1976) also reported small parasitic cells within the cytoplasm of phagocytic cells in rainbow trout. When all cell-doublets are free within the host cell cytoplasm, they pierce the host cell plasmalemma and enter the extracellular space (Fig. 18). These now extracellularly situated cell-doublets may penetrate neighbouring epithelial cells or may migrate deeper into the dermis and subcutis layers and penetrate new host cells, where they start the cycle all over again.

In the subcutis, aggregates of more than 30 cell-doublets were observed 2 days post-exposure. While the number of observed parasitic stages in the subcutis declined until 4 days post-exposure, they increased considerably in the nervous tissue especially of the central nervous system. Most parasitic stages were found in the spinal cord between 6 and 14 days post-exposure, and in brain between 16 and 24 days. Apparently a rising migration of the parasite stages into the cranial part of the central nervous system occurred. In any case the parasite stages were found interaxonally in the nerve tissues in the form of free as well as of membrane-bound aggregates of cell-doublets. These cell-doublets repeat the cycle in the subcutis and nerve tissues, but only intercellularly.

The lack of tissue reaction in the nervous system, in contrast to the epidermis, is not surprising, even if the parasite acts as a foreign body; however, no evidence could be found for contact of the parasite with the blood or immunocompetent cells, which could trigger an immune response. Therefore, the migration of the parasite via the nervous system to the cartilage is understandable. Careful examination of light and electron microscopic sections revealed that entry of the parasite stages into the central nervous system occurred along the peripheral nerves.

Stensaas *et al.* (1967) showed light and electron micrographs of similar aggregates in the axon of toad *Bufo arenarium*. However, they suggested that the observed organism was probably a sporozoan of the order Eucoccidia; further identification of the parasite was not attempted. Ferguson *et al.* (1985) also observed intra-axonal aggregates in the common shiner *Notropis cornutus* (Mitchill), which had a typical myxosporean structure of a primary cell containing a secondary cell. They assumed that these organisms might represent presporogonic stages of a concurrent *Myxobolus* sp. infection in the muscles.

Occurrence of myxosporean sporogenesis in the CNS of fish were studied ultrastructurally in fathead minnow *Pimephales promelas* Rafinesque (Mitchell *et al.*, 1985) and in bullhead *Cottus gobio* L. (Lom *et al.*, 1989; El-Matbouli *et al.*, 1990). A typical observation is the lack of cellular reaction in myxosporeans affecting the CNS (Hoffmann *et al.*, 1991). Most of these myxosporeans finish sporogenesis in the CNS in contrast of *M. cerebralis*.

In whirling disease transfer of the parasitic cell-doublets from nervous tissue into the cartilage was observed 20 days post-exposure. The primary cell grows and its nucleus divides to produce numerous vegetative nuclei, whereas the enveloped cell divides to produce many cells termed generative cells (Fig. 28). This stage is called a plasmodium. According to our observations, after disintegration of the enveloping cell, each enveloped cell either repeats the cycle producing numerous plasmodia or unite with another cell (Fig. 30), one becoming the pericyste envelope and the inner one forming the sporogonic cell; this unit generates pansporoblasts with two spores.

The general events of sporogenesis and the features of the pansporoblast comply with data existing on other *Myxobolus* species. In addition to the sporogonic phase of *M. cerebralis* occurring in the cartilage, this study also demonstrates the existence of purely proliferative and multiplicative phases in the epidermis and CNS of rainbow trouts. Since these multiplicative and proliferative phases take place before the sporogonic phase starts, we prefer to call it the presporogonic phase. The fact, that *M. cerebralis* uses the CNS for its development is also of interest with regard to the nomenclature. Whereas Hofer (1903) chose the species name ' cerebralis ' supposing spores could be situated in the brain, he wanted to change it into ' chondrophagus ' when Plehn convinced him of the real localization of mature spores in cartilage (Hofer, 1904). However, the present study proves that the CNS is in fact involved in the development of *M. cerebralis* and thus the name can be regarded as correct.

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Whirling Disease in Trout

WHIRLING disease, long recognized as a serious problem in the intensive rearing of salmonid fish in Europe, has now been found in Scotland. In October 1968, the myxosporidian protozoan parasite *Myxosoma cerebralis*, the aetiological agent of the disease, was identified in rainbow trout (*Salmo gairdnerii*) from two trout farms. In recent years the disease has appeared in North America, USSR and Japan, but the causative parasite has not previously been identified in Great Britain.

The following symptoms (which have been described previously[1–4]) were observed in the thirty-nine 6–8 month old rainbow trout examined: blackened tails, spinal curvature and exhaustive tail chasing movements; at the same time mortalities occurred. Microscopical preparations of wet mounts of head cartilage (Fig. 1) revealed the presence of the spores of *M. cerebralis* from which polar filaments were extruded following the application of 5 per cent NaOH to the wet mount material. Further, numerous spores were found in sections of head cartilage (Fig. 2). Infection occurs during the first year of life[2], usually in the first few weeks of active feeding and before the formation of bone; at this stage the cartilage is most susceptible and the effects most severe. Survivors become carriers of the disease as spores of the myxosporidian remain in the tissues for up to three years.

It is possible that the disease already existed in wild populations before the present diagnosis, but it is also possible that spores of *M. cerebralis* may have been introduced to Scotland via egg packing materials. In the United States the authorities consider that samples of frozen table fish, which were either accidentally fed or their viscera discarded into streams, were the direct cause of the initial outbreak in North America. In the four months subsequent to this first record of an outbreak of whirling disease in Scotland, the parasite was found in trout from two other fish farms and, on a symptomatic basis, it is not impossible that a further two fish farms are also affected. This illustrates how rapidly a previously unrecognized disease, which may or may not have already existed in the wild in Britain, appeared over a period of months in several widely spread fish farms apparently from a single source.

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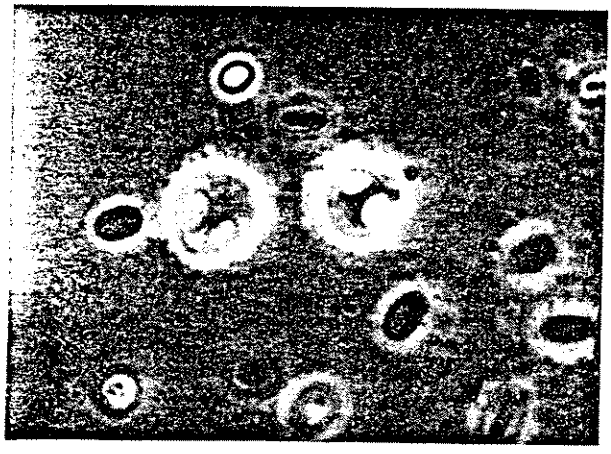

Fig. 1. Photomicrograph showing two spores of *Myxosoma cerebralis* from posterior part of the cranium. Wet mount by phase contrast microscopy (× 1,200).

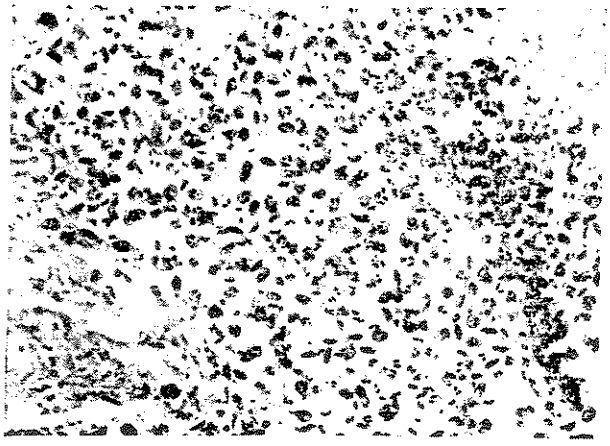

Fig. 2. Photomicrograph of *Myxosoma cerebralis* in cartilage of young Rainbow trout. Giemsa's stain (× 250).

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REVIEW

THE MYXOSPOREA OF FISH: A REVIEW

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INTRODUCTION

Fish farming is one of the fastest growing agricultural industries in Britain today. Over 30 000 tonnes of salmon and 15 000 tonnes of trout are produced annually by fish farms in the United Kingdom, representing over 50 million individual fish (MAFF figures). Similarly, the intensive culture of carp in Europe has shown tremendous growth in recent years. The Myxosporea, which are protozoal parasites of the phylum Myxozoa, cause a number of fatal diseases of fish, important both for their economic and welfare implications (Ferguson, 1988). This review describes the present views on the biology of the Myxosporea, and considers the more important myxosporean diseases in some detail.

MYXOSPOREAN LIFE CYCLES

The Myxosporea are transmitted from host to host by means of spores, multicellular spheroid structures of approximate diameter 10 μm. These spores have a characteristic morphology, which forms the basis of the taxonomy of the Myxosporea. Each comprises up to six polar capsules—typically two—containing extrusible coiled filaments; an infective sporoplasm, either single and binucleate or double and uninucleate; and a shell encasing the whole, which may have projections leading to distinctive shapes, or a mucous coat which aids in flotation (Fig. 1).

The pathogenic potential of Myxosporea was recognized at the beginning of this century, when spores had been identified as the transmissible agents of myxosporean disease. It was also known that cyst-like formations up to several millimetres in diameter (found in various tissues such as kidney, liver and spleen) were responsible for the acute inflammatory and granulomatous tissue responses which attended heavy myxosporean infections. These cyst-like structures, representing a trophic phase of the life cycle, contained numerous mature spores.

About 70 papers were devoted to myxosporean life cycles in fish hosts, often proposing complicated schemes which included alleged gamete formation (see review by Lom, 1987). However, electron microscopy revealed that the pre-spore stage of the life cycle was formed by the union of two generative cells, and photometry appeared to confirm that autogamy—fusion of haploid nuclei in the sporoplasm—was a primitive, and the only, sexual process in the myxosporean life cycle. The known facts fitted a simple life cycle, thought to be common to all

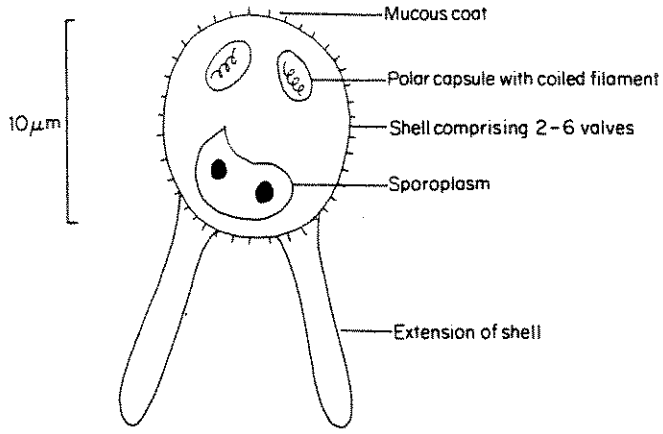

Fig. 1. Diagrammatic representation of a myxosporean spore.

Myxosporea. This life cycle, described below, remained unchallenged until the late 1970s (Fig. 2).

Spores were thought to be ingested by the host fish, and to hatch in its digestive tract. Hatching involved the violent uncoiling of the polar filaments, fracturing of the spore shell, and release of an amoeboid sporoplasm into the gastrointestinal lumen. The sporoplasm would penetrate the epithelium of the gastrointestinal wall and undergo the process of autogamy to produce a trophozoite. The trophozoite was thought to reach the final site of infection in an unknown way, and to become a multicellular sporogonic (spore-forming) structure called the plasmodium; the plasmodium was in fact the cyst-like structure described by earlier workers. Formation of the plasmodium involved the process of endodyogeny, or 'internal budding', giving rise to two populations of nuclei—one present freely within the cytoplasm of the primary host cell (vegetative nuclei), and the other present within secondary cells which were themselves contained within the cytoplasm of the primary cell (proliferative cells). (See inset, Fig. 2, for diagram illustrating endodyogeny; this arrangement of cells enveloped one within the other is a characteristic feature of the Myxosporea.)

Adjacent generative cells would then unite, one enveloping the other. A pansporoblast, or spore-forming structure, would thus be formed, the inner (sporogonic) cell dividing to give the number of cells necessary to complete two sporoblasts, which then matured to become spores—this process involving a one-step meiosis to reach the haploid state. The life cycle was then completed either by the rupture of plasmodia and the liberation of spores into the environment (in the case of superficial lesions), or by the ingestion of tissues containing infective plasmodia following death of the host or the act of cannibalism (deeper lesions).

This life cycle was typical of *Myxobolus cerebralis*, a species known to be responsible for Whirling disease of young salmonids (considered later). Certain other species, including *Sphaerospora renicola*, did not conform to this pattern, producing small plasmodia—or pseudoplasmodia—comprising just one vegetative nucleus that produced one or two spores only. In yet other species, again including

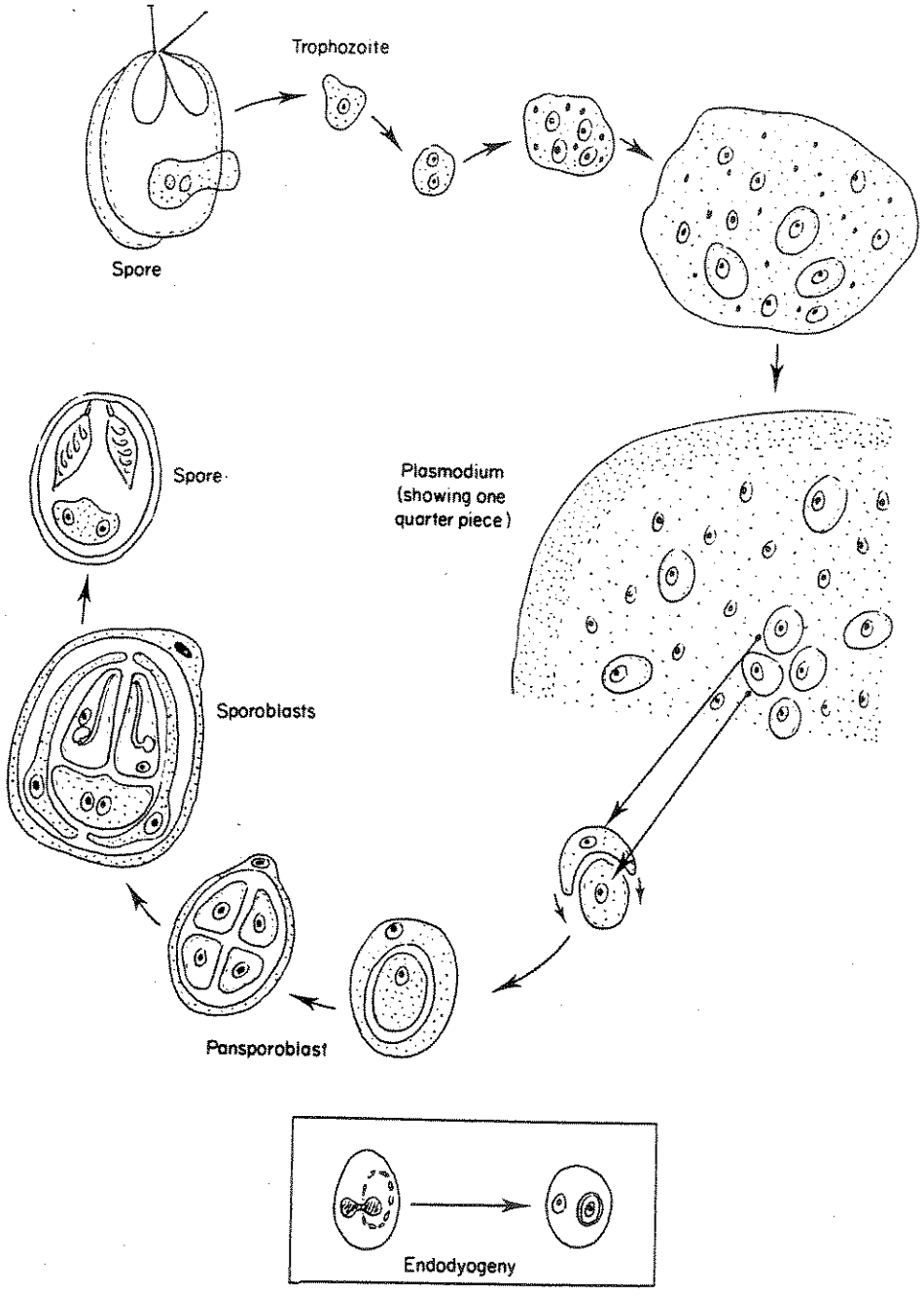

Fig. 2. A conventional idea of a myxosporean life cycle.

S. renicola, spores were produced without pansporoblasts by direct division of generative cells. These differences, however, were considered to involve minor details only, and the 'conventional idea' of the life cycle remained unchallenged for several years.

CONTROVERSIAL FINDINGS FUEL A HEATED DEBATE

It was not until the early 1980s that the 'conventional idea' of the myxosporean life cycle was called into question. Wolf *et al.* (1986) presented some remarkable data on the life cycle of *Myxobolus cerebralis*, which could—if confirmed—lead to a complete revision of the current general concept of the life cycle and taxonomy of the Myxosporea.

It had been known for some time that orally administered spores of *M. cerebralis* would fail to produce infection in experimental fish unless the spores underwent an extended period of 'maturation' at the bottom of earthen ponds. Wolf *et al.* (1986) explained this observation by suggesting the necessity of an oligochaete intermediate host, which inhabits mud at the bottom of ponds. Hence, the worm *Tubifex tubifex* was reported to ingest spores of *M. cerebralis;* these spores were observed to develop into a *Triactinomyxon*, an organism belonging to another class of the phylum Myxozoa, the Actinosporea; and only spores produced by this organism, when released from the worm and ingested by the experimental fish, would initiate a *M. cerebralis* infection.

These findings revealed an unparalleled case of an organism alternating two different life cycles, each with its own sexual and asexual processes occurring at different points in the cycle. Clearly, if these findings could be confirmed, and this alternation of life cycles demonstrated in other myxosporean species, the ramifications would be great indeed.

'UNIDENTIFIED BLOOD ORGANISMS'

In 1976, a fish pathologist in Hungary reported a curious organism in the blood of fingerlings of common carp (*Cyprinus carpio*). Csaba (1976) found amoeboid organisms which showed continuous rotating movements, each organism consisting of a cell with up to eight inner cells. These organisms seemed to differ from all other protozoa—including apicomplexans, myxozoans and haplosporidians—and so were called 'unidentified blood organisms' (UBOs), or 'Protozoa' (from Csaba). Subsequent epizootiological and ultrastructural studies have indicated that these UBOs may represent a proliferative phase of the life cycle of *Sphaerospora renicola*, a common myxosporean parasite of carp in intensive culture. UBOs were also found in other fish species harbouring other species of *Sphaerospora*, and it came to be realized that the myxosporean life cycle was more than just a cycle of sporogenesis, as described in the 'conventional idea' mentioned earlier: a proliferative, or extrasporogonic, phase also existed, destined to increasing the number of parasites in the host. This phase was thought to occur both before and during the sporogonic stages.

Csaba's initial observations provide the basis of the modern concept of extra-

sporogonic development: namely, that the cycle always begins with a primary cell containing a secondary cell, such as is produced by the released sporoplasm; that the secondary cells undergo repeated divisions within the growing primary cell; that tertiary cells are formed by the endogenous division of secondary cells; and that the cycle begins afresh when the primary cell eventually disintegrates, thereby releasing the secondary and tertiary cells (which respectively form the primary and secondary cells of the subsequent cycle). These events are illustrated in Fig. 3.

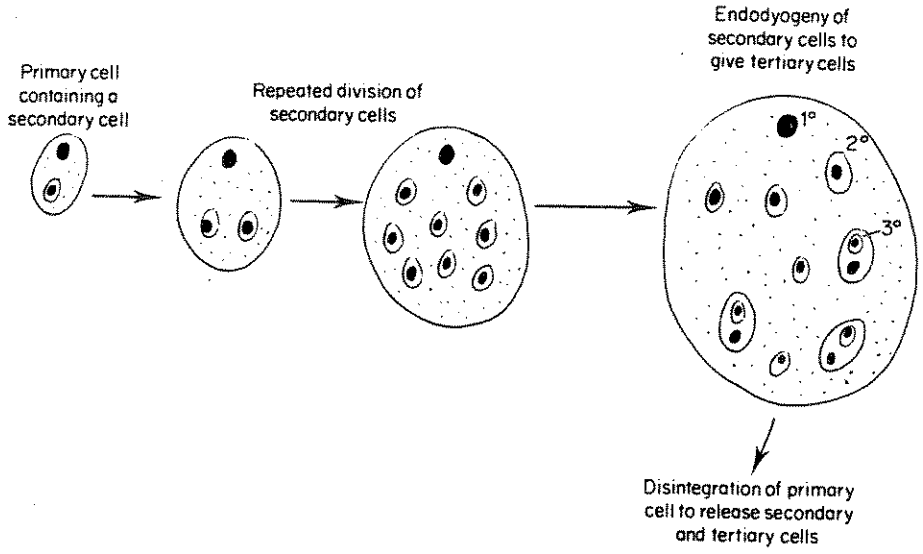

Fig. 3. Extrasporogonic development of the Myxosporea.

MYXOSPOREAN DISEASES

The myxosporeoses are as varied as they are numerous. Both sporogonic and extrasporogonic stages of the myxosporean life cycle are known to cause disease, and often myxosporean diseases are fatal (Ferguson, 1988).

Whirling disease

Whirling disease is a disease of young salmonids which occurs throughout the world, and in Britain is notifiable (Hoffman, 1990). It is characterized by anomalies of movement—in particular, a 'whirling' motion of fish—and is caused by a sporogonic stage of *Myxobolus cerebralis* (see earlier). The plasmodium of this myxosporean parasite digests the cartilage of the cranium and the spine of its host, destroying the labyrinth organ and causing spinal abnormalities; these lesions cause the loss of balance and erratic swimming characteristic of this disease. A granulomatous inflammatory response is established, and death follows anorexia due to an inability to catch and prehend food, or through cannibalism of affected fish. Fish over 6 months old do not develop the disease because the

plasmodia of *M. cerebralis* are unable to penetrate cartilage which has ossified. This disease is amenable to control since the transmission of *M. cerebralis* depends on development within an intermediate host, *Tubifex tubifex*, which lives in mud at the bottom of earthen ponds (Wolf *et al.*, 1986); control is therefore easily achieved by rearing fish in concrete ponds until older than 6 months. Contaminated earthen ponds must be drained and sterilized with calcium hydroxide before the reintroduction of fish. An alternative method of control is to kill the spores before they reach the fish by sterilizing the water flowing into the farm with ultraviolet light, although this is often not possible for economic and, or, practical reasons. In addition, the sterilizing efficacy of ultraviolet lamps deteriorates rapidly with time, necessitating regular changes of bulbs.

Studies carried out by Schmahl *et al.* (1989) have indicated that the symmetric triazinone drug, toltrazuril, has activity against all the developmental stages of *Myxobolus* sp. except the mature spores. Bream (*Abramis brama*) were naturally infected with *Myxobolus* sp. from the River Ruhr and the Lake of Kemnade, and were put into aquaria containing various concentrations of toltrazuril for periods of 2 or 4 h. Transmission electron microscopy of their tissues revealed that the sporogonic stages of the parasite suffered most damage following treatment with 10 μg toltrazuril/ml for 4 h. Since the drug was not found to have activity against spores, repeat treatments would be necessary after 2 or 3 months for effective control. These results offer exciting possibilities for the control of *M. cerebralis* in salmonids: although toltrazuril is not currently licensed for use in fish in the United Kingdom, this does not preclude its use by the veterinary surgeon for fish under his care.

More recently, equally encouraging results have been obtained by medication with Fumagillin dicyclohexylamine (DCH) (El-Matbouli & Hoffmann, 1991). Rainbow trout fry (*Oncorhynchus mykiss*) were exposed for 14 days to mesh capsules of tubificid worms heavily infected with *Triactinomyxon*; 30 days after the introduction of the worms (16 days after their removal), the fry were divided into two groups of 30, kept separately in 50-l tanks; one group received medicated pellets containing 0.1% Fumagillin DCH at a rate of 1.0% of body weight per day, while the other received the normal diet. Control groups of specific pathogen free (SPF) fish were also put onto these feeding regimens. Whereas 73.3–100% of non-medicated fish in two experimental series suffered severe infection, only 10–20% of medicated trout harboured spores of *Myxobolus cerebralis*, and then only to a very low degree (El-Matbouli & Hoffmann, 1991). In addition, spores from trout treated with Fumagillin DCH had distinct morphological defects, in contrast to those from medicated fish. El-Matbouli & Hoffmann (1991) therefore concluded that Fumagillin DCH seemed to be an effective drug for the control of whirling disease in salmonid fish. Like toltrazuril, Fumagillin DCH lacks a product licence for use in fish, but may nevertheless be prescribed by a veterinary surgeon for fish under his care. Definitive diagnosis of whirling disease depends upon the demonstration of spores within the cranial cartilage, which can be achieved by making a crush preparation of cartilage in water or saline (Bullock, 1989).

Renal sphaerosporosis

Renal sphaerosporosis is one manifestation of a disease caused by infection of

young cyprinids with *Sphaerospora renicola* (Lom *et al.*, 1985). Sporogonic pseudoplasmodia of this species develop within the renal tubules, causing distension of their lumina with consequent atrophy and necrosis of the tubular epithelium. These changes impair the excretory and haemopoietic functions of the kidney, rapidly leading to death. Sphaerosporosis caused by *S. renicola* has been reported in Hungary, Czechoslovakia, Germany, Poland, Bulgaria, Yugoslavia, the Union of Soviet Socialist Republics, and Israel (Dykova & Lom, 1988).

Another manifestation of sphaerosporosis, caused by an extrasporogonic stage of *S. renicola*, is *swimbladder inflammation*. This disease is seen in young cyprinids in their first or second summer, often in July, and is characterized by erratic swimming of affected fish on their sides, often in circles as they rise to shallower water. An extrasporogonic swimbladder infective agent (SBI)—consisting of primary, secondary and tertiary cells—develops within the wall of the swimbladder, leading to an exudative, proliferative inflammatory reaction. Thus, there is oedema of the connective tissue, infiltration of lymphocytes, haemorrhage, and hyperplasia of the lamina propria. This disease can be fatal, especially in 2–4-month old fry. It has previously been confused with spring viraemia of carp and ascites of bacterial origin (the former notifiable in Britain) (Ferguson, 1988).

Blood sphaerosporosis is a third clinical manifestation of infection with *S. renicola*. It is characterized by the presence in circulating blood of extrasporogonic stages of the parasite, the 'C-protozoa' of Csaba (see earlier) (Csaba, 1976). The pathogenicity of C-protozoa is currently unknown.

The final clinical manifestation of infection with *S. renicola* involves the intracellular development of extrasporogonic stages within renal tubular epithelium. Infected epithelial cells become hypertrophic, fusing to give rise to a syncytium which assumes the form of a 'cyst' of parasites. These extrasporogonic forms become structurally complex, but are then spontaneously degraded, becoming non-viable and eventually being eliminated from the host tissues. They thus seem to represent a 'blind alley' in the development of *S. renicola* but may nevertheless seriously compromise the excretory and haemopoietic functions of the kidney during their long span of development. While the blood and sporogonic forms are prevalent from spring through to the autumn, the intracellular forms are prevalent in the winter.

In summary, transmission of the species is achieved by the ingestion of spores which initiate an infection which is manifest in the blood and, or kidneys. While much is known about the individual clinical manifestations of sphaerosporosis, the true sequence of infection sites is not yet fully understood. A definitive diagnosis of this disease can be made by examining Giemsa-stained blood smears, which may show a variety of extrasporogonic forms from as early as 4 weeks after hatching in carp fry (Lom *et al.*, 1985). The possible treatment of *S. renicola* infections has been explored in recent years by Cirkovic (1986) and by Molnar *et al.* (1987). Cirkovic (1986) succeeded in preventing the outbreak of swimbladder sphaerosporosis in carp fry by administering food medicated with nitrofurazone: a concentration of 0.6 g/kg feed was used during the period from 14 to 29 days post-hatching, then raised to 2.0 g/kg until day 60 post-hatching, with two 7-day breaks. With equally encouraging results, Molnar *et al.* (1987) were able to prevent renal sphaerosporosis following the intraperitoneal injection of infective swimbladder

homogenate by feeding their fish a diet containing Fumagillin DCH at a concentration of 1.0 g/kg feed. In addition, the prevalence and intensity of spontaneous renal and swimbladder sphaerosporosis were greatly reduced in carp fry treated with Fumagillin DCH. Neither nitrofurazone nor Fumagillin DCH is currently licensed for use in fish in the United Kingdom, though this should not preclude their use by a veterinary surgeon for fish under his care.

Proliferative kidney disease

Proliferative kidney disease (PKD) is a disease of salmonids which has been reported throughout Europe and in North America (Clifton-Hadley *et al.*, 1984). It is a seasonal disease which in Europe reaches peak prevalence in July and August. Clinical signs become evident when water temperatures reach or exceed 15 °C, and include drowsiness, exophthalmia and anaemia, the last responsible for the high mortalities often experienced on commercial fish farms. Mortalities throughout the summer season may reach 90%, but usually remain below 50% (Clifton-Hadley *et al.*, 1984). Even in the absence of high mortality, morbidity rates may reach 100%, and fish with PKD show reduced food conversion, poor tolerance of environmental stress, and greater susceptibility to secondary infection (Clifton-Hadley *et al.*, 1984). Extrasporogonic PKX cells of *Myxidium lieberkuhni* develop in the lumina of renal tubules, inducing a granulomatous interstitial nephritis and tubular atrophy (Kent & Hendrick, 1986).* In heavily infected fish, PKX may also be found in the spleen, intestine, gills, liver and muscle, where it similarly invokes a granulomatous response. The PKX parasite most likely reaches these organs via the circulatory system and may adhere to vessel walls, provoking a necrotizing vasculitis. If fish survive PKD, the interstitial hyperplasia subsides in 12–20 weeks, and kidneys of recovered fish show little if any signs of previous infection. Such fish develop a solid immunity to the disease.

Two drugs, malachite green and Fumagillin DCH, have been shown to have efficacy against PKD (Clifton-Hadley & Alderman, 1987; Hendrick *et al.*, 1988a). Malachite green is an organic dye used in aquaculture as an ectoparasiticide and external fungicide on fish and fish eggs (Alderman, 1985). Clifton-Hadley & Alderman (1987) discovered that when rainbow trout *(Salmo gairdneri)* infected with PKX were treated with malachite green and formalin to cure an incidental infection with the ciliate *Ichthyophthirius multifiliis*, the subsequent development of PKD was delayed. Further experiments indicated that the therapeutic effect of the drug combination against PKD was due to the malachite green, and that repeated flush treatments using 1.0 p.p.m. malachite green eliminated PKX cells from the kidneys of clinically affected fish (Clifton-Hadley & Alderman, 1987). The results of preliminary studies on the pharmacokinetics of malachite green in rainbow trout were also mentioned by Clifton-Hadley & Alderman (1987) in the same paper; it was observed that the drug accumulates within exposed fish to levels greater than the initial exposure concentration, and that its main route of excretion is hepatic. Attention was also drawn to the persistence of malachite green within the tissues of fish, and to its potential acute or chronic toxicity. Its use as a chemotherapeut-

*Recent observations by Hendrick *et al.* (1988b) support the controversial view that PKX cells are, in fact, the trophozoites of a *Sphaerospora* sp.

ant against PKD was therefore discouraged until its pharmacokinetic properties had been further researched. Malachite green has since gained widespread use in the chemotherapy of PKD, being applied as a flush treatment sufficient to give 100 p.p.m. minutes* (Alderman & Clifton-Hadley, 1988). However, the initial reservations of Clifton-Hadley & Alderman (1987) should not be forgotten, since the drug has both a low safety margin—especially in fine scaled fish and fry—and a long tissue persistence, necessitating careful calculation of dosages and observation of a long recommended withdrawal period of 100 degree days† (Alderman & Clifton-Hadley, 1988).

Following the success of Molnar *et al.* (1987) in controlling renal sphaerosporosis of the common carp with the drug Fumagillin DCH, Hendrick *et al.* (1988a) were prompted to test its efficacy against PKX infections in chinook salmon (*Oncorhynchus tshawytscha*). The experimental fish were given intraperitoneal injections of kidney homogenate from infected chinook salmon, and then either fed the medicated diet immediately after the injections, or following a 7-day period of feeding a control diet lacking Fumagillin DCH. It was found that Fumagillin DCH was able to prevent the development of PKX and the accompanying renal pathology at concentrations in the feed as low as 0.5 g/kg, when fish were fed approximately 1% body weight per day. The drug was found to be effective even when first administered 1 week after experimental infection, thus showing promising potential for the control of established infections of PKX such as occur in the field. This is in contrast to the observations of Molnar *et al.* (1987), who found that Fumagillin DCH was only effective against renal sphaerosporosis of common carp when fed prior to, or immediately after, experimental infection of the fish. The question of the possible toxicity of Fumagillin DCH was also addressed by Hendrick *et al.* (1988a), who reported the unpublished results of preliminary trials with rainbow trout (*Salmo gairdneri*): levels of 0.5–1.0 g of Fumagillin DCH/kg feed, offered at 1.5% of body weight per day for 8 weeks, were found to cause mortality at the higher doses and depletion of haemopoietic tissues at all experimental doses. Fish receiving the highest dose became inappetent following 8 weeks of treatment, and showed severe splenic and renal hypertrophy. However, doses of 0.13–0.25 g/kg feed, offered at 1.0% fish body weight per day, were found to incur minimal toxic side effects. Hendrick *et al.* (1988a) concluded that although Fumagillin DCH had potent anti-PKD properties, further pharmacokinetic data were needed before it could be used with confidence in the field.

Other control strategies against PKD aim to prevent, or to palliate the effects of, the disease. Attempts at sterilizing the inflow water to the farm can be made using ultraviolet light, though this is often impracticable. During warm weather the stresses associated with intensive husbandry should be minimized. Hence, con-

*The unit p.p.m. minutes represents the integral function of the decay curve of concentration—measured in p.p.m.—against time—measured in minutes—and indicates the cumulative dose of drug to which the fish are exposed over a certain period of time. In practice, computer programs exist which calculate cumulative p.p.m. minutes for specific situations.

†The unit degree days is similar to that of p.p.m. minutes, representing the cumulative temperature to which the fish are exposed over a certain number of days. One can approximate its value by summing the mean daily temperatures for the evaluation period. This way of stating withdrawal periods recognizes the fact that elimination of a drug from the tissues of an ectotherm is a function both of environmental temperature and time.

sideration should always be given to the likely oxygen content of the water: stocking densities should be kept as low as possible, sprinkler systems or aerating devices should be considered to increase dissolved oxygen, and feeding should be minimized or stopped when water temperatures exceed 15 °C, this latter to eliminate the biological oxygen demand of digestion. However, prolonged periods of reduced feeding should be avoided, since they compromise growth and production and may themselves cause stress: clearly, the stockman must use his good sense to resolve the dilemma of maximizing production while at the same time minimizing digestive oxygen stress in warm weather. Current research is directed towards the development of a vaccine for PKD, which is likely to be useful because such an effective natural immune response is shown.

Kidney enlargement disease

One of the first myxosporean species to be described was *Hoferellus (Mitraspora) cyprini*, which causes kidney enlargement disease (KED) of young cyprinids. Ahmed (1974) established the course of the disease in a series of experiments carried out in a 2-year period from 1969 to 1971. Trophozoites settle on the epithelium of renal tubules and, or ureters and undergo endodyogeny, slowly becoming large plasmodia. Initially, the trophozoites congregate on the luminal surfaces of the epithelial cells, but are progressively incorporated within the cell layer as hyperplasia and papillary growth of the epithelium occurs. Some of the trophozoites undergo degeneration, giving rise to characteristic cells which have a large central vacuole and peripheral nuclei (ring type cells) (Ahmed, 1974); others continue development, giving rise to plasmodia which discharge spores into the lumen of the tubule. In the terminal stages of this infection cycle, the epithelial cells become a homogeneous mass of degenerating cytoplasm. The tubules show extraordinary enlargement, displacing the swimbladder and giving rise to marked abdominal distension. Affected fish thus lose equilibrium, and are thought to die from asphyxia, since they become unable to rise to the water surface to take gulps of air during periods of oxygen shortage. Fish affected with KED may also succumb to secondary pathogens such as *Aeromonas* spp. and *Saprolegnia* spp. In Ahmed's studies (1974) carried out at the University of Tokyo, fish which were hatched in April and May became infected with spores of *H. cyprini* from May to August; showed signs of abdominal distension from September onwards; and experienced heavy mortality from winter to early spring. This pattern is typical of the pathogen, although disease may generally be observed throughout the year. Those fish which survived the disease did not seem to show any subsequent immunity (Ahmed, 1974). KED is seen throughout Europe and Japan, and isolated cases of the disease have been observed in the UK (P. W. Scott, personal communication). At present, no drugs are known to be effective against this pathogen, and treatment aims to alleviate the problem of oxygen shortage in the water and to prevent secondary infection with bacteria or fungi.

Bi- and multivalvulid myxosporeans

Several myxosporean species are known to parasitize the muscle cells of fish. Multivalvulid myxosporeans with more than two spore valves and polar capsules are among the most important of these pathogens, developing within myocytes to

form oval pseudocysts which contain a great number of spores (Lom, 1984). These parasites are of great economic concern because they may render fish unsuitable for human consumption, either as a result of their unsightly appearance within fillets, or because of their ability to cause myodegeneration and softening of the muscles *post mortem*.

The most common multivalvulid genus is *Kudoa*, which has four spore valves and polar capsules. Various species of *Kudoa* exist, of which the most important are considered briefly in this review.

Kudoa clupeidae has been reported to parasitize yearling Atlantic herring (*Clupea harengus*) from the Maine coast of North America (Sinderman, 1970). Older herring do not seem to show evidence of infection and it has consequently been proposed that cysts of *K. clupeidae* burst open to the surface of the fish, thus releasing their spores, ensuring transmission of the species and freeing host fish from infection. Other *Kudoa* species have been reported to cause large, persistent cysts, such as those produced by *K. alliara* parasitizing southern blue whiting *(Micromesistius australis)* from the South Atlantic (Wootten, 1989). These large pseudocysts render fillets unacceptable to the consumer.

The association of pseudocysts of certain *Kudoa* species with post-mortem myodegeneration and development of softened muscle has been the subject of intense research (Patashnik *et al.*, 1982). Characteristically, fillets soften and lose their culinary value within 24 h of death or on cooking, despite refrigeration. Patashnik *et al.* (1982) sought to determine the reason for this myodegeneration in the fillets of Pacific whiting *(Merluccius productus)*, noting that this phenomenon alone was responsible for the lack of their exploitation for the food market. Several years before, Willis (1949) had suggested that the post-mortem myodegenerative effects of the myxosporean *Chloromyxum thyrsites* might be due to the extracellular release of powerful lytic enzymes. Patashnik *et al.* (1982) entertained a similar idea to account for the myxosporean-induced softening of whiting fillets. They discovered cysts which appeared as dark or white filaments between the myotomes, the darker ones being older and containing a brown pigment. Both types of cysts were found to have proteolytic activity, that of the younger, lighter cysts predominating. Examination of the morphology of the spores within the cysts revealed that two species of *Kudoa* were responsible for the myodegenerative changes. Patashnik *et al.* (1982) suggested that the proteolytic activity of the *Kudoa* spp. was localized within the cysts during life, but that on death of the fish, the enzyme(s) diffused out of the cyst and affected progressively more of the muscle biomass. However, no adverse effects on fillets were observed if the fish were rapidly chilled, processed and cooked, and consequently it was recommended that Pacific whiting be marketed as a fast-cooked or deep-fat-fried, portion-type product, limited to about 3/8 inch (0.95 cm) in thickness.

The two species of *Kudoa* described by Patashnik *et al.* (1982) had been identified by Kabata & Whitaker (1981). These were *Kudoa thyrsitis* and an uncharacterized species, for which the authors proposed the name *Kudoa paniformis*. Both species were thought to be involved in the myodegeneration of whiting, a view which was indeed confirmed a year later (Patashnik *et al.*, 1982). Pseudocysts of various forms could be identified, some appearing simply as white masses of spores within myocytes, and others appearing more degenerate: these latter would

be associated with melanin deposits and would assume a contracted, fragmented form, some even consisting of nothing more than a solidified mass of cell debris. The authors considered that the deposition of melanin granules around infected fibres was a host defence response, and that it led to the destruction of the contents of the pseudocysts. Moreover, the frequent coexistence of 'young' and 'degenerate' pseudocysts in the same host was taken to indicate that multiple, perhaps continuous, infections were not uncommon. *Kudoa thyrsitis* has also been reported to infect Atlantic salmon *(Salmo salar)* (Harrell & Scott, 1985). In this host species, the parasite seemed not to elicit the 'melanization' response characteristic of infection in Pacific whiting. However, pseudocysts caused extensive damage to surrounding muscle fibres, thought to be due to the same proteolytic enzyme as that released by cysts of *K. thyrsitis* in Pacific whiting (*M. productus*) (Patashnik *et al.*, 1982). Harrell & Scott (1985) reflected that the development of a defence mechanism against *K. thyrsitis* in Pacific whiting had led to the evolution of a stable relationship between parasite and host. The absence of any similar host response in the infections of Atlantic salmon was taken to indicate that this species was a relatively new host on the evolutionary scale, and that sufficient defences had not yet had time to evolve. Other examples of infections involving species of the genus *Kudoa* include *K. thyrsitis* in John Dory *(Zeus faber)* from South African waters (Davies & Beyers, 1947) and *K. musculoliquefaciens* in the Japanese swordfish (*Xiphias gladius*) (Matsumoto, 1954).

The bivalvulid genus *Henneguya* includes a number of pathogenic species which infect both warm and cold water species of fish in both the marine and freshwater environments. *Henneguya* species are noted for causing infections of the musculature and gills. For example, *H. zschokkei* causes 'boil' disease in a number of Pacific salmon (Petrushevski & Shulman, 1961). The parasites develop deep in the musculature, often near the spine. As the cysts enlarge to a diameter of 2–3 cm they rupture through the integument and discharge a white fluid rich in spores. Ulcers are thus formed, and may provide a portal for secondary infection which can kill the maturing salmon. Another species which parasitizes Pacific salmon is *H. salminicola,* which causes 'tapioca' disease (Wood, 1968). The parasites are contained in up to 50 small white cysts in the musculature, each 3–6 mm in diameter (Sinderman, 1970). *H. salminicola* is typically prevalent in up to 40% of fish, rendering fillets unsalable in heavy infection.

Several species of *Henneguya* parasitize the gills of fish. Dykova & Lom (1978), reflecting on the paucity of knowledge of tissue reactions and inflammatory processes in myxosporean infections of fish, sought to characterize the histopathological changes of fish gills infected with *Henneguya* species. Thus, studies were undertaken of the respective development of *H. psorospermica* and *H. creplini* in secondary lamellar arterioles of perch (*Perca fluviatilis*) and ruff (*Acerina cernua*), and of the development of *H. psorospermica* in the afferent filament arteries of pike (*Esox lucius*). Fish were caught from localities throughout Southern Bohemia, Czechoslovakia. Infection of perch with *H. psorospermica* was limited to the cold seasons of the year, first detected in the second half of December; cysts—comprising plasmodia located in the blood vessels at the bases of secondary lamellae—reached maximum size in April, when they would contain many mature spores (Dykova & Lom, 1978). Young plasmodia were about 0.35 mm in diameter

and took the form of small cysts, whose walls were formed by flattened host endothelial cells. As plasmodia grew in size they were further encased by flattened epithelial cells of the respiratory lamellae, with the disappearance of the stratified columnar epithelium between them. Spores were often released from the cysts following their maturation. Some cysts, however, were reduced and eliminated by a host defence reaction, which was characterized by invasion of the parasitic mass by macrophages and removal of spores by phagocytosis, followed by the infiltration of fibroblasts and histiocytes to create a granuloma (Dykova & Lom, 1978). This tissue response was minimal at the temperature of the natural habitat of perch (6–8 °C), but became marked in those fish transferred to water which was maintained at room temperature; this was interpreted to reflect the temperature dependence of inflammatory reactions in fish (Finn & Nielsen, 1971). Infections of *H. creplini* in ruff showed seasonal fluctuations similar to those of *H. psorospermica* in perch, but were never as heavy (Dykova & Lom, 1978). In addition, the cysts were always separated by regions of undamaged gill tissue. The structure of young and mature cysts, and the granulomatous tissue response leading to their elimination, were similar to those in perch. The temperature dependence of the host defence reaction was demonstrated once again (Dykova & Lom, 1978).

Species of the genus *Henneguya* also infect channel catfish (*Ictalurus punctatus*). McCraren *et al.* (1975) described seven possible disease manifestations of *Henneguya* in this species: these are described in the following paragraph. These diseases could be divided into three categories: those of branchial infection, those of cutaneous infection, and a third category including lesser known infections of the gall bladder, and of the connective tissue surrounding the mandibular teeth. Both intralamellar and interlamellar forms of branchial infection could be identified. The intralamellar form was commonly observed among cultured catfish, spores developing within capillaries of gill lamellae or blood vessels of gill filaments. This form was thought to debilitate fish without causing high mortality. The interlamellar form was characterized by the development of spores among basal cells *between* gill lamellae. Lamellar tissue was observed to undergo great hypertrophy with spore development, and would lose its original histological structure (McCraren *et al.*, 1975). This form was noted to be the cause of great mortality among young cultured channel catfish, with reports of epizootics involving losses of 95% of fingerlings less than 2 weeks old (Meyer, 1969). The consequence of interlamellar infection was a great loss of respiratory function, and fish would show signs of anoxia such as swimming at the surface of ponds with flared opercula. In addition, fish with interlamellar infection tolerated handling poorly. Of the cutaneous infections, papillomatous and cystic forms could be identified (McCraren *et al.*, 1975). The papillomatous form was characterized by the presence of white, granular growths on the dorsal, anal and caudal fins, and caudal peduncle, of cultured catfish; the largest growth recorded was 1 cm in diameter. The lesions were often haemorrhagic, and were associated with the presence of large numbers of trophozoites, sporoblasts and spores of *Henneguya*. The growths consisted of hyperplastic squamous epithelium and numerous goblet cells; tissue proliferation was superficial and did not extend beyond the basement membrane. The second cutaneous form of infection was characterized by the presence of visible cysts of diameter 2–4 mm in the connective tissue of the dermis. Neither trophozoites nor sporo-

blasts were noted in these lesions, suggesting that they represented an end-stage, or mature, state. The mandibular form of infection was characterized by the presence of cysts containing spores in the connective tissue surrounding the mandibular teeth, while the gall bladder form was associated with great enlargement of the gall bladder and the presence of spores in its lumen.

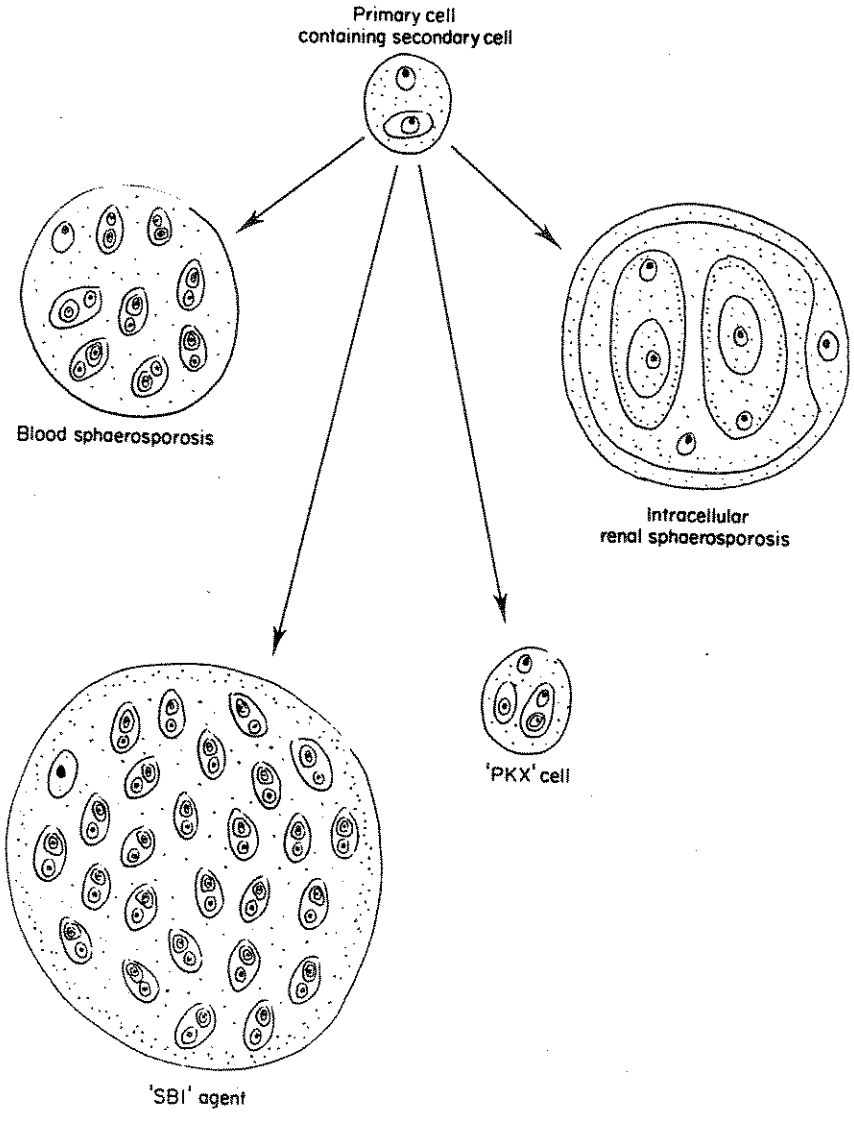

Fig. 4. Extrasporogonic agents of myxosporean disease.

Other myxosporean diseases

Two myxosporean diseases which are briefly considered in conclusion of this section are *ceratomyxosis*, and *sphaerosporosis* caused by *Sphaerospora molnari*. The latter is seen in yearling carp and has several clinical manifestations, in common with its counterpart caused by *S. renicola* (see earlier); these are respectively called branchial (or gill), cutaneous (or skin), and blood sphaerosporosis (Lom *et al.*, 1983). Branchial sphaerosporosis is the most important of these three states, being caused by the invasion of primary and secondary lamellar epithelium by pseudoplasmodia. This leads to epithelial hyperplasia and subsequent necrosis of branchial tissues, leading to the release of spores into the water. These changes greatly compromise the ability for gaseous exchange, resulting in tachypnoea, lethargy and even death. Diagnosis is based on the demonstration of developing extrasporogonic stages and spores in circulating blood. Little can be done to counter the disease, although antibacterial and antifungal agents may be used to prevent possible secondary infections. Branchial sphaerosporosis has been reported in Czechoslovakia, Hungary, Poland and Yugoslavia (Dykova & Lom, 1988).

Ceratomyxosis is an enigmatic myxosporean disease of young salmonids restricted in location to the western United States and British Columbia (Bower, 1985). It is caused by *Ceratomyxa shasta*, whose plasmodia give rise to granulomatous lesions in the spleen, liver, kidney, muscle and intestine. Fish affected by the disease show dropsy due to an abdominal accumulation of fluid rich in spores. Ceratomyxosis can be diagnosed by the post-mortem examination of sentinel fish, or the microscopical demonstration of spores in abdominal fluid collected from live fish by abdominocentesis. Chemotherapeutic agents for the disease are currently lacking. Indigenous fish seem to develop immunity to *C. shasta*, a situation rather similar to that for PKD (see earlier).

The various extrasporogonic agents of myxosporean disease are summarized in Fig. 4.

CONCLUSION

The Myxosporea are an enigmatic group of parasites. The diseases they cause are of great economic concern, compromising food conversion and causing direct loss. Control measures against the myxosporeoses largely aim to reduce the stresses of intensive culture which bring about infection, since few drugs have been shown to have activity against the Myxosporea. The last decade has seen some large leaps forward in our knowledge of myxosporean life cycles and modes of transmission.

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Myxosoma cerebralis: Detection of Circulating Antibodies in Infected Rainbow Trout (*Salmo gairdneri*)

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GRIFFIN, B. R., AND E. M. DAVIS. 1978. *Myxosoma cerebralis*: detection of circulating antibodies in infected rainbow trout (*Salmo gairdneri*). J. Fish. Res. Board Can. 35: 1186–1190.

Circulating antibody against spores of *Myxosoma cerebralis* has been detected for the first time in rainbow trout (*Salmo gairdneri*) affected with whirling disease. An indirect fluorescent antibody test (IFAT) was employed for detection of trout immunoglobulin. Most sera of fish from an infected experimental group were found to give a positive reaction in the IFAT, while the majority of specific-pathogen-free control sera gave no reaction. Reagent preparation as well as staining and evaluation procedures are given. These results provide additional information for understanding the host/parasite relationship and offer a potential for development of a nondestructive serological test for whirling disease.

Key words: indirect fluorescent antibody test, immune response, whirling disease

GRIFFIN, B. R., AND E. M. DAVIS. 1978. *Myxosoma cerebralis*: detection of circulating antibodies in infected rainbow trout (*Salmo gairdneri*). J. Fish. Res. Board Can. 35: 1186–1190.

Nous avons décelé pour la première fois chez des truites arc-en-ciel (*Salmo gairdneri*) affectées de la maladie giratoire un anticorps en circulation contre des spores de *Myxosoma cerebralis*. Pour la détection de l'immoglobuline des truites, nous avons utilisé un test d'anticorps à fluorescence indirecte (IFAT). Nous constatons que la plupart des sérums de poissons provenant d'un groupe infecté expérimentalement ont une réaction positive à l'IFAT, alors que la majorité des sérums de témoins libres de l'agent pathogène ne donnent aucune réaction. Nous décrivons la préparation des réactifs et les méthodes de coloration et d'évaluation. Ces résultats nous permettent de mieux comprendre la relation hôte/parasite et pourraient servir à mettre au point un test sérologique non destructeur de la maladie giratoire.

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THE myxosporidan parasite *Myxosoma cerebralis* causes the disfiguring, often crippling or fatal whirling disease of salmonids. The pathobiology of whirling disease has been reviewed most recently by Halliday (1976).

Lom (1969) suggested that poikilothermic animals generally show little, if any, response to myxosporidan infections. Neither Pauley (1974) nor Halliday (1974) was able to detect anti-*M. cerebralis* antibody in rainbow trout (*Salmo gairdneri*) through the use of serological techniques. Pauley's (1974) investigation of the spore antigens of *M. cerebralis* indicated that they mimic the antigens of rainbow trout cartilage even though electrophoretic differences were demonstrated. This host antigen mimicry apparently would account for the ease with which the parasite establishes itself in host tissues.

Halliday (1974), using rabbit antibody against whole trout serum in an indirect fluorescent antibody test was unable to detect specific antibody in rainbow trout with natural infections or in trout experimentally inoculated with spores of *M. cerebralis*.

The ability of various fish species to develop immunity and demonstrable antibody during some parasitic infections has been documented. Hines and Spira (1973) demonstrated acquired immunity as well as the presence of immobilizing antibodies in common carp (*Cyprinus carpio*) infected with *Ichthyophthirius multifiliis*. Molnar and Berczi (1965) detected precipitating antibodies to *Ligula intestinalis* in infected bream (*Abramis brama*). Other investigators have shown the presence of an immune response, indicated by increased levels of resistance to challenge (although not demonstrated by

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the presence of specific antibody) in several species: Kennedy and Walker (1969) in dace (*Leuciscus leuciscus*) infected with *Caryophyllaeus laticeps*; Beckert and Allison (1964) in white catfish (*Ictalurus catus*) infected with *Ichthyophthirius multifiliis*; and Nigrelli (1937) in Florida pompano (*Trachinotus carolinus*) and permit (*T. falcatus*) infected with *Epibdella melleni*.

The purpose of this paper is to report the development of an indirect fluorescent antibody technique for detection of trout antibodies to *M. cerebralis*, and to show by application of the technique a correspondence between the presence of *M. cerebralis* spores in fish tissue and specific antibody in their serum.

Materials and Methods

General Approach

Rainbow trout immunoglobulin was purified by elution from specific antigen–antibody complexes and used as antigen for the production of anti-trout immunoglobulin antisera in rabbits. Rabbit immunoglobulin G was purified by standard methods and labeled with fluorescein isothiocyanate for use in conventional indirect fluorescent antibody tests.

Infected and Specific-Pathogen-Free Rainbow Trout

Rainbow trout infected with *M. cerebralis* were obtained from the Lamar (Pennsylvania) National Fish Hatchery, and specific-pathogen-free rainbow trout controls from the Leetown (West Virginia) National Fish Hatchery. Fish were kept in 12°C spring water, the chemical characteristics of which were reported by Warren (1963).

Preparation of Antiserum

Immunoglobulin from high titer rainbow trout antiserum against *Aeromonas salmonicida* was purified by a modification of methods described by Cisar and Fryer (1974). Adult rainbow trout (25–30 cm fork length) were immunized by intraperitoneal inoculation of 0.5 mg (dry weight) of formalin-killed *A. salmonicida* in Freund's incomplete adjuvant, followed in 6 mo by an intraperitoneal inoculation of 1 mg of a similar bacterial preparation in phosphate-buffered saline, pH 7.2. The fish were bled 4 wk after the second injection and the sera from 30 animals were pooled.

The globulin fraction was collected after precipitation with an equal volume of cold saturated ammonium sulfate and centrifugation at 27 000 × *g* for 10 min at 4°C. The globulin proteins were dialyzed against two changes of 0.1 M phosphate buffered saline, pH 7.2 (globulin: buffer ratio = 1:100), and specific immunoglobulins were adsorbed for 1 h at 25°C onto formalin-fixed *A. salmonicida* cells (50 mg dry weight/mL serum). The bacterial cells had been washed with 0.1 M KCN, pH 11.0 to eliminate any bacterial components that might later be eluted along with the specific antibody. The cells were then equilibrated in 0.1 M phosphate buffered saline, pH 7.2. After immunoglobulin adsorption, cells were centrifuged at 27 000 × *g* for 10 min at 4°C. The supernatant was discarded and the cells were resuspended, washed, and centrifuged three times in 0.1 M phosphate buffered saline, pH 7.2. Immunoglobulins were then eluted from the cells by three sequential resuspensions and centrifugations in 0.1 M KCN; the volume of each wash was equal to that of the original serum. Immunoglobulins were precipitated from KCN by the addition of an equal volume of cold saturated ammonium sulfate, collected by centrifugation, and dissolved in and dialyzed against 0.1 M phosphate buffered saline, pH 7.2 at 4°C.

Immunoglobulin samples were applied to a 1.4- × 100-cm column of Sephadex G-100 (Pharmacia Fine Chemicals, Inc.) equilibrated in 0.1 M phosphate buffered saline, pH 7.2. The leading portion of the effluent void volume peak absorbing at 280 nm was collected and concentrated (by the addition of saturated ammonium sulfate and centrifugation) as before. After exhaustive dialysis against 0.1 M phosphate buffered saline, pH 7.2, the protein concentration was adjusted to 2 mg/mL. For quantitation, protein concentration was estimated on the basis of optical density, assuming an extinction coefficient of 1.6/mg/mL at 280 nm.

The immunoglobulin was emulsified with an equal volume of Freund's incomplete adjuvant. Rabbits were immunized by injection of 1.0 mL of the adjuvant mixture divided between two subcutaneous sites. A subcutaneous booster of 1 mg of protein in 1 mL of 0.1 M phosphate buffered saline, pH 7.2, was given 20 d later. Final bleeding followed 7 d after the booster. Rabbit anti-rainbow trout immunoglobulin prepared in this way gave a single line of precipitation in immunodiffusion against whole rainbow trout serum.

Fluorescent Labeling

Fluorescein isothiocyanate was conjugated to rabbit antibody by a modification of the methods of Johnson and Holborow (1973).

Rabbit antibody to rainbow trout immunoglobulin was mixed with an equal volume of cold, saturated ammonium sulfate; after centrifugation the precipitate was dissolved in and dialyzed against 0.015 M phosphate buffer, pH 8.0, and applied to a column of Sephadex DEAE, A-25 (Pharmacia Fine Chemicals, Inc.), equilibrated in the same buffer. Nonbinding protein was collected, then concentrated by adding an equal volume of saturated ammonium sulfate followed by centrifugation at 27 000 × *g* for 10 min at 4°C. The precipitate was dissolved in and dialyzed against 0.15 M NaCl and the protein concentration was adjusted to 10 mg/mL. Protein concentration was estimated on the basis of optical density, assuming an extinction coefficient of 1.4/mg/mL at 280 nm.

A 10% volume of 0.5 M carbonate–bicarbonate buffer, pH 9.5, was added and dry powdered fluorescein isothiocyanate (Isomer I, Sigma #F7250, Sigma Chemical Co.) was added at a ratio of 40 μg/mg protein. The mixture was shaken gently for 2 h at room temperature (pH maintained at 9.5 by addition of 0.5 M Na_2CO_3), and then applied to a 2.5 × 12 cm column of Sephadex G-25 equilibrated in 0.1 M phosphate-buffered saline, pH 7.2, for removal of unreacted dye. The fluorescein to protein ratio ranged from 0.4 to 1.1 in different preparations as indicated by absorption at 495 nm/280 nm. When a counterstain was used, Rhodamine (Difco #2340-56) was added at a 1:20 ratio. Conjugated rabbit anti-rainbow trout immunoglobulin antibody prepared in this way retained its biological activity and gave a single line of precipitation in immunodiffusion against whole rainbow trout serum.

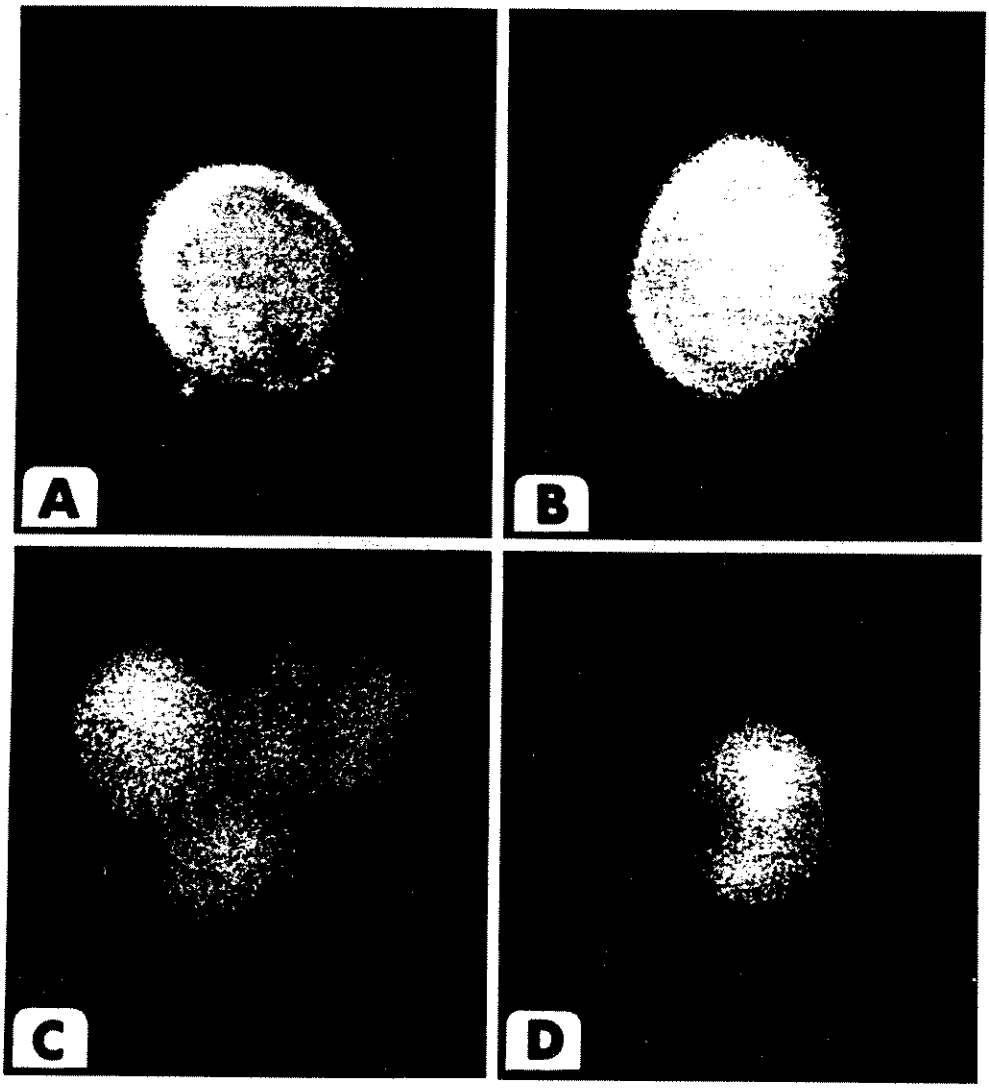

FIG. 1. Mature spores of *M. cerebralis* in the indirect fluorescent antibody test. A, spore after staining, using infected trout serum; B, spore after staining, using strongly positive, undiluted, infected trout serum; C, spores after staining, using serum from specific-pathogen-free fish; D, unstained spore, showing autofluorescence.

INDIRECT FLUORESCENT ANTIBODY STAINING

For the test, spores of *M. cerebralis* obtained from infected trout by the pepsin–trypsin–dextrose method (Markiw and Wolf 1974a) were spread on an acid cleaned slide and dried (60°C for 10 min). Test rainbow trout serum was added and slides were incubated 40 min at room temperature in a moist chamber. Slides were rinsed with a stream of 0.1 M phosphate buffered saline, pH 7.2, and immersed in the same buffer and shaken gently for 30 min at room temperature. The conjugate was added and after incubation for 30 min the slides were rinsed and washed as before. Stained slides were mounted in a mounting medium of 50:50 glycerol, 0.5 M carbonate–bicarbonate buffer, pH 9.5, and observed with reflected UV illumination at 500×.

To assess correlation between the presence of spores and specific antibodies, we microscopically examined an aqueous preparation of crushed cranial cartilage and bone from each fish for spores; the time limit was 5 min per sample.

Results

The indirect fluorescent antibody test revealed a correlation between the presence of spores in fish tissues and the presence of serum antibody against *M. cerebralis*. Sera from the group of fish infected with *M. cerebralis* gave fluorescence in 16 of 18 samples when tested undiluted and in 14 of 18 when diluted 1:3.

Spores were detected in 14 of the 18 trout when crushed cranial cartilage smear preparations were ex-

amined microscopically. There was not an absolute correlation between the presence of spores and the presence of antibody in individuals since in one case spores were detected but antibodies were not demonstrable in the serum of that animal and conversely antibody was detected in three sera from specimens in which spores were not detected. However, application of the sign test showed that the association of spores with serum antibody was statistically significant ($P = 0.01$). The undiluted control sera, on the other hand, yielded no fluorescence in 15 of 18 individual samples and when diluted 1:3 gave weak fluorescence in only one. Spores were not detected in the crushed cranial smears prepared from these specific-pathogen-free controls.

The test was specific for trout antibody to *M. cerebralis*. If spores were incubated with positive serum and then reacted with unconjugated rabbit anti-trout immunoglobulin before being stained with conjugate, specific fluorescence was not observed. Spores incubated with high-titer rainbow trout anti-*A. salmonicida* sera and then stained with the conjugate were also negative.

Fluorescence was usually apparent along the periphery of the spore with little staining of internal structures (Fig. 1A). Some undiluted sera gave bright confluent fluorescence over the entire spore surface (Fig. 1B); however, when these sera were diluted 1:3 the staining pattern was peripheral (as in Fig. 1A). Spores treated with sera from normal controls typically showed a dull autofluorescence after staining (Fig. 1C) that was characteristic of unstained spores (Fig. 1D). Polar filaments were often observed in the stained preparation but no consistent staining pattern was noted. The best fluorescence was obtained with conjugate preparations exhibiting a fluorescein to protein ratio ranging from 0.7 to 1.1. Rhodamine was not necessary since negative and positive preparations were readily distinguishable; however, the counterstain facilitated the identification of the bicapsular spore.

Discussion

The demonstration of circulating antibody in the present study does not support currently held opinions that rainbow trout do not mount an immune response to *M. cerebralis*. Halliday (1974) has suggested that the spores of *M. cerebralis* may occupy an immunologically privileged or guarded site in the cranial cartilage and bone of the infected animal, or alternatively that the absence of detectable antibody may be due to what he described as "non-pathogenicity of the spores." Failure to detect antibody in infected fish appears to have been the result of Halliday's use of a nonspecific fluorescent antibody conjugate rather than due to an insufficiency on the part of the infected animal. Similarly, Pauley's (1974) failure to demonstrate the presence of antibody may well have been due to the use of an insensitive assay — immunodiffusion in gel — rather than to the absence of antibody resulting from antigenic mimicry. Indeed, Pauley clearly illustrated molecular differences between spore material and normal fish tissue by electrophoretic analysis.

The detection of an immune response in trout infected with *M. cerebralis* provides additional background for understanding this host/parasite relationship. Dineen (1963a, b) suggested that specific antigenic disparity between parasite components and host tissues can serve to stabilize the relationship. The immune response to a limited range of parasitic antigens would control the parasite population to within tolerable limits that would not kill the host, thus enhancing survival of the parasite. Any strong display of critical parasite antigens which might lead to elimination of the infection through an intense host immune response would work against the long-term survival of the parasite. Antigenic disparity then, to best serve the host/parasite relationship, must be reduced but not eliminated.

Although the rainbow trout is not considered to be the natural host for *M. cerebralis* (Hoffman 1970), it is in the same genus as the natural host, the brown trout (*Salmo trutta*). The detection of antibody to *M. cerebralis* in infected rainbow trout makes plausible the argument of limited antigenic disparity in explaining the stability of the natural host/parasite relationship.

The inability of previous investigators to detect the immune response of trout infected with *M. cerebralis* has prevented consideration of a serodiagnostic test that is independent of finding spores for diagnosis, hence the methods of spore detection have been refined considerably. Markiw and Wolf (1974b) compared several of the currently available methods of detecting spores in an infected population and concluded that the pepsin–trypsin–dextrose centrifugation method (Markiw and Wolf 1974a) was most sensitive. The demonstration of circulating antibody to *M. cerebralis* spores in infected rainbow trout, as reported here, opens for investigation the use of the indirect fluorescent antibody test, or other serodiagnostic techniques, in the diagnosis of the disease. This test is nondestructive and can be performed in a matter of 2 to 3 h.

A number of factors required for a useful serodiagnostic test employing the indirect fluorescent antibody test are still to be investigated. These include an analysis of the range of cross-reactivity with other myxosporidans; a study of the age of the fish when antibody is detectable; an understanding of the influence of temperature on production of antibody; and an understanding of the predictive value of specific antibody as an indicator of the presence or absence of infection.

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From Ambulatory Clinic, Royal Veterinary and Agricultural University, Copenhagen, Denmark.

Studies on *Myxosoma cerebralis*, a Parasite of Salmonids.

I. The diagnosis of the infection

by *M. M. Halliday*

The problems of diagnosing infections of Myxosoma cerebralis are discussed. A method is described for concentrating the spores of the parasite.

The ability to accurately diagnose a disease in a population of fish is essential since:

a) It determines the probability of the detection of infected fish, thereby influencing the reliability of the estimate of the levels of infection and the assessment of experimental results, as well as the effectiveness of prophylaxis based on these estimates.

b) It is necessary for successful legislative control.

Current diagnostic techniques for whirling disease are based on the demonstration of spores of *M. cerebralis* which due to their resistance and longevity in the host are easily recognised compared with the pre-spore parasite stages. Clinical symptoms can only be used as an indication of the disease since many of the symptoms are also evident in other unrelated pathological conditions e.g.:

Symptom	*Disease or pathology*
Whirling, or abnormal swimming	Infectious pancreatic necrosis Viral haemorrhagic septicaemia.
Skeletal deformities	Genetic, nutritional, or unknown.
Black tail	Produced with different "shock" treatments e.g. electricity.

Nord. Vet.-Med. 1973, 25, 345—348.

Since the diagnostic techniques proposed by *Hoffman et al.* (1968) for the purposes of legislation, several more sensitive techniques have been described. (*MacLean* 1971, *Prasher et al.* 1971 and *Rydlo* 1971).

Although the diagnostic technique described in this paper is basically similar to some other techniques, it was developed by the author for an experimental study of *M. cerebralis* and will be referred to in future publications.

The biology of *M. cerebralis* has been described by *Hoffman et al.* (1962) and *Meyers & Scala* (1969).

Diagnosis by a direct method

a) Experimental methods.

The severed head was treated as follows:

Digestion with pepsin hydrochloric acid. Visual observation suggested that 0.5 % pepsin (E/g = 30,000) in 0.1 N hydrochloric acid resulted in satisfactory partial digestion of the head. The duration of digestion was dependent on the size of the head and it could be accelerated by incubation at higher temperatures. This allowed the cranial skeleton to be removed.

Homogenisation. The medium for homogenisation was distilled water, however with larger heads it was found necessary to use a tris hydrochloric acid buffer of pH 8.0 and include a divalent cation (magnesium chloride) to reduce interparticulate electrostatic effects (*Anderson* 1956). The cranial skeleton was minced with sharp scissors and then homogenised by means of an Ultra-turrax homogeniser. (Supplied by Bie & Berntsen, Copenhagen).

Filtration of the brei. After gauze filtration, stainless steel filters of 160, 250 and 400 mesh per linear inch were used. These filters were made by the author from stainless steel cloth. The debris retained by filtration was examined for spores after it had been washed with 10 ml of medium.

Concentration of the spores. The centrifugal characteristics required to sediment the spores were determined.

Examination. Phase contrast microscopy was used to determine the state of the spores.

b) Results of experimental methods.

Phase contrast microscopy indicated that the liberated spores were completely free from any obvious cellular debris. This probably indicates that contact between the spores and host tissue is only mechanical.

Homogenisation of the cranial skeleton by means of the Ultra-turrax homogeniser was successful. However, phase contrast microscopy always indicated that small fragments of bone remained incompletely homogenised. Only rarely were any spores identified within these fragments. Damaged spores were never observed.

Examination of the debris and filtrate

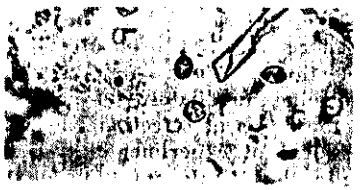

Fig. 1 (a).

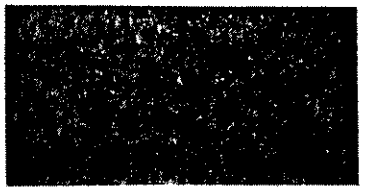

Fig. 1 (b).
The diagnosis of *M. cerebralis*.
Photomicrographs × 250.
a) Unstained. b) Malachite green staining.

fractions indicated that 10 ml of medium was sufficient to wash the spores through the filters.

Centrifugation at 1,000 rpm. on a standard bench centrifuge for 5 minutes was sufficient to sediment all the spores.

For examination, the pellet was suspended in the minimum volume of medium (between 0.2—0.5 ml) and examined as a wet preparation under 400 × magnification. The preparation can be stained by such stains as Leishman's, Giemsa, malachite green, and methylene blue to aid recognition of the spores. (Fig. 1).

Disinfection of the apparatus: It is essential for the consistently accurate diagnosis of infections that all equipment must be free of spores. For this reason the gauze was only used once and decontamination of the filters was achieved by thorough washing in boiling water containing a strong detergent. The homogeniser was difficult in this respect as minute particles of bone could become lodged between the blades. After each test it was dismantled and cleaned with a brush and finally by running it in a large volume of water.

Discussion

This technique is suitable for the large scale diagnosis of *M. cerebralis*. Its simplicity and cost both in monetary and labour terms recommend it as a standard diagnostic technique.

The sensitivity of this technique is likely to be inversely proportional to the size of fish examined. It is ideal for trout up to 15 cm in length but beyond this length some sensitivity is lost due to an increased amount of debris present with the spores.

The method of homogenisation and the small volumes of material from which examination is made suggest that this method is very efficient in collecting spores. However, although the presence of infection can be demonstrated its absence cannot be proved with present techniques.

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Summary

A method is described for the diagnosis of *M. cerebralis* spores from individual salmonids. By means of peptic digestion the cranial skeleton was obtained free from other tissue for homogenisation. This was carried out by means of an Ultraturrax homogeniser. The suspension was then filtered using several stainless steel filters and concentrated by centrifugation. Spores were detected by microscopy.

Sammendrag

Undersøgelser over Myxosoma cerebralis, en snylter hos laksefisk.

I. Diagnose.

Der beskrives en metode til påvisning af *M. cerebralis*-sporer hos inficerede laksefisk. Ved hjælp af pepsinfordøjelse befries hovedets skelet for andre vævsdele. Knoglevævet homogeniseres ved hjælp af en ultraturrax homogenisator. Suspensionen filtreres gennem flere finmaskede rustfri stålfiltre og koncentreres ved centrifugering. Sporer påvises ved mikroskopi.

Key words: Myxosoma, salmonids.

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Studies on *Myxosoma cerebralis*, a Parasite of Salmonids.

II. The development and pathology of *Myxosoma cerebralis* in experimentally infected rainbow trout (*Salmo gairdneri*) fry reared at different water temperatures

by *M. M. Halliday*

The different parasitic stages identified and the histopathology are described and discussed. High water temperatures increased the rate of parasitic development both morphologically and pathologically.

In general the outcome of protozoan infections of fish seems to be dependent on the action of the ecological variables influencing the relationship. Early accounts of myxosporidian pathology have been reviewed by *Plehn* (1910) and *Nigrelli & Smith* (1938), and to some extent the pathology appears to be dependent upon the host and/or organ parasitised. Little is known of the predisposing factors to infection.

The pathology of *M. cerebralis*, a cartilagophagous myxosporidian, is thought to be dependent on at least two variables:

a) The age of the host when it is first exposed to the pathogen.

b) The number of infective units to which the fish is exposed.

Descriptions of the histopathology vary and no really detailed accounts exist of the earlier stages of infection. Most workers have reported none or only slight destruction of the cartilage 40 days post infection (*Schäperclaus* 1954, *Hoffman et al.* 1962, *Lucky* 1970) but they identified granulation tissue 6—12 months post infection. *Roberts & Elson* (1970) did not identify any of these latter host reactions. *Lucky* (1970) concluded that the tissue response was dependent on the site of infection. With the exception of the work of *Schäperclaus* (1931) blood elements have not been identified around the infected areas.

Similar types of host reaction have been observed in other cartilagophagous myxosporidia. With infections of *Myxosoma cartilaginis n. sp.* in centrarchid fish an initial tissue reaction occurred 4—5 months post infection (*Hoffman et al.* 1965).

Since the ambient water temperature has been cited as an important factor in myxosporidian host-parasite relations (*Hoshina* 1952, *Leith & Moore* 1967, *Sanders et al.* 1970, *Lom* 1970). It was decided to investigate the effects of this variable on *M. cerebralis* infections. The observations were restricted to about four months post exposure to the infection.

Nord. Vet.-Med. 1973, 25, 349—358.

Materials and methods

Rainbow trout: Eyed eggs of rainbow trout were obtained from a Danish hatchery. They were reared in vertical hatching jars supplied with spore free water at 10°C. The fish were transferred to the experimental tanks once they had begun to feed.

Experimental tanks: The four tanks measured 100×20×20 cm.

Each tank had an independent spore free water supply, oxygen supply and thermostat. Water temperatures were maintained by a combination of a heating unit and a cooling system (HETO, Birkerød, Denmark). Before use, each tank had been thoroughly disinfected with formalin.

Mud samples: Mud was collected from two trout ponds in different fish farms. Both ponds had contained trout infected with whirling disease the previous year. The mud samples were pooled and stored for three weeks in polythene bags before equal quantities were placed in three of the tanks. The depth of mud in each tank was about 2 cm.

Water temperature: Since the alevins had been reared at a water temperature of 10°C, all tanks were initially adjusted to 10°C. After the fish had been introduced the water temperatures were adjusted to their experimental values over a period of 72 hours.

The experimental design was as follows:

Tank no.	No. of fry	Water temperature ± 0.5°C	Mud added
1	50	17	+
2	50	12	+
3	50	7	+
4 (Control)	50	12	—

Two days after the introduction of the fry the mud was stirred once daily for five days. Thus it was assumed that each experimental tank contained the same infection pressure.

Feeding: The fry were fed three times daily on "Chinook Mash" No. 0. As growth took place "Swim Up" No. 1, "Fry Fine" No. 2 and "Fry Coarse" No. 3 were used. (All food was supplied by Dansk Ørredfoder, Brande, Denmark).

Histological methods: Freshly killed fish were examined except where stated.

The heads were removed and fixed in 4 % formalin for at least 24 hours. They were then dehydrated, cleared and impregnated with 56°C melting point wax to which a little ceresin had been added. Sections were cut at six places in the head giving a total of twenty-four sections per head for examination.

Extreme caution must be exercised when examining this material due to the possible presence of histological artefacts.

Staining procedures:

I The investigation of normal and infected cartilage.
- a) Hæmatoxylin-phloxine-safran. (*Luna* 1968).
- b) Mayer's hæmatoxylin and eosin. (*Luna* 1968).

II The detection of *M. cerebralis* trophozoites, sporoblasts and spores.
- a) Mayer's hæmatoxylin and eosin. (*Luna* 1968).
- b) Ziehl-Neelsen method for acid fast bacteria (*Luna* 1968), with the following modifications.
  1) After staining in carbol fuchin for 24 hours, the sections were washed in running water.
  2) Decolourised with 25 % sulphuric acid. This consisted of literally just dipping the slide in.
  3) 1 % malachite green was used as a counterstain. Staining lasted 20 seconds.
- c) Giemsa (*Luna* 1968).
- d) Acridine orange (*Pearse* 1968). Unfortunately the tissue had been previously fixed and therefore no specific RNA reaction was observed.

Spore diagnostic methods: The method described by *Halliday* (1973) was used.

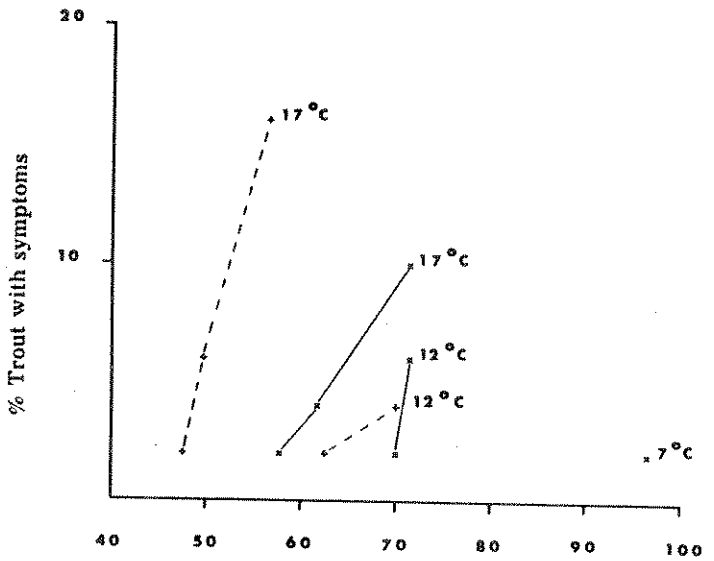

Fig. 1. Symptomatology of rainbow trout in tanks 1, 2, and 3 infected with *M. cerebralis* at different water temperatures.

Results

I) Gross mortality.

In such an experiment it is difficult to assign the cause of death especially in the preliminary stages. Some mortality occurred in all tanks. However, only two of the dead fish were shown to be infected with *M. cerebralis*. Both fish had been reared at 17°C.

II) The development of the infection

The results are illustrated in fig. 1.

a) Symptomatology: A trout was described as whirling if it exhibited these movements when disturbed. These could be differentiated from normal escape reactions.

Typical symptoms i.e. "whirling" and "black tails" were more prevalent at higher temperatures. Whirling was absent in those fish reared at 7°C and only one exhibited a black tail.

b) Development of the parasite: Great difficulty was experienced in identifying the preliminary parasitic stages. The illustrations (Plate 1) show some of the artefacts and organelles which were not considered to be trophozoites. Trophozoites of *M. cerebralis* are shown in fig. 5.

Table I. Examination of trout.

a) Killed for histological examination.

Days post exposure to the infection	Tank No. 1	2	3
20	—		
35	+	—	
52	+ +	—	—
60		+	—
71	+ +	+ +	—
85		—	—
90	+		
101		+	+
106	+		—
120		+	+

b) Killed and examined for spores 120 days post exposure to the infection

		Tank No. 1	2	3	4
No. examined		15	15	15	15
Spores	+	6	12	2	0
	—	9	3	13	15

+ positive infection
— no demonstrable infection

III) The parasitic stages of M. cerebralis.

The various parasitic stages were identified from histological section. Details of the dates and numbers of trout examined are shown in table I a. After 120 days exposure to the infection, fifteen remaining trout from each tank were examined for spores. (Table I b).

Table II displays the development of the parasite. The times of development can only be considered approximate as due to the small numbers of infected trout examined earlier stages in the parasites' development may have been missed.

Since most of the developmental stages were observed from fish infected at a water temperature of 17°C, the following description is taken from that material. Trophozoites were more evident when stained with hæmatoxylin and eosin while spores and sporoblasts were recognised by Giemsa or Ziehl Neelsen.

Trophozoites: Small multinucleate organisms are described by *Hoffman et al.* (1962) as trophozoites. From the examination of histological sections two likely stages of trophozoite activity are apparent.

a) A comparatively large multinucleate area. This could either be one or many trophozoites (fig. 5).

b) A smaller multinucleate organism of about 30 microns in maximum diameter, which is delimited by a membrane (figs. 6, 7).

In young fry there appear to be many necrotic foci in the cranial cartilage which could confuse the diagnosis of the early stages of *M. cerebralis* infection (fig. 3).

Sporoblast development: From observation it appears likely that an area of sporoblast formation follows from a proportionally dense area of trophozoite infestation. The nuclei of the trophozoite are thought to undergo rearrangement and form a multinucleate organism containing fourteen nuclei.

Thus fig. 6 may be an initial stage of sporoblast formation. Two groups of nuclei then become evident within the cell (fig. 7). During their rearrangement the nuclei appear normally stained by Giemsa and hæmatoxylin and eosin, however, as development proceeds the intensity of nuclear staining decreases markedly. Subsequently only the faint outlines of the sporoblast organelles are visible (fig. 8). This is supported by observations on fluorescently stained material (Plate 4). These results indicate that nuclear material (nucleic acids) is situated at the periphery of the developing spore wall and polar capsules.

Throughout their development each sporoblast is delimited by a membrane These membranes or similar structures form a network throughout the area of parasitic infestation (Plate 6).

PLATE I. Artefacts which could result in misidentification of *M. cerebralis* trophozoites.

Fig. 2. Histological artefact. Stain: Giemsa. Photomicrograph × 1250.

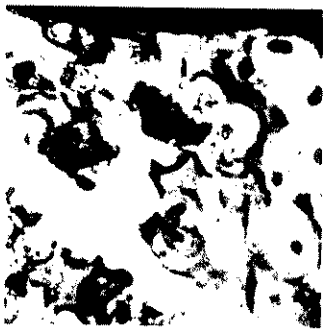

Fig. 3. Lesions in the cartilage of 2 month old rainbow trout. Stain: Hæmatoxylin-phloxine-safran. Photomicrograph × 1250.

Fig. 4. Giant multinucleate cells found in cranial sections. Stain: Giemsa. Photomicrograph × 200.

PLATE II. Trophozoites of *M. cerebralis* in cartilage.

Fig. 5. Infected cartilage 35 days post exposure to the infection. Stain: Haematoxylin and eosin. Photomicrograph × 900.

PLATE III. The development of the sporoblast of *M. cerebralis*.

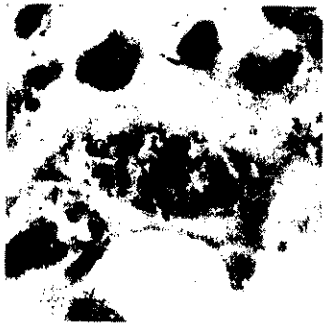

Fig. 6. Initial stages of sporoblast formation. Stain: Giemsa. Photomicrograph × 1250.

Fig. 7. Separation of nuclei within the sporoblast preparatory to the formation of two spores. Stain: Giemsa. Photomicrograph × 1250.

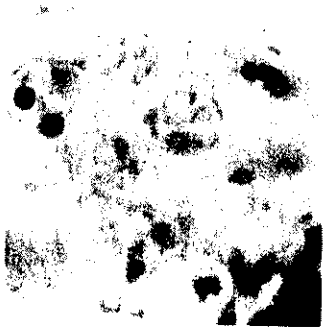

Fig. 8. Sporoblast containing two developing spores. Stain: Giemsa. Photomicrograph × 1250.

PLATE IV. Fluorescently stained parasitic stages of *M. cerebralis*.
Stain: Acridine orange. Photomicrographs × 1250.

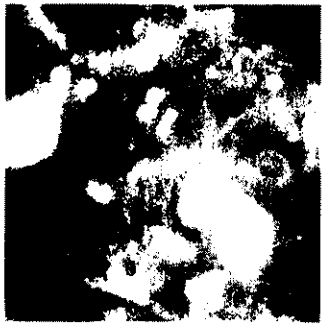

Fig. 9. Developing spore showing fluorescence around the periphery of the spore wall and polar capsules.

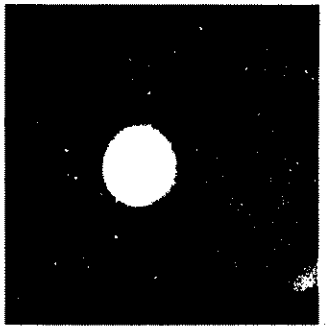

Fig. 10. More fully developed spore completely fluorescent.

PLATE V. Spores of *M. cerebralis*.

Fig. 11. Spores: polar capsules and some whole spores acid fast.
Stain: Ziehl Neelsen. Photomicrograph × 600.

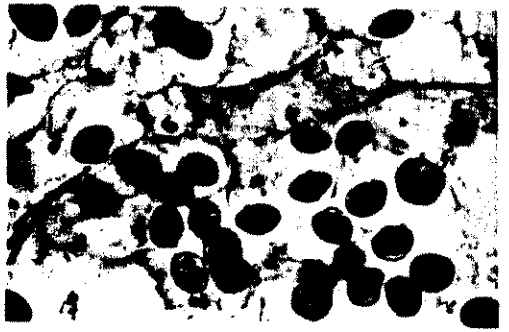

Fig. 12. Spores: most are completely acid fast.
Stain: Ziehl Neelsen. Photomicrograph × 600.

Fig. 13. Spores within the spinal column of a trout with a black tail.
Stain: Giemsa. Photomicrograph × 400.

PLATE VI. Membrane structures within the area of infection.

Fig. 14. An area of cartilage infested with developing sporoblasts. Stain: Giemsa. Photomicrograph × 500.

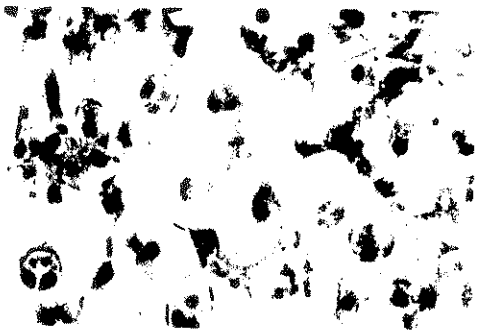

Fig. 15. Spore development taking place within a tissue network. Stain: Giemsa. Photomicrograph × 600.

PLATE VII. The pathology of *M. cerebralis*. Stain: Haematoxylin and eosin.

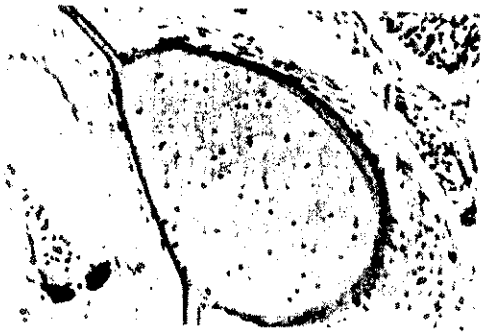

Fig. 16. Normal cartilage. Photomicrograph × 300.

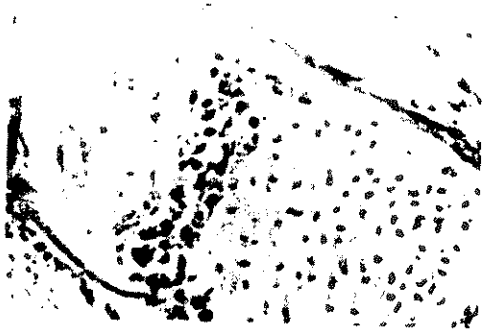

Fig. 17. Infected cartilage, 35 days post exposure to infection. Photomicrograph × 400.

Table II. Time scale of morphological development of *M. cerebralis* in days post exposure to infection.

Parasitic stage	Pathology	Water temperature (°C) 17	12	7
Trophozoites initially established		No observations		
Trophozoites	Extensive necrosis of cartilage cells in trout reared at 17°C	35	60	—
Sporoblasts initially formed		35	60	71
Sporoblasts	Necrosis evident at 12°C and to a lesser extent at 7°C	52	101	106
Sporoblasts and first "mature" spores		52	101	120
"Mature" spores		71	120	—
"Mature" spores in the spinal column		90	—	—

Spore development: Two spores develop from each sporoblast. The polar capsules appear to be the first spore organelles to become acid fast, subsequently the entire spore becomes acid fast (Plate 5); completely acid fast spores were called mature. The spores also fluoresce strongly when stained with acridine orange (fig. 10). In many cases the spores are retained within a network but sometimes they appear to be free, although this could arise from sectioning (cf. Plates 5 & 6).

IV) Pathological findings: By the time the earliest stages of infection were identified extensive necrosis of the cartilage had occurred (Plate 7, fig. 17). The pathology was temperature dependent. (Table II). No other reaction was consistently observed.

Discussion

Unfortunately like previous research on experimental infection with *M. cerebralis* no quantitative data on the number of infective units available to the trout were obtained.

(I) Development of the parasite:

Trophozoites were difficult to identify conclusively. Their description basically conforms to that of other authors (*Hoffman et al.* 1962).

The trophozoite is thought to be the multiplicative phase. From the point of view of the biological success of the parasite a high multiplicative potential is usually essential for survival. Thus it would seem likely that each established sporoplasm can produce many spores. This implies that the trophozoite is a large multinucleate organism. Therefore the smaller multinucleate organisms described are possibly the initial stages of sporoblast formation.

The results of fluorescent staining support those of other workers (*Chakravarty* 1962, *Maiti et al.* 1964, *Chaudhuri & Chakravarty* 1970).

An interesting feature of *M. cerebralis* spores is their acid fastness. *Sanders & Fryer* (1970) noted that the polar capsules of *Myxidium minteri* also appeared acid fast when stained with Ziehl Neelsen. In bacteria this property is either

attributed to a lipid or waxy substance in the cell wall, or else it is a property of the intact bacterium depending on the membrane structure (*Wilson & Miles* 1964). Acid fast bacteria are noted to be resistant to such solvents as strong alkaline solutions or mixtures of alkalis, sodium hypochlorite and also to digestive ferments however sodium hypochlorite and caustic solutions destroy *M. cerebralis* spores (*Hoffman* 1970). Nevertheless the structure of acid fast organisms may have some features in common. Osmium tetroxide-alpha napthylamine staining has indicated the possibilities of a phospholipid structure in the spore capsule (*Halliday* 1972).

(II) Pathological findings

Observations on the destruction of the cartilage suggest that it could either be a direct result of erosion or a necrotic reaction. However, a combination of both necrosis and erosion could be acting.

Erosion: Enzymes produced by the parasite may digest the cartilage and thus products may be used for metabolism.

Necrosis: The destruction of cartilage is reminiscent of an exaggerated host inflammatory response. In trout which have been infected for some months there is sometimes evidence of cartilage degeneration without any evidence of parasitic activity. If such a necrosis had an inflammatory basis it might be expected that leucocytes would be evident in necrotic areas. This was only rarely observed. Similar necrotic foci were also identified in healthy trout. Such necrosis may therefore be connected with the processes of calcification.

The network previously mentioned if it is of host origin is consistent with a process of fibrinous inflammation. In mammalian pathology the presence of fibrinous inflammation indicates that the necrosis has been severe. The tissue destruction is often so great that the animal will not survive (*Runnels et al.* 1965).

(III) The effect of temperature.

Environmental temperature is thought to play a very important role in poikilothermic host-parasite relationships.

Results of this study indicate that higher water temperatures enhance the development of the disease. The parasite not only develops at a faster rate but symptoms are more obvious. *Putz* (1970) has correlated the length of exposure and the numbers of infective units necessary to produce the gross symptom of "black tail". Since care was taken to ensure an equal distribution of infective units per tank it is assumed that the different rates of parasitic development are a direct result of temperature. The effect of temperature may be manifest at each, or all the following levels.

a) Non-parasitic stage: Since it has been shown that spores require about four months in which to become infective (*Putz* 1970), higher water temperatures may increase the numbers of infective spores. However, the ponds from which mud had been collected had been left free of trout for nine months, therefore it can be assumed that all the spores were infective.

b) Parasitic stage: Increasing water temperatures are likely to increase the parasites' activity. Results indicate that maturation is accelerated. In addition, it appeared from observations that those trout kept at 17°C had somewhat severer infections than those kept at lower temperatures. This could be explained in terms of an increased multiplicative potential which would also explain the symptoms being more obvious at those water temperatures.

c) Pathology: The extensive erosion of the cartilage observed at 35 days post infection when the trout had been reared

Table III.
Summary of previous research on the development of *M. cerebralis.*

thor	Water temperature	Time of initial symptoms	Time of initial identification of trophozoites	Time of initial identification of spores
häperclaus, 1931	10—18°C	2— 5 months		
häperclaus, 1954		40—60 days	40 days—3-4 months	4—6 months
uer, 1959		2 months		
ristensen, 1966	15°C	36—50 days		
lson, 1969			6—10 weeks	4—6 months
offman & Putz, 1969	12°C	71—73 days		3 months
tz, 1970	12°C	49 days		4 months
dd & Tubb, 1970		47 days		4 months
asutake & Wolf, 1970		trout six months old		6 months

at 17°C is probably another effect of temperature; possibly again related to an increased parasite activity. *Schäperclaus* (1954) and *Lucky* (1970) described only slight cartilage erosion at 40 days post infection. *Hoffman et al.* (1962) could not identify such changes at this time.

d) The host: If an immune response was to any degree protective then it might be expected that trout reared at lower temperatures may be more susceptible to the disease. This does not appear to be the case. Increasing temperatures will increase the host's metabolic rate and therefore may also allow host metabolic products to be available at a greater rate to the parasite.

In mammals, cartilage and bone are very sensitive to inadequate nutrition and react quickly to even minor variations in the diet. The faster the growth rate the greater is the probability that a bone disease will occur if the nutritional balance is incorrect. In contrast, a slow growth rate decreases the likelihood of any bone diseases (*Runnells et al.* 1965). It is likely that a similar relationship exists in fish. Thus nutritional factors in trout related to the increased growth rate occurring at higher temperatures may be important in determining the course of *M. cerebralis* infection.

There have been many studies on the effects of temperature on the pathogenicity and development of parasites. To the author's knowledge temperature has only been casually noted in some of the few accounts of the development of *M. cerebralis.* Table III displays the results of previous workers on this aspect.

In addition *Bogdanova* (1960) states that cold water (3—7°C) retards the development of the disease.

This study has shown that maturation of *M. cerebralis* is temperature dependent, spores being formed within 52 days at a temperature of 17°C while at 7°C they require about 120 days to develop.

The effect of temperature on other fish parasite systems is similar. Increasing temperature has been shown to increase the growth rate of diphyllobothrid plerocercoids (*Wikgreen* 1966), *Schistocephalus solidus* (*Sinha & Hopkins* 1967) and *Trypanosoma diemyctyli* (*Barrow* 1948 & 1958). It decreases the time of development for *Icthyopthirus multifilis* from 5 weeks at 50°F to 3—4 days at 70—75°F (*Meyer* 1969). The prepatent period of

the infection of young carp with *Eimeria carpelli* decreases from 17 to 7 days with increasing temperature (*Zmerzlaya* 1966). Temperature has also been shown to affect the pathogenic potential of protozoan parasites. *Sanders et al.* (1970) found that *Ceratomyxa shasta* infections of juvenile coho salmon only caused severe mortality when the water temperature exceeded 10—12°C. In addition a characteristic pathology only took 21 days to develop at 20°C. *Barrows* (1958) has shown that *T. diemyctyli* is only pathogenic at intermediate temperatures (about 15°C). At 20—25°C it was non-pathogenic. *Lom* (1970), in a study of *Henneguya psorospermia* in the gills of perch, found that at 20—24°C the trophozoites degenerated. This was either due to the parasites inability to withstand high temperatures or an accelerated host immune reaction.

Thus, *M. cerebralis* is no exception and like other poikilothermic parasites develops faster both morphologically and pathologically with increasing water temperatures.

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Summary

The different parasitic stages identified and the histopathology are described and discussed. High water temperatures increased the rate of parasitic development both morphologically and pathologically. At 17°C the first symptoms (whirling) were apparent by 47 days post exposure to the infection. Spores were apparently fully formed by 52 days post exposure to the infection while they took 101 days and 120 days at 12°C and 7°C respectively. Spore development in the host was considered complete when the spores were acid fast as shown by the Ziel Neelsen method for bacteria.

Sammendrag

Undersøgelser over Myxosoma cerebralis, en snylter hos laksefisk.

II. Udviklingen af Myxosoma cerebralis og de af denne snylter fremkaldte patologiske forandringer hos eksperimentelt inficerel regnbueørredyngel (Salmo gairdneri) ved forskellige vandtemperaturer.

De forskellige identificerede udviklingsstadier af *M. cerebralis* og de af denne fremkaldte histopatologiske forandringer beskrives og diskuteres.

Høje vandtemperaturer forkortede den parasitære udvikling såvel morfologisk som histopatologisk.

Ved 17°C opstod de første symptomer (drejesyge) 47 dage efter smitteoptagelse. Sporer var ved 17°C fuldt udviklede efter 52 dage, medens det tog 101 og 120 dage ved henholdsvis 12°C og 7°C.

Som tegn på fuld udvikling af sporerne blev anset syrefasthed ved farvning ad modum Ziehl-Neelsen.

Key words: Myxosoma, salmonids.

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Studies on *Myxosoma cerebralis*, a Parasite of Salmonids

III. Some studies on the epidemiology of *Myxosoma cerebralis* in Denmark, Scotland and Ireland

by *M. M. Halliday*

Subclinical infections of M. cerebralis were found in many of the trout farms investigated. The presence of these infections together with some experimental data are discussed in relation to the control of myxosomiasis.

The biology of *M. cerebralis* has been described by *Hoffman et al.* (1962) and *Meyers & Scala* (1969). Current diagnostic techniques have been discussed by *Halliday* (1973 a).

A method of ecological control of myxosomiasis has been proposed by *Rasmussen* (1965) and is used successfully in continental Europe (*Christensen* 1966, *Ghittino* 1970). Such control is based on the premise that once a trout has reached a length of 5—6 cm (i.e. approximately 4—6 months of age) it is generally resistant to infection because ossification of the skeleton prevents massive infection. Since ossification of the skeleton is a function of both the age and growth of the fish, these factors must be taken into account in control measures (*Rasmussen* 1967). *Hoffman et al.* (1962) consider that trout over four months of age may become infected, but normally not seriously.

In view of the international problems caused by this parasite these control measures were examined by the author using material from thout farms in Denmark, Ireland and Scotland, supplemented with experimental observations.

Nord. Vet.-Med. 1974, *26*, 165—172.

Material and methods

Farm-reared rainbow trout

i) Denmark: Samples of fish were obtained in August and November since it was considered that spores would have developed by this time. Samples were taken from each of the types of rearing tank used in nine farms, all of which were supplied with stream water.

Samples of rainbow trout 14 months old which had first shown distinct symptoms of whirling at 13 months of age were also examined. These fish had been bought from a farm at 6 months of age and subsequently reared in earthen ponds during the winter and spring. Six fish were examined histologically. Samples of rainbow trout from the original farm were also examined for spores in an attempt to determine the source and date of infection. Both farms were employing conventional control methods for myxosomiasis.

ii) Scotland: An outbreak of myxosomiasis among rainbow trout 15 months old was investigated. The farm employed conventional control methods.

iii) Ireland: Samples of fingerlings and older rainbow trout were examined from one farm employing conventional control methods.

Experimental infection of seven-month-old rainbow trout

A sample of seven-month-old rainbow trout was taken from a newly-constructed Danish trout farm. Spores of *M. cerebralis* could not be detected in 20 of the fish examined at the beginning of the experiment. Infection was attempted in the way described by *Halliday* (1973 b), with the following experimental design.

Tank number	Number of fish	Water temperature	Infective mud
1	40	17C	+
2	40	8C	+
3 (control)	50	12C	—

Fish in tanks 1 and 2 were exposed to the mud for 3 months, and then removed to spore-free environments where they were examined at intervals for the presence of spores. Fifty newly hatched rainbow trout were then exposed to the mud in tanks 1 and 2, again with an appropriate control. These fish gave an indication of the severity of the infection and were examined after three months for spores.

Examination of rainbow trout

The examination of rainbow trout was carried out as described by *Halliday* (1973 a) — detection of spores, and *Halliday* (1973 b) — histological methods.

Results

Farm reared rainbow trout

(i) Denmark: Danish fry: Results of the examination of fry from nine Danish trout farms are presented in Table I. Only two farms contained rainbow trout with clinical symptoms of myxosomiasis, and with very few exceptions, these were limited to fry which had been placed in earthen ponds soon after hatching. Trout from one farm only appeared to be free of myxosomiasis.

Danish yearlings: Two forms of *M. cerebralis* infection could be distinguished in each of the six fish examined.

In the first form, cartilage around the membraneous labyrinth was markedly destroyed and the area contained a large amount of degenerate cartilage, granulation tissue and many spores. In addition, bone which had been formed was irregular and appeared to have been affected by the presence of the parasite.

In the second form, trophozoites and sporoblasts were the only parasitic stages identified in other areas of cartilage. There was no host reaction to the parasite in these areas.

General histological observations on each of the fish indicated that considerable areas of cartilage were still intact. Four types of cartilage could be distinguished:

a) that in the process of forming bone
b) that undergoing calcification
c) that which appeared to be being transformed into fat cells
d) that which appeared to be inactive.

Origin of the infection: Two out of twenty fry from the farm where the above-mentioned yearlings were initially reared contained spores of *M. cerebralis*. Thus it is ligely that at least some of the rainbow trout had become infected as fry.

ii) Scotland:

Rainbow trout eggs, initally imported from Tasmania, were hatched and reared in concrete tanks supplied with river water. Once the fry were six months old they were transferred to earthen ponds. The first clinical symptoms of myxosomiasis became evident at the beginning of the winter when the trout were 15

Table I. Infection of rainbow trout with *M. cerebralis* in relation to rearing conditions in various farms in Denmark

Rearing unit	Farm	Number of fish examined	Age of fish (months)	Percentage of fish infected	Comments
Earthen ponds	I	20	5	100	clinical infections present
	II	9	5	78	
	III	8	5	0	fingerlings newly transferred to earthen ponds
	VII	19	5	0	
	VIII	25	14	100	
	II	31	17	61	
Concrete raceways	V	21	5	19	brown trout (*Salmo trutta*)
	VI	25	5	4	
	VII	15	5	1	
	VII	10	5	0	
	IX	20	7	10	
Fibre glass tanks	IV	9	5	0	
	VIII	8	5	0	all had spinal deformities not due to myxosomiasis
	I	20	7	10	large tank with great depth of water — some clinical infections
Hatching troughs	III	8	5	25	
	II	9	5	33	
	VII	16	5	0	

Infections were described as clinical when the symptoms of whirling and black tail were apparent.

months old. Diagnosis was confirmed by the identification of spores in the fish.

In the farms in Denmark and Scotland containing clinically infected yearlings no mortality attributable to the infection was evident.

iii) Ireland

As far as the author is aware there is no previously published evidence of the occurrence of myxosomiasis in Ireland. A sample of rainbow trout from a farm in Southern Ireland (Eire) obtained in December 1971 was found to be positive, although clinical symptoms were absent. Spores of *M. cerebralis* were detected in all eight fingerlings examined but not in older rainbow trout.

Experimentally-infected rainbow trout

Results of the fish examined are presented in Table II. Apart from the single case mentioned no symptoms were apparent in any of the other rainbow trout. Since clinical symptoms were on the whole absent and only a few spores were recovered from the fry and older fish, it was considered that the mud was only lightly infected. Nevertheless the results indicate that fish 7 months old can become infected.

Spores seemed to take longer to develop in older trout than in fry. The reason for this is not known. Temperature also influenced the rate of development of the parasite, the rate of development of spores being increased at the higher

Table II. The recovery of spores from rainbow trout exposed to infected mud

Water temperature (C)	Age of fish when first exposed to the infection	Examination number of days post-exposure	Examination number of fish	Results number infected. Percentage in brackets
17	7 months	50	1	1 (100) *
		100	10	1 (10)
		150	10	7 (70)
		200	12	8 (67)
17	1 week	85	10	7 (70)
8	7 months	100	10	0 (0)
		150	10	2 (20)
		200	14	4 (28)
8	1 week	120	10	3 (30)
12 (controls)	1 week	120	10	0 (0)
	7 months	200	20	0 (0)

* This was the only fish which displayed whirlin, first apparent 23 days post-exposure to the infecmtion. Trophozoites were identified in the cartilage.

water temperature. A more detailed investigation of the effects of temperature has been published by *Halliday* (1973 b).

Conclusions

a) Infections with *M. cerebralis* exist in two forms: Clinical, with a.o. whirling, and subclinical. These two forms are distinguished in the discussion below.

b) The fry-rearing tanks which have been employed to prevent infection largely eliminate clinical infections, but subclinical infection occurs.

c) Rainbow trout seven months old are susceptible to subclinical infection.

d) Clinical infections are usually restricted to fish in earthen ponds, which probably allow the infective agents to concentrate.

e) The onset of symptoms can occur in yearlings as well as in fry. Studies on clinically infected yearlings suggest that reinfection (new infections from spores in the environment) or autoinfection (spores formed in the cartilage releasing their sporoplasm, *Kudo* 1930) may occur.

Discussion

The severity of myxosomiasis depends on three factors- the susceptibility of the host, the density of infective spores and the probability of contact between the two.

Factors influencing the susceptibility of the host under farming conditions.

Due to a lack of biological information, any discussion of this aspect is inevitably theoretical.

Clinical myxosomiasis is unknown in natural populations of salmonids where infections are usually light (*Bogdanova* 1970). It is therefore likely that the outbreak potential of the parasite is elevated under conditions of artificial rearing. The stocking density and growth rate of fish in farms are higher than those of

wild fish. A high density of fish may cause stress which can increase their susceptibility to disease (*Wedemeyer* 1970). A high growth rate, at least in mammals, can increase the probability of bone diseases should any malnutrition occur (*Runnells et al.* 1965).

The parasite infects only cartilage, which during the growth of the fish is replaced by bone formation, calcification, or with the formation of fat cells. The occurrence of these processes are related to the age and growth rate of the fish (*Rasmussen* 1967), however, the more exact temporal aspects of these relationships are unknown.

The present results indicate that clinical symptoms can initially appear when the rainbow trout are about one year old, although fish of this age are thought to be resistant. This does not necessarily imply that fish of this age are susceptible to clinical infection as the symptoms could be caused by either of the following:

a) The maturation of an earlier infection which has remained at a low level of activity during the winter.

b) A host reaction to an earlier infection which produces extensive granulation tissue. Such a reaction was observed in all six yearling rainbow trout examined from the Danish farm. The granulation tissue appeared to cause pressure on the membraneous labyrinth thus affecting the sense of balance.

The observation that symptoms can be apparent in yearlings may not invalidate the present theories of control but further investigation is required.

Factors influencing the probability of contact between host and parasite under farming conditions.

In fish farms the main factors in the prevention of diseases are the nature of the water supply, the rate of elimination of pathogens from holding facilities and the isolation of infected fish, the latter being unimportant in myxosomiasis. The nature of the water supply is probably the most important factor in the prevention of myxosomiasis. Four types of water supply can be used. They are —

i) Pumped well water artificially aerated.

ii) Spring water taken directly from the source.

iii) Stream water, the farm being the first on the water course.

iv) Stream water which has been used by other farms.

On a theoretical basis, farms using either pumped well water or water taken directly from a spring source should be able to remain absolutely free from myxosomiasis. With stream water there is a risk of spores being released from wild fish and/or other farms.

The accepted methods for control of myxosomiasis consist of keeping fry in tanks or raceways until they have become clinically resistant to the parasite. Under these conditions of rearing reinfection within the tank is unlikely to occur as, according to current knowledge (*Hoffman & Putz* 1969), spores require 3—6 months to develop in the host and additional 4 months "ageing" before they are infective. As the fry rearing tanks are only in use for a limited time each year and are, or should be disinfected between use, any infections arising in these tanks are due to spores in the water supply. Thus, the water flow characteristics of the rearing facilities can affect the infective potential of these spores. In tanks, the eddies produced tend to prevent the accumulation of waterborne pathogens if the filters are kept clean. Raceways have a "dead area" located at the bottom of the lower end of the channel, even under conditions of maximum water flow. The flushing action is dependent on the depth of water present and its rate of flow. It may be uneconomical to use an efficient flushing

action, and under those conditions clinical infections of *M. cerebralis* have been observed to occur. Some studies on disease prevention have indicated that a raceway with a high flow rate is superior to a tank, e.g. rearing salmonids in raceways reduces the effects of *Ceratomyxa shasta* (*Leith & Moore* 1967).

Consideration of these factors in salmonid production..

i) Production of fry and fingerlings. In a trout farm or hatchery using a spore-free water supply, fingerlings can be produced completely free from myxosomiasis as long as it is kept as a completely closed unit — no introductions of eggs and fry which might be carriers of the infection — and as long as other ways of possible introduction of the parasite are controlled e.g. sporecontaining dust, bird transmission and non-hygienic management.

When the farm is using stream water which has not been contaminated by other farms, myxosomiasis can be limited to a subclinical level by adopting standard control measures which take into account the age and growth of the fish. In countries such as Denmark, fry cannot be reared in farms which use water which has passed through several other farms as clinical myxosomiasis results even with standard control measures (pers. comm. *Christensen* 1973). These farms must use a spore-free water source for rearing fry. When the fingerlings are exposed to stream water they will probably become subclinically infected.

ii) Production of portion size fish. Once the fry have grown sufficiently they are considered to be resistant to myxosomiasis, and are placed in earthen ponds for final rearing. However, the results suggest that at least some of these fish are susceptible to subclinical infections of *M. cerebralis*. Once fish are exposed to the milieu in countries where *M. cerebralis* is established there is a risk of myxosomiasis from introductions of other fish, and transmission by birds and humans even if the water supply is completely free of spores. In earthen ponds reinfection can occur and the spores can accumulate. Newly constructed fish farms may remain free from the disease for several years before it occurs.

In conclusion then in countries where *M. cerebralis* is established fish which have been exposed to the milieu cannot be considered completely free from myxosomiasis unless they have been reared in a closed unit with spore free water and under strict hygienic control.

International implications of myxosomiasis.

An ecological approach to the control of myxosomiasis has the advantages of retaining any natural resistance to the pathogen within a population of fish. It has no apparent adverse effect on production despite the presence of subclinical infections. In view of the extreme resistance and longevity of the infective agent and its host range, total eradication of *M. cerebralis* is likely to be impossible with existing farming techniques, and ecological control may have to be accepted. However, ecological control raises problems when trout are exported to those countries where *M. cerebralis* has not been identified.

Certification of exports has been introduced to control the international spread of fish diseases and can apply to either individual farms or to particular shipments. Since it is difficult to detect *M. cerebralis* spores in older fish, legislation might be more effective if based on an annual examination of fry and fingerlings in individual farms rather than on an examination of portion-sized fish in export shipments. On the basis of the previous discussion, it is thought that in those countries where *M. cerebralis* is

known to be well established, trout which have been reared in stream water should not be certified free from myxosomiasis. Trout from these countries can only be certified free from myxosomiasis if they have been reared as a closed unit with spore-free water and are negative for the parasite on examination. This closed system may be rarely practical.

Certification can discriminate either against fish with clinical symptoms of the disease or against all infected fish. Future methods in trout farming may indicate that one of those methods is more appropriate than the other. Should trout farming become more intensive and change from an open system to one isolated from the milieu (e.g. by ultraviolet sterilization of incoming water) then the parasite could eventually be eradicated from farms, assuming that an adequate diagnostic test was available to enforce legislation. However, present farming methods usually employ untreated river water and in those countries where *M. cerebralis* exists it seems likely that fish are being exposed to the parasite without any harmful effects. Thus, certification of freedom from clinical infections as opposed to all levels of infection is likely to be sufficient. Countries where *M. cerebralis* does not occur can only remain free of the parasite by either rejecting imports of fish and fish products from countries where the infection occurs or by imposing strict certification of freedom of the parasite from the trout farms in question.

Certain fishery operations involving natural waters may act as amplifiers for any infections of *M. cerebralis*, no matter how slight; thus certification against all levels of infection may be necessary in, for example the following projects.

i) The Columbia River System, USA (*Ellis & Noble* 1959). The extensive hatchery projects aimed at supplementing salmon stocks in the Columbia River could be susceptible to such an oportunist pathogen as *M. cerebralis*.

ii) The adoption of "put and take" harvesting utilizing the natural productivity of small lakes for seasonal operations with salmonids (*Johnson et al.* 1970).

These dangers of amplification can only be assessed once the factors which can elevate the outbreak potential sufficiently to produce clinical infections are known.

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Summary

Studies on several trout farms indicated that clinical infections of *M. cerebralis* were largely confined to earthen ponds, but that subclinical infections occurred in fry which were reared in tanks designed to prevent whirling disease.

Seven-month-old rainbow trout were shown to be susceptible to subclinical infection. Thus it is possible that additional infection might occur when fingerlings are transferred to earthen ponds after having been reared in tanks for about six months.

Two atypical outbreaks of whirling disease are described in which the onset of symptoms occurred at 13—15 months of age.

The data are discussed in relation to existing control methods and legislation.

Sammendrag

Undersøgelse over Myxosoma cerebralis, en snylter hos lokalfisk
Myxosomiasis' epidemiology i Danmark, Skotland og Irland

Undersøgelser på flere ørreddambrug viste, at klinisk manifeste infektioner med *Myxosoma cerebralis* overvejende var begrænset til jorddamme, medens subkliniske infektioner forekom hos ørredyngel, der var opdrættet i bassiner, der var konstrueret med det formål at forebygge drejesyge.

Syv måneder gamle ørreder fandtes at være modtagelige for subklinisk infektion. Det er derfor muligt, at infektion kan udvikles, når sættefisk overføres til jorddamme efter at være blevet holdt i bassiner i op til 6 måneder.

To atypiske udbrud af drejesyge beskrives, i hvilke de første symptomer iagttoges i alderen 13—15 mdr. Disse observationer diskuteres i relation til de eksisterende forebyggelsesmetoder og i forhold til gældende lovgivning.

Key words: Myxosoma cerebralis, epidemiology, salmonids.

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Studies on *Myxosoma cerebralis*, a Parasite of Salmonids

IV. A preliminary immunofluorescent investigation of the spores of *Myxosoma cerebralis*

by *M. M. Halliday*

Antibodies to Myxosoma cerebralis were detected in a rabbit inoculated with M. cerebralis spores but no antibodies could be detected in rainbow trout, either naturally infected or inoculated with spores.

Fluorescent antibody (FA) methods have been used in many aspects of microbiology. The methods and their applications have been discussed by *Goldman* (1968). In research on fish diseases the indirect fluorescent antibody (IFA) test has been used to diagnose viral haemorrhagic septicaemia (*Jørgensen & Meyling* 1972) and to detect the presence of *Aeromonas liquefaciens* (*Lewis & Savage* 1972). For the latter pathogen the direct method also has been used (*Lewis & Allison* 1971).

Because the spores of *M. cerebralis* lodge in the tissues of salmonids for long periods they may be a source of antigen for antibody production. Initially the antigenic properties of intact spores were investigated by inoculating them into a rabbit and assessing the results by means of the IFA test. As in any immunodiagnostic test, the quality of the antigen is an important factor in its success. For this reason the investigation of *M. cerebralis* was confined to the spore stage, because it was the only stage in the life cycle which could be obtained in a relatively pure state.

Material and methods

Rainbow trout

For the production of rabbit anti-rainbow trout serum, serum was taken from fifteen healthy eighteen-month-old rainbow trout which had been reared in the laboratory at a water temperature of 15°C. Six similar rainbow trout were inoculated with *M. cerebralis* spores.

Samples of yearlings naturally infected with whirling disease were obtained in May and June from a Danish trout farm. Six of these fish which were examined histologically contained all stages of the parasite, and in addition some granulation tissue.

Collection of rainbow trout serum

After anaesthetizing the trout with either chlorobutol (2.0 g/l) or MS 222 (3.75 g/l), blood was taken from their dorsal aorta with a 2 ml plastic syringe and a 38 mm og 19 gauge needle (*Schiffman* 1959). The freshlydrawn blood was allowed to stand at room temperature and was then left to clot at 4°C for 4 h. The serum was separated by centrifuga-

Nord. Vet.-Med. 1974, *26*, 173—179.

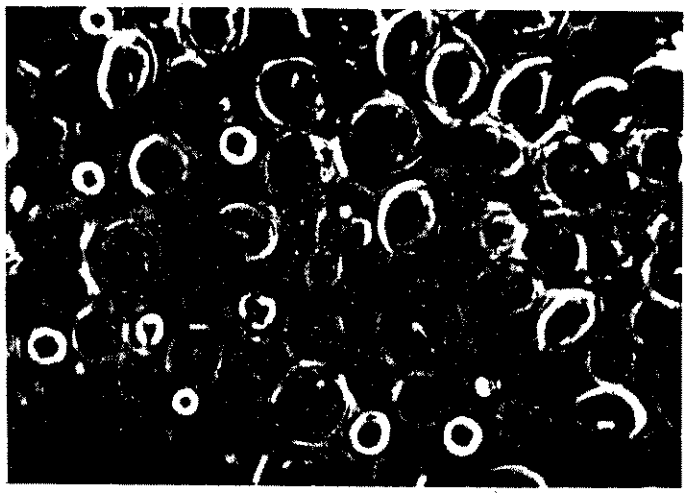

Fig. 1. Spore preparation of *M. cerebralis* Photomicrograph × 1000.

tion at 1500 g for 15 min and samples stored at — 20°C until required.

Preparation of spores for use as antigens

A suspension of spores of *M. cerebralis* was obtained from rainbow trout of lengths 6—10 cm. These trout had been shown by the method of *Halliday* (1973) to contain a very high number of spores. Satisfactory purification was achieved by centrifugation of the spores suspended in 1.1 M sucrose 5 mM Tris-hydrochloric acid buffer pH 8.0 5 mM magnesium chloride, through 1.2 M sucrose with similar additives, for 20 min at 20 g. Centrifuge tubes were of 50 ml capacity. The surface millilitre was discarded and the supernatant which contained the majority of the spores, collected. The spores were concentrated by centrifugation at 1300 g for 10 min. By repeating this procedure high concentrations of spores with little contamination could be prepared (fig. 1). The degree of separation of spores attained by this method was variable, separation being most successful when the initial suspension contained a very high concentration of spores. The spores were washed twice with phosphate-buffered saline (pH 7.2) before being stored in distilled water at —20°C.

By the technique described above a suspension of spores of *Myxobolus spp.* (probably *M. pseudodispar)* from the spleens of roach (*Rutilus rutilus*) was prepared to give some indication of the specificity of antiserum prepared against *M. cerebralis.*

Preparation of antisera

i) Rabbit anti-rainbow trout serum. Four rabbits were immunized with a pooled sample of whole normal rainbow trout serum. Two rabbits were immunized by method 1 and the other two by method 2.

Method 1. In week 1, 0.5 ml of whole normal rainbow trout serum and 0.5 ml of Freud's complete adjuvant were injected intramuscularly (IM). In week 6, an IM injection of 0.5 ml whole normal rainbow trout serum was given. The rabbits were bled in week 7.

Method 2. 1 ml of whole normal rainbow trout serum was IM injected into the rabbits every second day. A total of eight injections was given. The rabbits were bled one week after the final injection.

The results of immunization by both methods were assessed by immunoelectrophoresis.

ii) Rabbit anti-*M. cerebralis* spore serum

Only one rabbit was inoculated subcutaneously. In week 1, 1×10^6 *M. cerebralis* spores in 0.5 ml saline and 0.5 ml Freud's complete adjuvant were injected. This was followed by an injection of 1×10^6 *M. cerebralis* spores in 0.5 ml saline in week 6. The rabbit was bled in week 7 and the results assessed by immunofluorescence.

iii) Rainbow trout anti-*M. cerebralis* spore serum

The six rainbow trout were inoculated intraperitoneally at monthly intervals. The initial inoculation consisted of 1×10^6 *M. cerebralis* spores in 0.5 ml saline and 0.5 ml Freud's complete adjuvant. Thereafter, 1×10^6 *M. cerebralis* spores in saline were inoculated. Three trout were kept at a water temperature of 18°C and three at 12°C. Prior to each inoculation a blood sample was taken, the initial sample acting as a control. Each sample was tested by immunofluorescence. Due to deterioration in the condition of the rainbow trout only three injections were given and the fish killed after 15 weeks.

Immunoelectrophoresis

This was carried out in 1.5 % agar gel using a veronal buffer pH 8.6. Fish serum was electrophoresed for 1½ hours at a constant voltage of 5 v/cm. After the application of rabbit antiserum optimal immunoprecipitates formed within 48 hours.

Preparation of the reagents for immunofluorescence

Phosphate-buffered saline: 0.15 M sodiumphosphate in physiological saline, pH 7.2.

Preparation of rabbit anti-rainbow trout fluorescein isothiocyanate (FITC).

The method used was as follows, being based on that of *The & Feltkamp* (1970).

1 ml of saturated ammonium sulphate at 0°C was added to an equal volume of rabbit anti-rainbow trout serum also at 0°C. The mixture was kept for two hours at 0°C. It was then centrifuged at 6000 g for 20 minutes at 4°C. The pellet obtained was resuspended in its original volume using 0.9 % saline, and an equal volume of saturated ammonium sulphate added. The mixture was left for two hours at 0°C and centrifuged as previously described. The procedure of precipitation was repeated.

The final pellet was partly redissolved in a minimum volume of phosphate-buffered saline and transferred to a dialysis bag. It was dialysed against phosphate-buffered saline for 18 hours with three changes of buffered saline.

The final protein concentration was determined by spectrophotometry (spectrophotometer: Hitachi-Perkin-Elmer 139 UV-V15. Cells: type 1.5 mm Patt. Length. Spectrosilspectrophotometer cells code Q). The protein content was adjusted to 4 ml containing 27.281 mg/ml immunoglobulin by ultrafiltration through 47 mm Pellicon type PSED filters, 1000 molecular weight cut-off (Millipore U.K.).

Conjugation of FITC to the immunoglobulin:

A solution of 0.15 m disodium hydrogenphosphate (pH 9.0) containing 1.0 mg FITC (Sigma Chemical Company (U. K.) per ml was made up. Because the total protein content of the solution amounted to 109.124 mg, 1.091 ml of the FITC solution was added (calculation

based on the criteria of *The & Feltkamp* 1972) while stirring at room temperature. The pH was adjusted to 9.5 by the addition of 0.1 M sodium phosphate and the reaction allowed to continue for one hour at room temperature. The pH was maintained at 9.5.

Separation of the unconjugated dye:

This was separated on a G 25 medium column (Pharmacia Fine Chemicals Ltd.). The column was equilibrated and eluted with phosphate-buffered saline.

From a nomogram the F/P molar ratio was found to be 0.40—0.45.

Absorption of non-specific FITC:

The procedure adopted is outlined by *Goldman* (1968). Absorption was carried out using rabbit liver powder (Sigma Chemical Company U.K.). Finally 1 ml samples of the conjugate were stored at — 20°C in plastic tubes.

Glassware:

Only clean grease-free-microscope slides 0.8—1 mm thick, and pasteur pipettes were used. They were cleaned in chromic acid for 24 h, rinsed for 3 h in running tap water and twice in distilled water, then air dried.

Swine anti-rabbit serum FITC was obtained from —

Nordic Pharmaceuticals and Diagnostics, London. Code SwAR/FITC

Packing 2 MLLYOF and Dako Immunoglobulins a/s Copenhagen. Code F 2190

Both were stored at 4°C

Mounting medium:

Glycerine buffered at pH 9.0 with a carbonate-bicarbonate buffer.

Fixation of antigens

Since the specificity of the IFA test can be affected by the method used to fix the antigen, several methods were investigated — fixation by ethanol, methanol, 4 % formalin, acetone and air-drying.

Standard IFA test procedure

Six drops of the purified spore suspension (antigen) were individually placed on a clean microscope slide and allowed to dry. The spores were fixed in ethanol at —20°C for 5 min. The antigens were isolated by surrounding each drop by nail varnish. All the serum used was diluted with phosphate-buffered saline. About 0.05 ml test serum was allowed to mix with antigens for 40 min at room temperature in a humid chamber. The slide was then washed twice with phosphate-buffered saline, each washing lasting 5 min. After drying the slide by blotting it gently with filter paper, about 0.05 ml conjugate serum was applied and allowed to mix with the antigens for 40 min. at room temperature in a humid chamber. The slide was washed and dried as before. The antigens were mounted in buffered glycerine and examined by means of either a Nikon or Leitz fluorescent microscope with filters B. 0.

When testing the serum from rainbow trout which had received injections of *M. cerebralis* spores or were naturally infected, an additional procedure was used:

After the test serum was applied to the antigens, rabbit anti-rainbow trout serum was next applied, and finally anti-rabbit conjugate. The slide was washed and dried as described above between serum applications.

Results

a) Methods

Use of FITC:

Reference to control slides in which phosphate-buffered saline, normal rabbit serum or fish serum was used instead of a test serum, indicated that both anti-rabbit conjugates and the antirainbow trout conjugate should be used at a dilution of 1/20 to minimize non-specific fluorescence.

Fixation:

Fixation by ethanol at —20°C for 5 min was found to be most suitable.

Evaluation of fluorescence:

The degree of fluorescence was measured visually on a four-pointscale varying from 1 — faint, to 4 — very bright. The degree of fluorescence are illustrated in fig. 2.

+/— was used to indicate fluorescence which was very faint and only just detectable.

b) Test sera

Rabbit anti-*M. cerebralis* spore serum: The antibody titres of this serum, when measured by the IFA test using swine anti-rabbit FITC from Nordic Pharmaceuticals, are shown below.

Titre of test serum	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560
Degree of fluorescence	4	4	3	2	2	1	1	+/—	+/—

When spores of *Myxobolus* spp. were used as the antigen, no significant cross-reaction was observed with rabbit anti-*M. cerebralis* spore serum.

Rainbow trout anti-*M. cerebralis* spore serum:

Non-specific staining was higher when using rabbit anti-rainbow trout FITC (+ 1) than with anti-rabbit FITC (+/—). However, antibody to the spores of *M. cerebralis* could not be detected with either conjugate in any of the serum samples from rainbow trout reared at 18°C and 12°C.

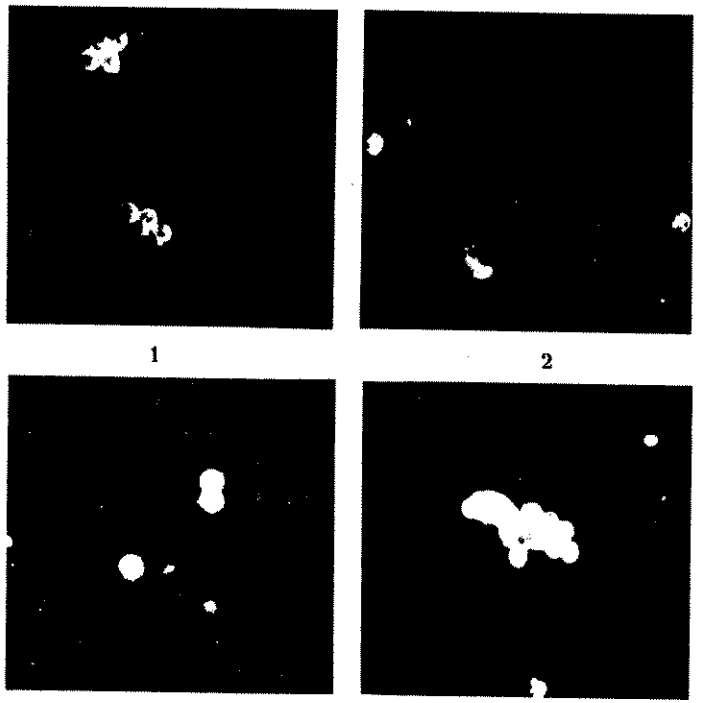

Fig. 2. Varying degrees of fluorescence of *M. cerebralis* spores when treated with rabbit anti-*M. cerebralis* spore serum followed by swine anti-rabbit FITC. Photomicrographs × 350.

Serum from rainbow trout naturally infected with whirling disease:

Neither fluorescent method indicated the presence of any antibody response to the infection in any of the fish examined.

Summary of results

i) Spores of *M. cerebralis* are capable of eliciting a specific antibody response in a rabbit when inoculated subcutaneously. This response is detectable by the IFA test.

ii) There was no demonstrable cross-reaction between antiserum prepared specifically against *M. cerebralis* spores and the spores of *Myxobolus* spp. from roach.

iii) Antibody could not be detected in rainbow trout receiving monthly injections of *M. cerebralis* spores.

iv) Antibody could not be detected in rainbow trout naturally infected with clinical whirling disease.

Discussion

Teleosts are capable of producing a vigorous and anamestic immune response (*Cushing* 1970, *Finn* 1970, *Lom* 1969), the efficacy of which is dependent on the ambient water temperature (*Avtalion* 1969 a, b, c).

Although it was shown that the spores of *M. cerebralis* can induce antibody formation in a rabbit, there were no detectable levels of antibody in rainbow trout naturally infected with the parasite. The absence of detectable levels of antibody might arise from either the site of infection, i.e. predominantly cranial cartilage, sheltering the parasite from exposure to the host's immunological defences, or from the non-pathogenicity of the spores. Nor could antibody be detected in rainbow trout inoculated with spores. It is thought that this indicates a technical inadequacy rather than reflecting the inability of the recipient to respond to the antigens. The fact that all the rainbow trout were in poor condition after fifteen weeks may have been a major factor in the failure of the fish to produce detectable levels af antibody.

Although the IFA test may be of no value in the diagnosis of *M. cerebralis* it may be taxonomically useful. The classification of myxosporidia is currently based on spore characteristics, since the trophozoites have little definable structure. Taxonomic confusion arises because a very large number of species is classified according to a very few characteristics, some with an inherent natural variation. However, scanning electron micrographs suggest that additional, morphological structures may be used in the taxonomic of the myxosporidia (*Lom & Hoffman* 1971). Results from the present study suggest that species-specific antisera can be produced against spores; thus a serological study of spores may also be of value in clarifying the taxonomy.

The existence of geographical variants within a species, suggested by *Lom & Hoffman* (1970), might be best detected by an IFA test using antisera prepared from either whole or disrupted spores. Once the amount of intra-specific variation likely to occur in a clearly-definable species can be estimated, the value of immunology in the classification of less well-defined species can be assessed.

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Summary

By means of the indirect fluorescent antibody technique antibodies were detected in a rabbit inoculated with *M. cerebralis* spores. However, no antibodies could be detected in rainbow trout either naturally infected or inoculated with spores. Thus this technique could not be used for the diagnosis of whirling disease.

Antibodies appeared to be species specific as no cross reaction was evident between rabbit antiserum produced against *M. cerebralis* spores and spores of *Myxobolus* spp. from roach. This technique therefore may be useful in classifying the myxosporidia.

Sammendrag

Undersøgelser over Myxosoma cerebralis, en snylter hos laksefisk

Ved hjælp af indirekte immunofluorescensteknik påvistes antistof hos en kanin, der var podet med Myxosoma cerebralis sporer. Imidlertid kunne der ikke påvises antistoffer hos naturligt inficerede ørreder eller ørreder podet med sporer. Metoden kan således ikke anvendes til at diagnosticere drejesyge.

Antistofferne synes at være artspecifikke, da der ikke kunne påvises nogen krydsreaktion mellem kanin antiserum mod sporer af M. cerebralis og mod sporer af Myxobolus spp. fra skaller.

Teknikken kan derfor være nyttig ved klassificering af myxosporidier.

Key words: Myxosoma cerebralis, salmonids, immunofluorescence.

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The biology of *Myxosoma cerebralis:* the causative organism of whirling disease of salmonids

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The literature describing the biology and control of *Myxosoma cerebralis* (whirling disease) is reviewed. New data on the world distribution of the parasite are presented. It is concluded that the presence of *M. cerebralis* is not an important limiting factor in salmonid farming *per se* but only limits methods of production.

I. INTRODUCTION

THE CURRENT PROBLEM OF WHIRLING DISEASE

Whirling disease is caused by the protozoan *Myxosoma cerebralis* [Hofer, Plehn, 1904 emend. Kudo, 1933 syn. *Lentospora c.* (Hofer, Plehn) and *Myxobolus c.* Lom & Hoffman, 1970]. The transcontinental dissemination and economic importance of *M. cerebralis* have paralleled the increased development in salmonid fish farming. The disease has therefore been included in the International Office of Epizootics list of major infectious diseases of freshwater fish. However, despite its economic importance, there is a lack of biological information on the parasite concerned; this arises from research difficulties, some of which are outlined by Hoffman & Putz (1970*a*).

Infections with *M. cerebralis* have been refered to as ' Myxosomiasis '. However, as there are many species of *Myxosoma* which may cause different diseases, or perhaps no disease, this is not the most appropriate name for the disease. It is suggested that the name ' whirling disease ' (*Myxosoma cerebralis*) of salmonids be continued to avoid future confusion. Whirling disease is known in French as *Tournis*; in Spanish as *Torneo*; in Russian as *Vertesch*; in German as *Drehkrankeit*; in Italian as *Capostorno* or *Lentosporiasi*; in Danish as *Drejesyge*, and in Swedish as *Kringsjuka*. Short reviews of the biology of *M. cerebralis* have been published in the following languages—Danish by Christensen (1966); English by Hoffman *et al.* (1962); French by Christensen (1966); German by Schäperclaus (1954*a*); Italian by Ghittino (1970*a*); and Russian by Bogdanova (1960) and Hoffman (1971).

The first confirmed outbreak of whirling disease occurred in Germany in 1903 among rainbow trout (*Salmo gairdneri*), which are not indigenous to Europe (Hofer, 1903). From 1903 until 1952 whirling disease had been reported from Germany, France and Denmark (Bruhl, 1926; Schäperclaus, 1931; Luling, 1952; Vanco, 1952).

Whirling disease is indicative of an imbalance in the relationship of *M. cerebralis* and its host. The international importance of this disease has led to speculation as to

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the factors which have caused the imbalance. The following two explanations have been suggested.

(i) Whirling disease originated from feeding rainbow trout with raw marine fish, which were infected with spores similar to *M. cerebralis* (Bergman, 1922; Plehn, 1924). However, it is probable that the spores were those of *Myxobolus aeglefini* (Schäperclaus, 1954*a*; Hoffman, 1970*a*).

(ii) It has been stated that brown trout (*Salmo trutta*) become infected but not diseased (Hoffman *et al.*, 1962). Thus Hoffman (1970*a*) suggested that brown trout are a natural host of *M. cerebralis*, and that whirling disease in rainbow trout has arisen from their contact with naturally infected brown trout. He suggests that other European and Asian salmonids may also be natural hosts.

It has not been firmly established that brown trout are resistant to whirling disease. According to Schäperclaus (1931); Bauer (pers. comm., 1973) and O'Grodnick (pers. comm., 1973), brown trout appear to be less susceptible to clinical disease than rainbow trout. However, Havelka & Volf (1970) have reported 80–90% mortality in brown trout caused by *M. cerebralis*. Brown trout can therefore become clinically infected, but are less susceptible than rainbow trout to lower levels of infection.

An additional explanation of the host-parasite imbalance is that the increase in disease may be a by-product of intensive salmonid husbandry. The severe clinical manifestations of the disease only arise where a high rate of infection occurs (Putz & Hoffman, 1970; Halliday, 1974*a*). Therefore, the problem of whirling disease may have arisen from the introduction of an unnatural host into an area where *M. cerebralis* is endemic and/or from the introduction of *M. cerebralis* into an environment where an high infective potential can build up.

II. DISSEMINATION OF *M. CEREBRALIS* AND CURRENT GEOGRAPHICAL DISTRIBUTION

DISSEMINATION

In general the large-scale spread of *M. cerebralis* is thought to have arisen from the shipping of infected fish and fish products to areas where the parasite did not occur naturally. Any attempt to explain the spread of *M. cerebralis* is complicated by the fact that the numerous intercontinental shipments of ova from brown trout and other salmonids occurred prior to the first description of *M. cerebralis* (MacCrimmon & Marshall, 1968; MacCrimmon & Campbell, 1969; MacCrimmon, 1971). The possibility that viable spores can be transferred as a contaminant of egg shipments has been suggested by Schäperclaus (1931), Keiz (1964) and Putz & Hoffman (1970).

In addition, almost all observations of the parasite occur in trout farms, and from the few observations on natural populations of salmonids (Uspenskaya, 1957; Bogdanova, 1960, 1964, 1966, 1968, 1969, 1970 and 1972), it is difficult to ascertain if the infection was naturally endemic or introduced. There is therefore little evidence from which to define the original distribution of *M. cerebralis*. Nevertheless attempts have been made using the following criteria.

(i) The observation that *M. cerebralis* was first described in Europe, and some 50 years elapsed before it was identified in other continents.

(ii) The assumption that brown trout are a natural host.

(iii) The assumption that where *M. cerebralis* has been found among natural populations of European and Asian salmonids, these represent original foci of the parasite.

According to these criteria, *M. cerebralis* is thought to have had a natural distribution which included central Europe and North East Asia (Hoffman, 1970*a*; Bogdanova, 1972). Schulman (1966) supposes that the parasite has a holarctic distribution. In view of the relatively recent appearance of *M. cerebralis* in the U.S.A. and South America and its virtual

absence from Canada, it seems likely that the parasite was introduced to America recently although shipments of brown trout ova from Germany to America began in the 1880's.

CURRENT GEOGRAPHICAL DISTRIBUTION

Table I shows the current geographical distribution of *M. cerebralis*. Information could not be obtained from some countries. Inconsistencies are evident in the literature which it is not always possible to resolve.

TABLE I. The current world distribution of *M. cerebralis*

Continent	Country/state	*M. cerebralis* Reported (+) Not Reported (−)	Origin of Report
Africa	Cape Province	+	Wyk (1968), Smith (pers. Comm., 1973)
	Morocco	+	Preudhomme (1970)
	Transvaal	Suspected	Pott (pers. comm., 1973)
America	Canada	+	Bogdanova (1969)
		−	Margolis (pers. comm., 1972)
	South America		
	Columbia	Under investigation	Conroy (pers. comm., 1973)
	Ecuador	+	Meschkat (1971)
	Mexico	+	Osaka (pers. comm., 1973)
	Venezuela	−	Meyers (1969*a*, *b*)
	U.S.A.		
	California	+	Hnath (1970)
	Connecticut	+	Hnath (1970)
	Massachussets	+	Hoffman (pers. comm., 1973)
	Michigan	+	Hnath (1970)
	Nevada	+	Yasutake & Wolf (1970)
	New Jersey	+	Hoffman (1968)
	Ohio	+	Tidd & Tubb (1970)
	Pennsylvania	+	Hoffman *et al.* (1962)
	Virginia	+	Hoffman (1970*b*)
	West Virginia	+	Meyers (1969*b*)
Asia	China		
	Taiwan	−	Chen (pers. comm., 1973)
	Japan	−	Hoffman (1970*a*)
	Lebanon	+	Hoyek (pers. comm., 1973)
Australasia	Australia	−	Francois (pers. comm., 1973)
	New Zealand	+	Anon. (1971), Hewitt & Little (1972)
Europe	Austria	+	F.A.O. (1972)
	Belgium	+	F.A.O. (1972)
	Bulgaria	+	Margaritov (1960)
	Cyprus	−	F.A.O. (1972)
	Czechoslovakia	+	Dyk (1954), Volf (1957), Havelka & Volf (1970) & Lucky (1970)
	Denmark	+	Bruhl (1926) Schäperclaus (1954*b*, *c*) Rasmussen (1967), Christensen (1972*a*)
	Eire (Southern Ireland)	+	Halliday (1974*a*)
	Finland	−	F.A.O. (1972)

TABLE I.—*continued*

Continent	Country/state	*M. cerebralis* Reported (+) Not Reported (−)	Origin of Report
	France	+	Vanco (1952)
	Germany	+	Hofer (1903), Plehn (1904, 1924), Heuschman (1940, 1949), Schäperclaus (1931, 1954*a*)
	Greece	−	F.A.O. (1972)
	Hungry	+	F.A.O. (1972)
	Iceland	−	Palsson (pers. comm., 1973)
	Israel	−	Sarig (pers. comm., 1973)
	Italy	+	Scolaria (1954), Ghittino (1962 & 1970*b*)
	Liechtenstein	+	Luigg (pers. comm., 1973)
	Luxemburg	+	F.A.O. (1972)
	Netherlands	+	F.A.O. (1972)
	Norway	+	Håstein (1971)
	Poland	+	Kocylowski (1953)
	Portugal	−	Silva Leitão (pers. comm., 1974)
	Spain	+	Cordero-del-Campillo & Alvarez-Pellitero (pers. comm., 1974)
	Sweden	+	Johansson (1966)
	Switzerland	−	Thomson (1972)
	United Kingdom		
	England	−	Mawdesley-Thomas (pers. comm., 1973)
	Northern Ireland	−	Vickers (pers. comm., 1973)
	Scotland	+	Elson (1969), Roberts & Elson (1970)
	Wales	−	Mawdesley-Thomas (pers. comm., 1973)
	U.S.S.R.	+	Uspenskaya (1955 & 1957), Bogdanova (1960, 1966, 1968, 1969, 1970)
	Yugoslavia	+	Tomasec (1960)

(*i*) *Canada*

Bogdanova (1969) has found spores of *M. cerebralis* in wild *Salvelinus malma* from British Columbia. However, Margolis (pers. comm., 1972) examined over 500 fish from the same source, of which about half were *S. malma*. All were negative for the parasite, and he is sure that whirling disease has not been identified in Canada.

(*ii*) *Korea*

Whirling disease has often been quoted as occurring in Korea by Tinkina (1962). However, the report only states that the usual preventative measures are adopted against whirling disease. It cannot therefore be accepted that the disease occurs in Korea. No information could be obtained to clarify this point.

(*iii*) *South America*

Reports have been published stating that whirling disease has occurred in Venezuela

(Anon., 1969*a*; Martinez, 1966 cited by Bogdanova, 1968). The report by Martinez (1966) is not mentioned in her bibliography. According to Meyers (1969*b*) the authorities deny its occurrence; this discrepancy was apparently due to problems in translation.

III. THE HOST RANGE OF *M. CEREBRALIS*

The parasite has been mainly identified in farm-reared salmonids and, with the exception of work by Uspenskaya (1957) and Bogdanova (1960, 1964, 1966, 1968, 1969, 1970 & 1972) in the U.S.S.R, has only rarely been detected in the wild.

Table II illustrates the known host range of *M. cerebralis* in both farms and natural populations of salmonids (Uspenskaya, 1957; Hoffman, 1970*b*; Havelka & Volf, 1970; Yasutake & Wolf, 1970; Bogdanova, 1972; Christensen, 1972*b*; Yoder, 1972).

TABLE II. The occurence of *M. cerebralis* in salmonids in hatcheries and natural waters

Species	Europe and Asia and Far East		America	
	Natural waters	Hatcheries	Natural waters	Hatcheries
Salmo clarki	−	−	−	+++
S. gairdneri (*S. irideus*)	−	+++	+	+++
S. ischchan isp. aestivalis	−	+	−	−
S. ischchan isp. gegerkuni	−	+++	−	−
S. salar	+	+++	−	−
S. trutta	+	+++	+	+++
S. trutta labrax	+	+++	−	−
Salvelinus fontinalis	−	+++	+	+++
S. leucomasnis	+	−	−	−
S. malma	+	−	+	−
S. namaychush			0	
Oncorhynchus gorbushcha	+	+	−	−
O. keta	+	+	−	−
O. kisutch			0	
O. masu	+	+	−	−
O. nerka	−	+	−	−
O. tschawytscha				0

\+ *M. cerebralis present.*
+++ Whirling disease recorded.
0 Experimental infection.
— Fish not examined.

M. cerebralis has been recorded from seventeen species of the genera *Salmo*, *Oncorhynchus* and *Salvelinus*. The parasite has also been reported from grayling (*Thymallus thymallus*) by Volf (1957), Havelka & Volf (1970) and Bogdanova (1971); tench (*Tinca tinca*), gudgeon (*Gobio gobio*), pike (*Esox lucius*) and perch (*Perca fluviatilus*) by Ramirez-Medina (1962). Dannevig & Hansen (1952) reported infections of *M. cerebralis* in young aquarium-reared herring (*Clupea harengus*). These reports of *M. cerebralis* in coarse fish and herring are possibly errors in identification due to the many species of *Myxosoma* and *Myxobolus*.

Bogdanova (1970) stated that all salmonids participated in the distribution of the parasite in nature, but that nonanadromous forms were the most important hosts. Infection only took place in fresh water. The incidence of infection could reach 100% but the intensity was low and no disease symptoms were observed.

Little is known of the differential susceptibility of salmonids. Rainbow trout are most seriously affected, brook trout less severely and brown trout seem to be more resistant to clinical infection (Hoffman *et al.*, 1962). Although current information indicates that the parasite is generally limited to salmonids, other species of fish may be natural or potential hosts.

IV. THE LIFE CYCLE, DEVELOPMENT AND TRANSMISSION OF *M. CEREBRALIS*

LIFE CYCLE

Information is scarce on the means of infection. Experiments designed to infect susceptible fry have been unsuccessful or inconclusive. Hoffman & Putz (1970*a*) state that in 26 attempts over an 8 year period only seven aquaria yielded positive infections. Since Schäperclaus (1954*a*) and Hoffman *et al.* (1962) were unable to infect fish by exposing them to spores, they supposed that either an intermediate host and/or different water conditions were necessary for infection to occur. However, Uspenskaya, 1966*b* (pers. comm., to Walliker) was able to produce an infection by ageing the spores for 4 months in a non-freezing stream before introducing them into the fishes' stomachs. Hoffman & Putz (1969), Putz (1970) and Putz & Herman (1970) confirmed the spores aged in the presence of mud for 3·5 to 6 months became infective. In addition, they demonstrated that the infective agents were retained by a 12 μm filter. Schäperclaus (1931) and Halliday (1973*b*, 1974*a*) were able to produce infections by exposing rainbow trout to mud taken from earthen ponds which had previously contained infected fish.

The factors, organic or inorganic, influencing the infectivity of the spores are unknown. It is generally assumed that the spores are ingested by the host, although infection by the gills is also possible.

Initial penetration of the host's tissues is assumed to take place in the intestine. This assumption is based on the observation that the action of the digestive juices, caused the spores of several species of myxosporidia to extrude their polar filaments, split along the suture line and liberate their sporoplasm (Erdman, 1971; Kudo, 1922). The polar filaments are thought to anchor the spore during its passage through the alimentary canal (Kudo, 1930). Lom (1964); Hoffman, *et al.* (1965); Plehn (1904) and Uspenskaya (1957) found that acid (unspecified), glycerine, alcohol, ether and artificial gastric juice did not cause extrusion of the filaments of *M. cerebralis* whereas 1–2% potassium hydroxide was successful (Meyers, Scala & Simmons, 1970).

Halliday (unpublished results) failed to release consistently the sporoplasm from *M. cerebralis* spores which had been freshly collected or stored for 4 months at 4° C by treating them with digestive juices collected from various parts of the alimentary tract of rainbow trout. It is possible that the ageing process required before the spores are infective may affect the release of the sporoplasm. To the author's knowledge no published work exists on this aspect of the biology of *M. cerebralis*.

After release, the sporoplasm is thought to pass between the cells of the intestinal tract and reach the cartilage by means of the blood, lymph, and/or coelomic fluid. In the cartilage it develops into a multinucleate amoeboid trophozoite. The subsequent development of *M. cerebralis* (from histological observations), has been described by several authors (Plehn, 1904; Schäperclaus, 1931; Hoffman, *et al.*, 1962; Lucky, 1970; Halliday, 1973*b*; O'Grodnick & Gustafson, 1974). Although the trophozoites

can be stained with haemotoxylin and eosin, better results are obtained with the Mallory Heidenhain (Casson modification) (O'Grodnick & Gustafson, 1974). As far as the author is aware electron microscopy has not been used in the study of the development of *M. cerebralis*.

Once established, the trophozoite grows by nuclear division and cytoplasmic growth. It may reach a maximum of 1 mm in diameter (Hoffman, *et al.*, 1962) with 50 or more nuclei (Bykhovskaya-Pavlovskaya *et al.*, 1962). Nutrients are assumed to be absorbed at the surface of the trophozoite, since pinocytosis and phagocytosis have been observed in other species of *Myxosporidia* (Lom & Puytorac, 1965; Uspenskaya, 1966; Lom, 1969).

In some species of *Myxosporidia*, division of the nuclei is accompanied by plasmotomy (Minchin, 1922; Noble, 1944). If plasmotomy is a regular feature of the development of *M. cerebralis* trophozoites, clinical infections could result from initial infections of a single trophozoite. However, Putz (1970) suggests that there is a relationship between the duration of exposure of fry to *M. cerebralis* and the severity of the infection. The production of clinical symptoms seemed to require an accumulation of infective agents in the host. Thus it seems likely that the infective potential of a single spore is limited.

The factors regulating the growth of the trophozoite are unknown, but at some stage the process of spore formation begins. Within the trophozoite pansporoblasts are formed which contain 12–14 nuclei (Hoffman, *et al.*, 1962; Christensen, 1966). Two spores are formed within each pansporoblast. The nuclear products are incorporated into the structures of each spore as follows—two nuclei contribute to the shell valves, two nuclei contribute to the polar capsules, and two nuclei form the sporoplasm nuclei. Nucleic acids have been demonstrated in spores of *M. cerebralis* by Halliday (1973*b*).

The final feature which has been observed in the parasitic development of the spores of *M. cerebralis* is their acquisition of acid fastness (Halliday, 1973*b*). Completely acid fast spores have been called ' mature ' by Halliday (1973*b*). However, it is not known if this feature indicates that spore development in the host is complete. The final development of the spore necessary for infection takes place after the spore has been released.

DEVELOPMENTAL TIME SCALE

Observations on the initial stages of the parasite are limited. However, it is generally thought that trophozoites are evident from about 40 days post infection until 3 months (Schäperclaus, 1931; Hoffman, *et al.*, 1962). Spores can be demonstrated in the cartilage of infected fish 4–6 months after infection (Schäperclaus, 1931, Hoffman & Putz, 1969; Putz, 1970; Tidd & Tubb, 1970). Most of these observations were taken from hatchery epizootics but have now been substantiated by experiment (Halliday, 1973*b*; O'Grodnick & Gustafson, 1974).

Bogdanova (1960) and Schäperclaus (1931) have noted that the water temperature affects the rate of parasitic development. The effects of temperature on development have been studied by Halliday (1973*b*). From experimental infection of rainbow trout, he found trophozoites were apparent 35 days after exposure to infection and spores were completely acid fast by 52 days after infection when the fish were reared at 17° C. At water temperatures of 12 and 7° C, spores took 101 and 120 days respectively to become acid fast.

In addition to water temperature, the results of Halliday (1974*a*) suggest that parasitic development may be retarded in older fish. In experimentally infected rainbow trout 7 months old, spores were first identified at 100 and 150 days after infection at 17 and 8° C respectively.

TRANSMISSION

As in other species of *Myxosporidia*, the details of transmission are unknown. It is generally assumed that the spores are the infective stage. The spores are thought to be released after the death of the host. However, Uspenskaya (1957 & 1964) and Rydlo (1971) have found spores in other parts of the body and they suggested that spores may be released in the faeces. Schäperclaus (1954*a*) Meyers *et al.* (1970) have implicated avian vectors in the transmission of *M. cerebralis* by showing that spores are found in the faeces of the great blue heron (*Ardea lerodias*). But Hoffman & Putz (1970*b*) could not infect fry with heron faeces containing *M. cerebralis* spores aged 4 months. However it is not known if the spores were viable. The possibilities of cannibalism have been investigated by Putz and Hoffman (1970). They could not infect trout by feeding them with ground fish heads containing various parasitic stages.

Transovarian transmission of the parasite has not been demonstrated, O'Grodnick & Gustafson (1974) but viable spores may be transferred with eggs, (Schäperclaus, 1931; Keiz, 1964; Putz & Hoffman, 1970).

An important factor in the transmission of the parasite is the resistance of the infective agent to environmental conditions and disinfectants, but studies on the survival of *M. cerebralis* spores are faced with the problem that no reproducible tests exist for determining viability. In studies on *Myxosporidia*, a commonly accepted criterion of spore viability is the extrusion of the polar filaments. However, the variety of treatments which produce extrusion of the filaments suggests that this property may not be an indicator of spore viability. McKinney & Bradford (1970) propose that the viability of *M. cerebralis* spores may be determined by measuring their respiration. Other workers, e.g. Hoffman *et al.* (1962), examined the morphology of the spores and noted if any abnormalities arose from the treatments.

According to Plehn (1904, 1924) and Schäperclaus (1931, 1954*a*), the spores are resistant to drying and freezing and have a long survival time. Bauer (1959) suggests that they retain their viability for 12 years, Hoffman *et al.* (1962) found a maximum of 3 years storage, while Funk (1968) believes that they are viable for 30 years. Hoffman & Putz (1969) found keeping spores at a temperature of 60–100° C for 10 min is sufficient to kill them. Thus, Hoffman (1970*b*) suggests that spores would be killed if trout were smoked. However, the spores retain their infectivity after freezing at —20° C for 2 months (Putz, 1970; Hoffman & Putz, 1971).

Transmission of *M. cerebralis* may therefore be effected by shipments of live trout, frozen trout, eggs and by birds. Halliday (1974*a*) suggests that human agency and wind may be additional vectors. In conclusion, the complete life cycle of *M. cerebralis* has not been established. Observations have been limited to the development of the parasite once it is established in the cranial cartilage.

V. PATHOLOGY OF *M. CEREBRALIS* IN RAINBOW TROUT

SYMPTOMATOLOGY

The symptomatology of whirling disease has been described by Plehn (1904), Schäperclaus (1931, 1954*a*), Uspenskaya (1957), Hoffman *et al.*, (1962), Christensen

(1966), Bogdanova (1968), Havelka & Volf (1970) and Meyers & Scala (1969). Clinical symptoms are evident only in young fish. The most obvious clinical sign is a tail chasing movement which occurs 2–3 months after infection (Schäperclaus, 1931). According to Hoffman *et al.* (1962), whirling can last a year after infection. However, Halliday (1974*a*) has studied outbreaks of the disease where whirling initially appeared in yearlings. Whirling was at one time thought to be caused by toxins released by the parasite (Plehn, 1904, 1924), but it seems more likely to be caused by erosion of the cartilage surrounding the auditory organ (Hoffman & Dunbar, 1961). In addition, observations by Christensen (1966) and Halliday (1974*a*) suggest that granulation tissue, produced by the host between 8–12 months after infection (Lucky, 1970), can cause pressure on the auditory capsule and thus induce whirling. Halliday (1974*a*) suggests that when this is produced in response to an earlier subclinical infection, it could initiate whirling in yearlings and prolong whirling in survivors. In some fish which display whirling the symptoms can disappear (Schäperclaus, 1931; Halliday—unpublished observations). This is assumed to be caused by ossification of the auditory capsule alleviating the effects of the parasite. Excessive whirling can cause severe exhaustion, malnutrition and death.

The parasite can also infect the cartilage of the spinal column, and when this occurs posterior to the 26th vertebra, it causes pressure on the caudal nerves which control the pigment cells in the tail. This produces a black tail in the fish (Plehn, 1904; Schäperclaus, 1954; Hoffman *et al.*, 1962; Hoffman, 1966) and causes permanent spinal deformities in survivors (Hoffman, 1966; Havelka *et al.*, 1971).

The additional deformities listed below have been associated with infections of *M. cerebralis*:

(i) Cranial deformities—due to interference with the process of osteogenesis (Hoffman *et al.*, 1962; Christensen, 1966; Hoffman, 1970*b*).

(ii) Deformities of the jaws and opercula (Christensen, 1966; Havelka & Volf, 1970; Lucky, 1970).

(iii) Disintegration of the fins (Havelka & Volf, 1970).

(iv) Opercular cysts (Taylor & Haber, 1974).

The symptomatology of epizootics varies. Schäperclaus (1931) has described whirling as the most common symptom, Uspenskaya (1957) black tails while Bauer (1959) has attributed high mortalities in fish which did not display any abnormal signs. These observations may be explained in terms of variations in the diasthetic condition of the host. Bauer (1959) has postulated that symptoms are manifest when the critical phase of the infection has been overcome. A decrease in growth rate can occur among survivors of an epidemic (Uspenskaya, 1957; Rydlo, 1971; Hoffman, 1974).

It is generally considered that susceptibility to the parasite decreases with increasing age of the host, (Hoffman *et al.*, 1962; Rasmussem, 1965; Putz & Hoffman, 1966). However, there are several reports of older fish becoming infected (Schäperclaus, 1954*a*; Bradford & McKinney, 1969—quoted by Hoffman, 1970*b*; Halliday, 1974*a*). Halliday (1974*a*) suggested that reinfection is possible.

Water temperature is another factor which is known to influence the symptomatology of whirling disease. Schäperclaus (1931) reported that the course of whirling disease was mild at water temperatures of 20–25° C. This was attributed to the more intensive growth rate of trout at these temperatures. Halliday (1973*b*) observed that symptoms (whirling and black tails) were more frequent in rainbow trout infected at 17° C than those at 12 and 7° C. In addition he suggested, that should there have been

any suboptimal nutrition, bone and cartilage may have been more susceptible to disease at high growth rates. The observations of Schäperclaus may equally be interpreted as the parasite being unable to withstand very high water temperatures (Lom, 1970).

HISTOPATHOLOGY

The histopathology of *M. cerebralis* is dependent on at least three variables.

(i) The age of the host when first exposed to infection (Hoffman *et al.*, 1962).

(ii) The number of infective units to which the fish is exposed (Putz, 1970).

(iii) The ambient water temperatures (Halliday, 1973*b*).

The trophozoite is the pathogenic stage of the parasite, and, after an incubation period (40–60 days) which is temperature dependent (Halliday, 1973*b*) it directly or indirectly destroys the cartilage (Schäperclaus, 1954; Hoffman *et al.*, 1962; Lucky, 1970). Osteogenesis is disrupted and irregular bone formation producing permanent deformities can result. The main reaction to the infection is the production of granulation tissue (Hoffman *et al.*, 1962; Lucky, 1970; Taylor & Haber, 1974). Lucky (1970) concluded the tissue response was dependent on the site of infection. Roberts & Elson (1970) did not identify any host defence reaction in rainbow trout 4–6 months old. Halliday (1973*b*) and Runnells, Monlux & Monlux (1965) have suggested that a process of fibrinous inflammation may occur in an infected area.

In general, whirling disease is mainly a disease of fry. The increased resistance of older fish is assumed to be due to a decrease in the amount of cartilage produced by osteogenesis, and calcification of existing cartilage. The details of these processes remain to be investigated but they are dependent on both the age and growth of the fish.

VI. DIAGNOSIS OF THE INFECTION

The need for an accurate diagnostic technique has been emphasised by the European Inland Fisheries Advisory Commission (Christensen, 1972*b*). Direct demonstration of the presence of the parasite is the only certain method of diagnosis (Hoffman *et al.*, 1962, 1968) as symptoms similar to those associated with whirling disease are found in other diseases (Halliday, 1973*a*). Due to the difficulties of conclusively identifying the amoeboid stages (Hoffman *et al.*, 1962; Halliday, 1973*b*), the spores are considered as the diagnostic criterion (Hoffman *et al.*, 1968).

Techniques for the routine examination of fish for *M. cerebralis* have been outlined by Hoffman *et al.* (1968). The spores can be demonstrated by either maceration of the cranial skeleton or scraping the bones surrounding the auditory capsule, and examining a sample of the preparation microscopically. Since these techniques were proposed, more sensitive methods have been described (MacLean, 1971; Prasher *et al.*, 1971; Rydlo, 1971; Halliday, 1973*a*; Landolt, 1973; Tidd, Tubb & Wright, 1973; O'Grodnick, 1975). In general these techniques concentrate the spores (if present) by homogenizing the skeleton, and centrifuging the homogenate with or without prior filtration. The preparation can then be stained by Giemsa, malachite green or methylene blue to aid recognition of the spores. The sensitivity of these methods, as pointed out by Halliday (1973*a*), is likely to decrease with older fish due to ossification of the skeleton making homogenization of the cranium difficult. A more detailed investigation of the recovery of *M. cerebralis* spores from infected fish has been carried out by Contos & Rothenbacher (1974) and Markiw & Wolf (1974). Either technique is

suitable for diagnosis and the preparation of spore antigens for immunological studies.

Halliday (1974*b*) has investigated the possibilities of using an indirect fluorescent antibody test on the basis that since spores lodge in the host's tissues for a considerable time they may be a source of antigen. However, although antibody to spores of *M. cerebralis* could be detected in a rabbit inoculated with a suspension of spores, it could not be detected from rainbow trout inoculated with spores or from clinically infected yearlings. Pauley (1974) has shown that spore antigens mimic antigens of rainbow trout.

VII. IDENTIFICATION OF *M. CEREBRALIS*

The classification of the *Myxosporidia* relies entirely on features of the spore (Kudo, 1920). The large number of species described by this system indicates its limitations. The most modern description of the spores of *M. cerebralis* is given by Lom & Hoffman (1970). The average dimensions (in μm) are as follows.

Spore	Length 9·7	Breadth 8·5
Polar capsules	Length 4·2	Breadth 3·1
Polar filaments	5–6 coils	

The spores are usually oval. Polar capsules are normally of equal size. There is no regular intercapsular process but only a thickening on the shell border. Shell markings are absent from the frontal view. The scanning electron microscope has revealed a deep ridge separating the vaulted surface of the shell from the valve border, a distinct opening of the canal for the discharge of polar filaments, as well as a mucous envelope on the posterior half of the spore (Lom & Vavra, 1963; Lom & Hoffman, 1970, 1971). These characteristics are considered as being species specific. Furthermore, Lom & Hoffman suggest that parasites of American and European origin should be compared. Halliday (unpublished results) has examined spores of *M. cerebralis* from Denmark with a scanning electron microscope and identified the same structures as described by Lom & Hoffman. No additional morphological characteristics were observed. Immunological techniques may be used as a further means of clarifying the problems on inter- and intra-specific variation (Halliday, 1974*b*; Pauley, 1974).

Hoffman *et al.* (1965) have prepared a list of the cartilophagous *Myxosporidia* of the genus *Myxosoma* from North American fish. They conclude that *M. cerebralis* has the smallest spores. Among species of the genus *Myxobolus* listed by Bykhovskaya-Pavlovskaya *et al.* (1962), *Mxyobolus neurobius* invades the spinal cord and brain of salmonids. The author has identified this parasite in brown trout (unpublished). However, in view of its large size, different spore shape and polar capsules, confusion with *M. cerebralis* is unlikely.

VIII. CONTROL AND TREATMENT

Disinfection, husbandry and water treatment, have been used with varying success to control whirling disease (Hoffman *et al.*, 1962; Rasmussen, 1958, 1961 and 1965; Brierly & Scott, 1965; Hoffman & Putz, 1969; Ghittino, 1970*a*; Hoffman, 1970*b*, *c*; Hoffman & Hoffman, 1972). Hoffman *et al.* (1962) and Schäperclaus (1954) recommend destroying all fish which have been exposed to the infection. However, Bauer (1959), Rasmussen (1965) and Bogdanova (1968) suggest that only those fish which exhibit symptoms should be destroyed.

DISINFECTION

After removing the infected fish, the holding facilities can be disinfected by one of the following methods (Schäperclaus, 1931; Tack, 1951; Scolari, 1955; Rasmussen, 1958; Hoffman *et al.*, 1962; Hoffman & Putz, 1969; Meyers & Scala, 1969; Werdelin, 1970; Hoffman, 1970*b*, *c*; Hoffman & Hoffman, 1972).

(i) *Hatchery facilities and rearing tanks*

Calcium hydroxide	0·5–2·0%
Calcium oxide (quick lime)	0·25–2·0%
Chlorination	
Sodium hypochlorite	1600 p.p.m.
Chlorine	200 p.p.m.
Potassium hydroxide	
Roccal (alkyl dimethyl benzyl ammonium chloride)	200–800 p.p.m.
Sodium hydroxide	1–2%

(ii) *Earthen ponds*

Calcium oxide	1250 kg/ha
Calcium cyanamide	50000–10000 kg/ha

Schäperclaus (1954*a*) states that calcium cyanamide is more effective than quicklime. Since earthen ponds are difficult to disinfect (Hoffman, 1970*b*), they are not recommended for rearing fry. Schäperclaus (1931) suggests after treatment with calcium cyanamide, the upper layers of mud and silt should be removed. Disinfection should be repeated with flushing between treatments. Tack (1951) allowed 6 weeks before adding fingerlings. The success of the treatment can be assessed by using susceptible test fish in floating cages (Hnath, 1970).

HUSBANDRY

Prevention of the disease is based on the premise that once a fish has reached a length of 5–6 cm (i.e. approximately 4–5 months of age) it is generally resistant to infection because ossification of the skeleton will prevent massive infection. Since ossification is a function of both the age and growth of the fish, these factors must be taken into account in control measures (Rasmussen, 1967). This method is used successfully in Europe (Christensen, 1966; Rasmussen, 1961, 1967; Ghittino, 1970*b*). The technique is summarised as follows.

Eggs are hatched in spore-free water. After hatching, the fish are reared in either tanks or raceways. These replace the earthen ponds which were a source of infection. The fish are placed in earthen ponds only when they are considered to be clinically resistant to the parasite.

The success of this technique in Denmark has been assessed by Halliday (1974*a*). His results showed that where trout farms used stream water, subclinical infections existed in many of the tanks and raceways used to prevent the disease. It was considered that transmission of the infection within these rearing facilities was unlikely and the infections were due therefore to spores entering the tanks in the water supply. Subclinical infections were also prevalent among yearlings in earthen ponds.

As in Denmark, trout farmers in the U.S.A. have been able to reduce signs of whirling disease by rearing fry in concrete raceways and restocking cleaned ponds. Eradication of whirling disease from two fish hatcheries has been achieved by these procedures and the convertion to well water. The carrier problem certainly exists in

the U.S.A. but there has been voluntary control of the spread of carriers (Hoffman pers. comm., 1973).

A risk of whirling disease limits the methods which can be used to produce fry particularly when the farm is receiving water which has already passed through other farms. The problem of whirling disease is amplified to an extent which makes fry-rearing impossible (Christensen, pers. comm., 1973).

The production of trout free from infection with *M. cerebralis* is only essential when the fish are being exported to countries where the parasite has not been reported or which have legislation against its introduction. In addition to normal farming procedures, restocking operations and ' put and take ' harvesting may be susceptible to such an opportunist pathogen as *M. cerebralis* (Halliday, 1974*a*). Therefore its introduction to such systems should be avoided where possible.

WATER TREATMENT

Several types of filtration systems have been developed to eradicate the problem of whirling disease. Sand-charcoal filters have met with some success in France (Hoffman *et al.*, 1962). Recently a method of irradiating hatchery water supplies with ultra-violet light has been developed. This treatment, in addition to destroying other fish pathogens kills the spores of two important myxosporidians—*Ceratomyxa shasta* and *M. cerebralis* (Leith & Moore, 1967; Anon., 1969*b*; Bedell, 1971; Flatow, 1971. Fryer, 1971; Sanders, Fryer, Leith & Moore, 1972; Hoffman, 1970*c*, 1974). 2537 Å units of UV at dosages of 35 000, 43 000 and 112 000 MWS/cm^2 prevented infection. According to Ivanov *et al.* (1968) (cited by Bogdanova, 1972) this treatment has been used in Russia with promising results.

CHEMOTHERAPY

No proven chemotherapy is available at present. However, some control has been achieved with the drug acetarsone (N-acetyl-4-hydroxy-m-arsanilic acid or stovarsol) at 10 mg per kg of fish, (Vanco, 1952; Scolari, 1954; Bauer, 1959; Ghittino, 1970*b*). Fish are treated daily on three consecutive days with weekly intervals between each course. Hoffman *et al.* (1962) concludes that further work is required to evaluate this treatment. Ten drugs have been tested by O'Grodnick and Gustafson (1973, 1974) for action against *M. cerebralis* in rainbow trout. The drug amprolium hydrochloride showed promise of success causing a reduction in spore numbers in test fish but could be toxic. Taylor *et al.* (1973) have shown the drug furazolidone reduces spore formation. Further studies are in progress to determine the minimum effective dose.

INTERNATIONAL LEGISLATION IN THE CONTROL OF WHIRLING DISEASE

A lack of national legislation is a major factor in the spread of fish diseases. A recent E.I.F.A.C. survey in 1969 (cited by Hjul, 1971) stated that 38 of 86 countries had no regulations governing the imports and exports of fish and fish eggs. They further state that the most reported disease was whirling disease. Disease regulations to prevent the spread of *M. cerebralis* are being emphasised at the state, national and international levels (Hoffman *et al.*, 1968; Tunison, 1969; Hoffman, 1970*a*, *b*; Canadian Committee on Fish Diseases, 1972; Mackelvie, 1972). An F.A.O. report (F.A.O., 1972) concludes that the most practical disease control system is one of inspection and certification at the source. Systems of farm disease records have also

been proposed by Tunison (1969) and the Canadian Committee on Fish Diseases (1972).

The effectiveness of certification in controlling whirling disease is dependent on the number of fish examined and the efficiency of the diagnostic technique. Hoffman *et al.* (1968) suggest that a minimum of 1 fish per 1000 should be examined. The U.S.A. Division of Fish Hatcheries (Anon., 1969*c*) proposes that hatcheries should be sampled to detect an incidence of more than 4%. Two inspections for *M. cerebralis* are proposed, both to take place during the first year of life. Halliday (1974*a*) suggests that an annual examination of both fry and fingerlings would give a more accurate result rather than the examination of export shipments. With this system farms could be granted an export licence. Since the present diagnostic techniques for whirling disease can only demonstrate the presence of an infection but not its absence, the local or national incidence of the parasite should also be taken into account for the purposes of legislation. Thus, Halliday (1974*a*) suggests that in those countries where *M. cerebralis* is known to be established, salmonids should only be certified as free from the parasite if they have been reared in isolation from other fish in spore-free water, and tests for the parasite are negative.

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Studies on the proposed role of *Tubifex tubifex* (Muller) as an intermediate host in the life cycle of *Myxosoma cerebralis* (Hofer, 1903)

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Abstract. In a recently proposed hypothesis for the transmission of *Myxosoma cerebralis*, the causative agent of salmonid whirling disease, it was suggested that there was a developmental cycle in tubificid worms culminating in actinosporean spores, which were infective to the fish. Results are presented here which do not support the actinosporean involvement in the life cycle. On addition of *M. cerebralis* spores to *Tubifex tubifex* colonized in sterilized medium, no significant change in the prevalence of *Triactinomyxon dubium* (i.e. *T. gyrosalmo*) was found. Although it is shown that these worms are capable of ingesting *M. cerebralis* spores, neither hatching of the spores nor further development within the worm has been observed. Field observations on the distribution of actinosporean species show no obvious correlation between the occurrence of *T. dubium* and *M. cerebralis*.

Introduction

The exact mode of transmission of *Myxosoma cerebralis* (Hofer, 1903), the causative agent of whirling disease in salmonid fish, has long remained problematical. Uspenskaya (quoted by Hoffman & Putz 1969) reported direct transmission of the disease but only after *M. cerebralis* spores had undergone some type of ageing process. More recently, Prihoda (1983) produced similar results. However, a more radical hypothesis for the transmission has been suggested by Markiw & Wolf (1983) and Wolf & Markiw (1984). They proposed that *M. cerebralis* spores were not in themselves infective to salmonids but that the spores passed into *Tubifex tubifex*, an oligochaete worm, and there underwent further development. The phase in *T. tubifex* corresponded to the life cycle of an actinosporean species which they named *Triactinomyxon gyrosalmo*. At the end of this cycle typical actinosporean spores were produced, which in turn infected juvenile fish. Thus *M. cerebralis* in fish and *T. gyrosalmo* in tubificied worms were considered to be parts of a single life cycle.

Most recently, Wolf, Markiw & Hiltunen (1986) have shown that on exposure to *M. cerebralis* spores, the prevalence of *T. gyrosalmo* in *T. tubifex* increases significantly. This paper examines the roles of Actinosporea and tubificid worms in the life cycle of *M. cerebralis* by means of prevalence studies, and also presents the results of histological studies which were undertaken to detect the uptake of *M. cerebralis* spores into *T. tubifex*. In addition, field observations on the distribution of various actinosporean species are included.

In their description of *T. gyrosalmo*, Wolf & Markiw gave spore dimensions as: epispore, 36 μm long, containing between 32–50 sporoplasms, surmounting a style, 90 μm in length, with projecting arms reaching 170 μm further. These characteristics and the published illustration do not differ substantially from a previously described species *T. dubium* Gra-

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nata, 1924. The nine described species of *Triactinomyxon* have been reviewed by Marques (1984) and, after comparison of *T. gyrosalmo* with these, we believe that *T. gyrosalmo* is, in fact, synonymous with *T. dubium*.

Methods and materials

Field observations on Actinosporea

The six sources listed in Table 1, all but one of which was in the British Isles, were sampled for the presence of *Tubifex* worms infected with actinosporean species.

Isolation of oligochaetes and identification of Actinosporea

Mud was passed through a 56 μm filter to remove all oligochaetes for examination. Identification of the worms, according to the key of Brinkhurst (1971), was made by taking subsamples. Individual worms were then smeared on glass slides and examined for the presence of Actinosporea under bright field and phase contrast illumination. Actinosporean species were identified according to descriptions given by Mackinnon & Adam (1924), Janiszewska (1955, 1957), Wolf & Markiw (1984) and Marques (1984).

Tubifex *infection experiments*

Cultures. Worms derived from a local sewage farm were identified and examined for the presence of Actinosporea as above. They were then added in batches of 1000 to culture vessels containing 5 l of sterile water over a mixture of heat sterilised soil and sand to which nutrient agar was periodically added to provide an organic input. The mud substrate was stirred at weekly intervals during the course of each experiment.

M. cerebralis spores. Spores were obtained from the heads of young rainbow trout, *Salmo gairdineri* Richardson, (3–7 months) originating from a serious outbreak of whirling disease on a fish farm in Brittany, France. The heads were kept cool in transit and on arrival were immediately washed in phosphate buffered saline (PBS), pH7. Each was thoroughly macerated in 10 ml PBS. Spore numbers were estimated using a haemocytometer and at least 1×10^6 spores in this crude suspension were added to each *Tubifex* culture. At no point were the spores exposed to any chemical treatments. Cultures to which spores had not been added served as negative controls.

Initially, three pairs of tanks, to which *M. cerebralis* spores had been added, were maintained at temperatures of 10, 15 and 20°C, respectively. Each pair had a corresponding negative control culture. Infection with Actinosporea in the worms was monitored for up to 30 weeks after the addition of the spores. Subsequently, these experiments were repeated at temperatures of 10°C and 15°C using *Tubifex*, which contained a range of actinosporean infections derived from other sewage farms. In total, over a period of 20 months, 18 worm cultures were exposed to *M. cerebralis* spores, derived not only from the Brittany outbreak but also from two infected farms in West Germany, all with the appropriate controls.

Exposure of T. tubifex *to* M. cerebralis *spores*

A sample of 80 *T. tubifex* were washed thoroughly in PBS, then left overnight in running dechlorinated water. The worms were placed in batches of 10 into eight plastic tubs, containing a heat sterilized mud substrate with nutrient agar added, covered by 10 ml of heat sterilized dechlorinated tapwater. After equilibrating for 48 h in a dark box, freshly isolated *M. cerebralis* spores in PBS were added to four of the tubs in volumes equivalent to 10 000 spores per tub. PBS only was added to the remaining 40 worms, which served as controls. Ten worms, five from the test tubs and five from the controls were sieved out at intervals of 2, 5, 7, 14, 21, 28, 35 and 42 days after the addition of spores. Worms were fixed in Carnoy's fluid for 45 min and were then transferred to 95% ethanol, containing 1% eosin, for 1 h. After immersion in absolute ethanol for 2 h, followed by xylene for 1·5 h, the worms were embedded in Paraplast wax. Sections cut at 7 μm were stained by the Giemsa Colophonium method (Shortt & Cooper 1948). Slides were mounted in Green Euparal for examination.

Results

Table 1 shows the prevalence rate of actinosporean species from the sites from which mud samples were taken. Tables 2, 3 & 4 show the prevalence rate of Actinosporea, including *T. dubium*, over a period of months after the addition of *M. cerebralis* spores to *Tubifex* cultures. There were no significant changes in prevalence rate of any of the three Actinosporea during the period of observation. In the largest experiment (Table 2), there were no significant changes in prevalence in any of the three Actinosporea during the period of observation. The highest test prevalence recorded was 3%, as against 2·5% in control

Table 1. Prevalence of actinosporean species from the sites from which mud samples were taken

Source	Actinosporean present	Prevalence rate (per 200 worms unless otherwise stated)
Cambrian Fish Farm*	(a) Inlet: *Echinactinomyxon radiatum*	1·0
	Synactinomyxon tubificis	1·5
	(b) Outlet: *S. tubificis*	2·0
Hammer Fish Farm*	(a) Ponds: *Triactinomyxon ignotum*	0·5
	(b) Outlet: *E. radiatum*	0·5
Brittany Fish Farm†	(a) Ponds: None found	— (n=107)
	(b) Outlet: None found	— (n=42)
Stratfield Saye, Fish Farm	(a) Outlet: *E. radiatum*	0·5 (n=187)
	T. ignotum	1·5 (n=187)
M.A.F.F.‡ (Weymouth)	*E. radiatum*	9·5
	S. tubificis	2·0
	T. ignotum	2·0
	T. dubium (i.e. *T. gyrosalmo*)	0·5
	Auractinomyxon sp.	0·5
Sewage Farm	*E. radiatum*	0·5
	T. dubium (i.e. *T. gyrosalmo*)	0·5
	S. tubificis	1·0

*Previously diagnosed by M.A.F.F. and Water Authority inspectors as whirling disease positive.
†Whirling disease diagnosed by authors.
‡Whirling disease being cycled in an experimental infection system.

Table 2. Prevalence of actinosporeans 2, 3·5, 6, 7 and 8 months after adding *Myxosoma cerebralis* spores to *T. tubifex* cultures held at 10, 15 and 20°C (figures in italics represent prevalences in test cultures)

	T. dubium (*T. gyrosalmo*)		*S. tubificis*		*E. radiatum*	
	Control (%)	Test (%)	Control (%)	Test (%)	Control (%)	Test (%)
***2 months* (n=200) after spore addition**						
10°C	0·5	*0* *0·5*	0·5	*0* *0·5*	0	*0·5* *0*
15°C	1·0	*2·5* *1·0*	0·5	*1·0* *0·5*	1·5	*0·5* *1·0*
20°C	0·5	*1·0* *0*	0	*1·0* *0*	0	*0* *0*
***3·5 months* (n=200)**						
10°C	1·0	*1·0* *0*	0·5	*3·0* *1·0*	1·0	*1·0* *1·5*
15°C	2·5	*0·5* *1·5*	1·0	*0* *0·5*	0·5	*1·0* *0*
20°C	0·5	*3·0* *1·5*	0·5	*0* *0*	0	*1·0* *1·0*
***6 months* (n=200)**						
10°C	0·5	*0* *0·5*	0	*0·5* *0*	0·5	*1·5* *2·0*
15°C	0	*1·0* *0*	2·0	*0* *1·0*	0	*1·0* *0·5*
20°C	1·5	*1·0* *1·0*	1·5	*1·0* *0·5*	0·5	*0* *0·5*
***7 months* (n=100)**						
10°C	0	*0* *0*	2·0	*1·0* *0*	1·0	*0* *1·0*
15°C	1·0	*0* *0*	0	*1·0* *0*	2·0	*2·0* *1·0*
20°C	0	*1·0* *0*	1·0	*0* *2·0*	1·0	*0* *0*
***8 months* (n=+)**						
10°C	0 (*n*=61)	*0* *0* (*n*=16,+)	0	*0*	1·6	*0*
15°C	0 (*n*=96)	*0* *0* (*n*=43,91)	1·0	*0* *1·1*	1·0	*0* *1·1*
20°C	2·2 (*n*=44)	*0* *0* (*n*=57,39)	0	*0* *0*	0	*0* *0*

Table 3. Prevalence of actinosporeans in the original sample, and at 3 and 6 months after adding *Myxosoma cerebralis* spores to *T. tubifex* cultures held at 10 and 15°C

	T. dubium (i.e.*T. gyrosalmo*)		*T. ignotum*		*Auractinomyxon* sp.	
	Control (%)	Test (%)	Control (%)	Test (%)	Control (%)	Test (%)
***Original sample* (n=200)**						
10°C	0·5	*0* *1·0*	0	*0·5* *0*	1·0	*0* *0·5*
15°C	0	*0* *0·5*	0	*0* *0*	0·5	*0·5* *1·5*
***3 months* (n=200)**						
10°C	0·5	*0·5* *0·5*	1·0	*0·5* *0·5*	2·0	*0* *1·0*
15°C	0·5	*0* *1·0*	0	*0* *1·0*	0	*0·5* *1·0*
***6 months* (n=200)**						
10°C	0	*2·0* *0*	0·5	*0* *0*	0·5	*0* *0·5*
15°C	1·0	*0·5* *0·5*	0·5	*1·0* *0·5*	1·5	*1·5* *0*

Table 4. Prevalence of actinosporeans in the original sample, and at 6 months after adding *Myxosoma cerebralis* spores to *T. tubifex* cultures held at 5 and 10°C

	T. dubium (i.e.*T. gyrosalmo*)		*E. radiatum*		*S. tubificis*		*Auractinomyxon* sp.	
	Control (%)	Test (%)	Control (%)	Test (%)	Control (%)	Test (%)	Control (%)	Test (%)
***Original sample* (n=200)**								
5°C	1·0	*0·5* *0·5*	0	*1·0* *2·0*	1·0	*0* *0·5*	0	*0·5* *0*
10°C	0	*0* *1·5*	2·5	*0·5* *0*	0·5	*1·0* *0*	0	*0* *0*
***6 months* (n=200)**								
5°C	1·5	*1·0* *2·5*	0·5	*0·5* *1·0*	0·5	*0·5* *0*	0	*0* *0*
10°C	1·0	*0·5* *0*	1·0	*0·5* *0*	0	*1·0* *0*	0	*0* *0*

Figures 1 & 2. *Myxosoma cerebralis* spores (arrows) within the gut lumen of *Tubifex tubifex*.

cultures. Similarly, there were no significant changes in actinosporea prevalences in either of the other experiments (Tables 3 & 4).

In contrast to the first experiment (Table 2), in the other two smaller experiments, the *Tubifex* worms were not sampled to extinction.

Figures 1 & 2 show *M. cerebralis* spores within the gut lumen in sections of *T. tubifex*. Although *T. tubifex* is clearly capable of ingesting *M. cerebralis* spores, there was no evidence despite extensive histological examination of worms over a period of weeks that the spores develop further; i.e. there is no eversion of polar filaments nor signs of hatching. Neither was it possible to discern an accumulation of spores within worms with time.

Of the oligochaete worms isolated from field samples, 75% or more were *T. tubifex* and the majority of the remainder were *Limnodrilus hoffmeisteri*. Of the worms observed from a local sewage farm and used in the *Tubifex* cultures over 90% of those identifiable were *T. tubifex*, though again the presence of *L. hoffmeisteri* was noted.

Discussion

Actinosporea were widely distributed but in low prevalence in all the tubificid samples collected in the British Isles and it was difficult to locate *T. tubifex* populations in which they were not present. It is possible that they would have been found from the French site, if more worms had been examined. Wolf & Markiw (1984) and Wolf, Markiw & Hiltunen (1986), make no reference to the occurrence of actinosporean species other than *T. gyrosalmo* in their sample populations, whereas we have found five commonly occurring species. Of particular note is the apparent absence of *T. dubium* (i.e. *T. gyrosalmo*) in three out of four sites in which whirling disease had recently been diagnosed (Table 1). In the whirling disease infection system, at the Ministry of Agriculture, Fisheries and Food, Fish Diseases Laboratory, Weymouth, England, four other actinosporean species were found in addition to *T. dubium*, two of which were in higher prevalence. *Triactinomyxon dubium* was also found in *T. tubifex* colonizing the tanks of a sewage farm after filtration. All *M. cerebralis* spores would have been removed by filtration and no trout could have been present in the tanks. Our conclusion does not concur with Wolf & Markiw's hypothesis. Their results are further complicated by an apparent difference in the actinosporean species illustrated in the reports of Wolf & Markiw (1984) and Markiw (1986). There are obvious size and morphological differences.

A possible explanation for the stable prevalence of actinosporean infections in the tubificid worms, to which the *M. cerebralis* spores had been added, is that the spores were not viable. However, spores had not been subjected to chemical treatment or physical disruption and there is little reason to question their viability in these experiments. The other actinosporean species present in the *Tubifex* cultures appeared to follow a similar pattern in prevalence to that of *T. dubium*. It is not known whether the stability in actinosporean prevalences are due to long lived infections or to a regular turnover between worms. The conditions for infection of tubificids are not known; attempts to show direct transmission of Actinosporea between tubificids have been inconclusive (Granata 1922).

Tubificids ingest mud particles as part of their feeding process (Wavre & Brinkhurst 1971) and it would follow that *M. cerebralis* spores would be present in the mud in whirling disease habitats. As there was no sign of accumulation or hatching of these spores, it is likely that they are passed out without change.

In the light of these observations it is difficult for us to agree with Wolf & Markiw's hypothesis. There are several theoretical objections to the hypothesis, such as the difference in size of spores of *M. cerebralis* and *T. dubium*, and the presence of two polar capsules in the former and three in the latter. Also, if there is a direct relationship between these species, it is to be expected that other Myxosporea are transmitted in the same way. To date there are far fewer actinosporean species described, only 30 species (Marques 1984) compared with several hundred Myxosporea. This may, of course, be due to the greater depth of study of the fish parasites.

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SHORT COMMUNICATION

The production of mouse anti-*Myxosoma cerebralis* antiserum from Percoll®-purified spores and its use in immunofluorescent labelling of Historesin®-embedded cartilage derived from infected rainbow trout, *Salmo gairdneri* Richardson

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Myxosoma cerebralis is an important myxosporean parasite which destroys the cartilage of infected juvenile salmonids. Heavy infections may cause gross skeletal deformity of the skull and backbone, and damage to the otoliths may lead to the condition known as whirling disease (Schaperclaus 1954). The diagnosis of this pathogen has relied principally on the isolation of the spore stage; cartilage is removed from around the brain and gill arches and, in the simplest procedure, crushed and smeared for examination. More complex purification and concentration regimes have been developed to enhance the detection of spores (Markiw & Wolf 1974; Kozel, Lott & Taylor 1980).

However, pre-spore stages cannot be detected with any degree of reliability with these techniques. Early stages of development have been detected using conventional paraffin wax embedding and sectioning (Lucky 1970; Hoffman 1970; Halliday 1973) though the descriptions of these stages are not definitive. The detection of pre-spore stages is of particular significance in fish which have low levels of infection or have only recently become infected, since in these cases spores will not be easily identifiable.

Immunofluorescence has been used to great effect in localizing hypnozoites of malaria parasites, which are small and sparsely distributed in mammalian liver, and barely detectable by conventional staining techniques (Krotoski, Garnham, Bray, Krotoski, Killick-Kendrick, Draper, Targett & Guy 1982). This paper deals with attempts to localize pre-spore stages and spores within sections of rainbow trout, *Salmo gairdneri* Richardson, cartilage using anti-*M. cerebralis* antiserum via immunofluorescent labelling.

Trout heads were obtained from three localities where *M. cerebralis* was known to occur: infected rainbow trout farms in West Germany and France and an experimental infection system used at Imperial College, London, England. Infected fishes were processed in batches of 10. Cartilage, removed from around the brain, particularly from the otolith region and from the gill arches, was mechanically disrupted, suspended in phosphate buffered saline (PBS) and filtered through 64 and 25 μm meshes. The crude extract was concentrated by centrifugation at 10000 g for 5 min, and the pellet was resuspended in 1 ml PBS. One millilitre of cartilage extract was added to the top of a column consisting of 2 ml layers of 25, 50, 75 and 100% Percoll (Pharmacia Labs, Milton

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Keynes, UK), made up in PBS, in a universal tube. The column was centrifuged at 300*g* for 5 min and cleaned spores were then removed and washed in 5 ml PBS at 1000*g* for 5 mins.

The pellet was then resuspended in PBS to a volume of 2 ml and filtered through a 0·8 μm Millipore filter (Millipore S.A., France). Spores were retained on the filter, which was then washed in 10 ml PBS. After centrifugation at 1000*g* for 5 min, filtration and washing were repeated twice, followed by a final centrifugation and resuspension of the pellet in 1 ml PBS. Spore density was estimated using a haemocytometer, and the spores were stored at 4°C for subsequent use as antigen. A total of 2×10^7 cleaned spores in 5 ml PBS were sonicated on ice intermittently for a total of 30 min using a M.S.E. 150 W sonicator with a 9 mm probe at an amplitude of 12 μm peak to peak. After centrifugation at 1000*g* for 5 min the pellet was resuspended in 0·25 ml PBS and mixed with 0–75 ml Freund's complete adjuvant (FCA), until a water in oil emulsion was formed. Five Balb C mice each were inoculated intraperitoneally with 200 μl of the antigen preparation.

A second intraperitoneal inoculation of 200 μl of the antigen in FCA was given 20 days later. After a further 7 days, the mice were exsanguinated. The pooled blood was allowed to clot for 30 min at room temperature and at 4°C overnight. The samples were then centrifuged at 1000*g* for 10 min and the serum collected. Serum was stored in aliquots at −20°C for subsequent use. The mouse antiserum was absorbed by incubating with macerated cartilage and bone fron uninfected rainbow trout for 2 h at 4°C, followed by overnight incubation at 10°C. The absorbed antiserum was centrifuged at 10000*g* for 10 min and the supernatant was passed through a 0·22 μm Millipore filter, previously washed with a 1% solution of porcine serum albumin in PBS, to prevent antibody sticking to the filter. The antiserum was filtered a second time, then titrated, using freshly extracted *M. cerebralis* spores in a fluorescence antibody test (Markiw & Wolf 1978) and stored at −20°C for further use.

Cartilage from infected rainbow trout was dissected from the otolith region and fixed overnight in aqueous 3% glutaraldehyde. Specimens were dehydrated, via an ethanol series, and embedded in LKB Historesin, after overnight infiltration at 4°C. The resin was polymerised for 1 h at 40°C. The blocks were sectioned at 0·5–2·0 μm using glass knives on a Reichert Jung Supercut 2050 microtome and were floated on to microscope slides for drying on a hotplate. Cartilage sections were incubated with absorbed mouse antiserum at dilutions of 1:25, 1:50 and 1:75 for 40 min, at 37°C. After washing three times in PBS, fluorescein conjugated swine anti-mouse IgG (Sw α M/FITC, Nordic Labs, Maidenhead, UK) was added at dilutions of 1:40 or 1:80 and the slides were incubated again as above. After a further three washes with PBS, the slides were rinsed with a 1% solution of Evans Blue in PBS, mounted in Citifluor low fluorescence mounting medium and examined using dark ground illumination on a Zeiss microscope with u.v. illumination at 330–500 nm. Normal mouse serum was used as a negative control, along with controls in which PBS was substituted for the mouse serum or the conjugate. Some of the slides were also stained using the Giemsa/Colophonium technique.

Preparations of *M. cerebralis* spores contaminated with only very small amounts of cartilage were obtained by phase separation of triturated infected cartilage on the discontinuous Percoll gradient. Clean spores were found at the interface of the original cartilage extract and the 25% Percoll band. On this basis, the density of the spores was

Table 1. Number of spores isolated (spores/ml) from individual infected trout

Percoll band (%)	7-month-old trout from German farm	18-month-old trout from French farm	5-month-old infected at I.C.
×/25	250000	350000	15000
25/50	50000	125000	3000
50/75	10000	35000	—
75/100	—	—	—

approximately 1·08 g/ml. Spores were also found at the 25/50% and at the 50/75% interfaces, but with a higher level of contamination. The latter were effectively purified after reprocessing through the gradient. Bacteria were largely confined to the band containing the original extract at the top of the column. Table 1 shows numbers of spores isolated from given bands on the gradient for individual fish.

The mouse anti-*M. cerebralis* antiserum, raised against spores separated on the Percoll gradient, titred out at 1:120 in an IFAT against freshly isolated *M. cerebralis* spores (using fluorescein conjugated IgG swine anti-mouse antiserum at 1:40). The polar capsule region of the spores was less reactive than the remainder of the spore wall, which appeared as a pronounced fluorescent halo (Fig. 1). At dilutions >1:120, the fluorescence pattern became indistinguishable from the background: firstly, the fluorescence around the polar capsule disappeared, followed by the fluorescence from the spore wall itself. When aged spores were used in the IFAT (those which had been previously extracted and stored at 4°C for several days) the antibody titre was only 1:60. When spores were incubated with normal mouse serum, the fluorescence was barely detectable, and the same was found when spores were incubated with PBS in place of antiserum or conjugate.

Using the Historesin embedding technique, semi-thin sections of infected cartilage, and even of the heavily ossified cartilage of older fish, were easily cut for immunofluorescence examination. The absorbed mouse anti-*M. cerebralis* antiserum, in combination with fluorescein conjugated swine anti-mouse antiserum, reacted most strongly with spores in the sections. Figures 2 & 3 show spores in which both the spore wall and the polar capsules show a strong fluorescence. These spores occurred mainly on the margins of damaged cartilage, which appeared as dense fibrous areas in Giemsa/Colophonium stained sections. These areas in turn showed pronounced fluorescence compared with the surrounding normal cartilage (Fig. 4). The fluorescence pattern was more clearly seen in the younger fish (5 months old), which had been experimentally infected, than in the older fish sampled from fish farms. Similarly, brightly fluorescent presumptive pre-spore stages (Fig. 4) could be seen within areas of damaged cartilage derived from the younger fish, but they were seldom seen in cartilage from older fish. The fluorescence patterns described above were clearest when mouse anti-*M. cerebralis* antiserum was used at 1:50, and the swine anti-mouse antiserum FITC at 1:40. Background levels of fluorescence, as assessed by the use of normal mouse sera and PBS in place of the fluorescent conjugate, were significantly lower, as shown in Fig. 5 in the case of damaged cartilage and Fig. 6 in the case of a spore in section.

It is difficult to estimate the relative efficacy of the numerous methods, developed principally for direct diagnosis, which have been used to isolate spores of *M. cerebralis*,

because of the variation in intensity of infection between samples used in previous studies. For example, O'Grodnick (1975) recovered an average of 91000 spores per fish using his plankton centrifuge method, and Tidd, Tubb & Wright (1973) recovered between 15300 and 2885000 spores per fish from the head region alone, using their wash, filter and centrifuge technique. The spore counts obtained in the present study, using Percoll, of between 16000 spores per fish in young experimentally infected fish and 510000 spores per fish (heads only) in older, naturally infected fish, compare reasonably well with the previous studies. However, the principal advantage of this method is that it avoids the use of acid or enzyme treatments, as has been used by some authors (Markiw & Wolf 1974), because these could substantially alter the antigenicity of the spores for use in immunological studies. The importance of this is clearly illustrated by the fluorescent corona, which is seen in freshly isolated spores used in the IFAT. Griffin (personal communication) has observed that if the IFAT is performed on spores that have been enzymatically treated the corona effect is greatly reduced, in much the same way as on spores which have been stored before use. The spores of *M. cerebralis* have a pronounced surface coat, particularly around the posterior half (Lom & Hoffman 1971), and it may be damage or loss of this by ageing or removal by enzymes which reduces the immunological reactivity of the spore. It is, therefore, advisable that only freshly isolated spores be used in immunological studies in *M. cerebralis*. There are a number of advantages to Historesin over wax embedding techniques which make it an excellent embedding medium for cartilage. Firstly, Historesin is effective with hard specimens which are difficult to cut, so that the decalcification techniques necessary for processing the highly ossified cranial cartilage of older fish, which might disrupt or destroy pre-spore stages, are avoided. Secondly, thin sections (0·5–2μm) are easily cut and, as a result, resolution is much higher. Thirdly, it is unnecessary to remove the resin prior to staining, as is the case when using paraffin wax embedding.

The absorbed mouse anti-*M. cerebralis* antiserum reacted strongly with spores within the Historesin sections when used in the IFAT, indicating that the relevant antigens had survived fixation and embedding. In turn, the antiserum reacted with pre-spore stages showing that antigens are shared between the pre-spore and spore stages. The generalized labelling of areas of damaged cartilage, in which the pre-spore stages were found, suggests that parasite antigens are released into this area. These antigens may represent enzymes released by the parasite which might be responsible for the destruction of the cartilage.

Figure 1. *M.cerebralis* spore fluorescing in an IFAT using anti-*M. cerebralis* antiserum (×400).

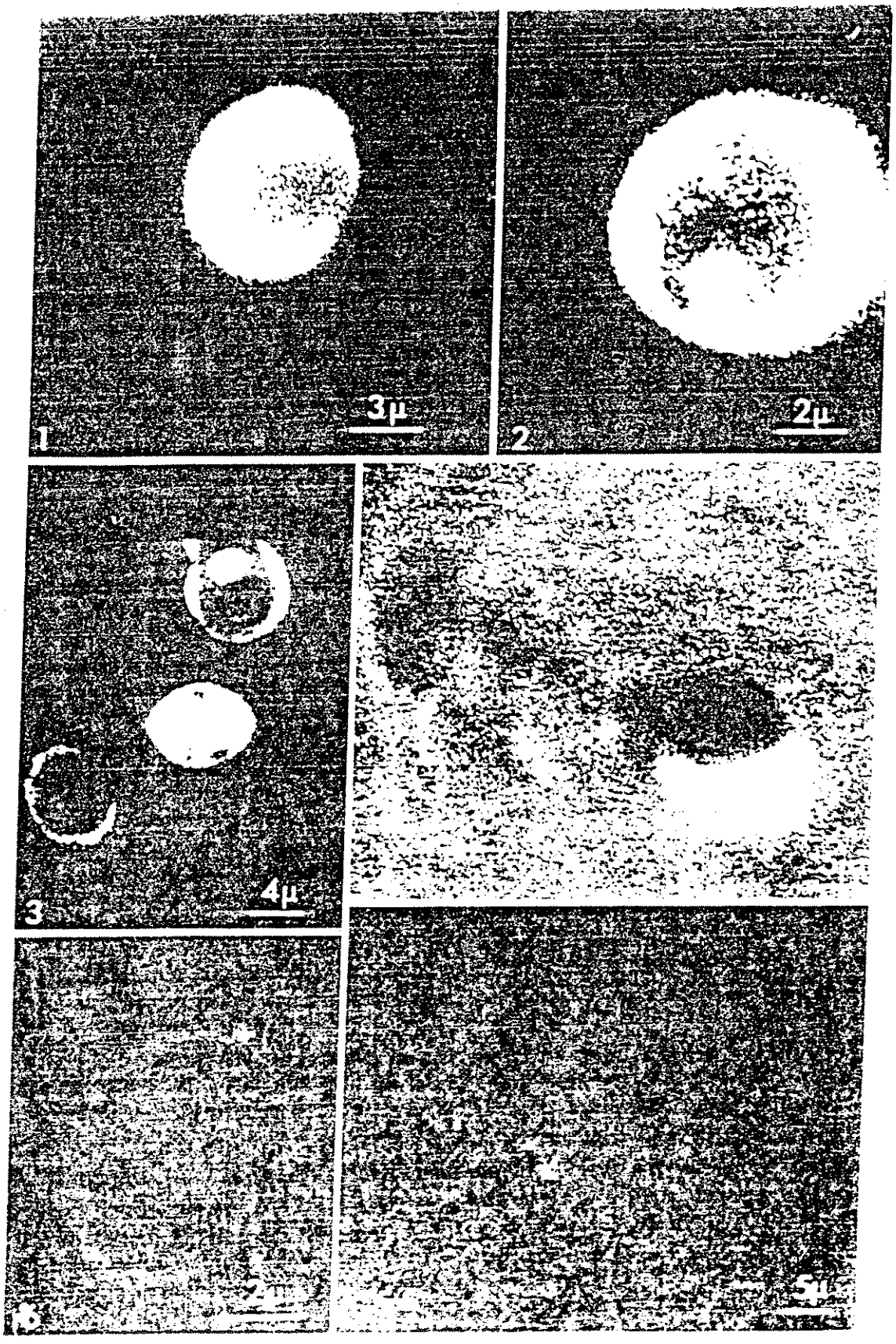
Figure 2. Immunofluorescent labelled *M. cerebralis* spores in Historesin section (×400).

Figure 3. As for Fig. 2 (×1000).

Figure 4. Infected cartilage incubated with mouse anti-*M. cerebralis* antiserum. Note generalized labelling and presumptive pre-spore stage (arrowed) (×250).

Figure 5. Infected cartilage section incubated with normal mouse serum and fluorescein conjugate (negative control) (×400).

Figure 6. Spore incubated with normal mouse serum and fluorescein conjugate (negative control) (×400).


3μ
1
2μ
2
4μ
3

The detection of such labelled areas in young infected fish, in which spores have not yet developed, may be of some use in the early diagnosis of *M. cerebralis*.

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Brief Communication

THE OCCURRENCE OF WHIRLING DISEASE (MYXOSOMIASIS) IN NORWAY

Whirling disease caused by Myxosoma cerebralis is a widespread and feared disease in fingerlings of hatchery reared rainbow trout (Salmo gairdneri). Since the first description of Whirling disease *(Hofer* 1903), it has been diagnosed in many countries in and outside Europe.

In Norway whirling disease was first detected in 1970. Investigations, however, showed that the disease probably had existed for several years in a fish farm near Oslo without confirmation of the diagnosis.

In the following a short description is given of whirling disease in rainbow trout in connection with outbreaks of the disease in three Norwegian fish farms.

The disease was first diagnosed in a freshwater fish farm in connection with an inspection of the farm required by the Norwegian fish disease legislation. A great deal of the fry that were held in earth ponds showed blackened tail, deformities of the vertebral columns, misshapen heads with shortened gill covers and shortened, twisted lower jaw. Some of the fishes had a tendency to whirling movements. The mortality was minimal, probably because the fry had been held in glassfiber-armed plastic throughs until they reached a length of 5—10 cm before they were stocked in earth ponds. Previously the fry had been transferred directly from the hatching troughs to the earth ponds with a resulting heavy mortality. The older fish in the farm (fingerlings to brood fish) showed more or less the same pathological picture as described above, but the frequency of misshapen fish was not so high. Except for the protracted stocking of the earth ponds, nothing had been done to control the disease.

The disease was spread to two other fish farms which had bought 5000 fingerlings each from the fish farm mentioned above. In these farms there were heavy losses of fish the first

two days after the transportation; the mortality was estimated at 5—600. After this initial mortality, the death rate was about 15—20 fish per day in the following two months before fish-pathologists were informed. In October 1970 the National Veterinary Institute received some fingerlings from these farms located on the western coast of Norway. In accompanying letters, the owners told that the fish had not gained sufficient weight compared with other fishes in the farms and some fish had twisting movements, black tails, twisted spines and deformed heads with shortened gill covers. The shortened gill cover had resulted in Saprolegnia infection of the gills.

The diagnosis in the three cases was confirmed by the demonstration of spores and trophozoites in the cartilaginous tissue of the head. As method the scraping technique (*Hoffman et al.* 1968) was used. The scrapings from the cut surfaces were partly mounted as wet preparations in a drop of sterile saline, partly in glycerin albumen. The wet mount preparations were examined at once, while the glycerin albumen preparations were allowed to dry and then stained with May-Grünwald-Giemsa and Hematoxyline-Eosin.

As no effective treatment exists for control of the disease, necessary steps to avoid further spread of the disease were taken according to the Norwegian fish disease legislation. These steps included prohibition of selling live fish to other fish farms and for stocking to natural waters until the diseased fish had been destroyed and ponds, trough, and equipment had been thoroughly disinfected.

DISCUSSION

The symptoms, pathological changes and the parasitological findings described above, are in accordance with the characteristics of whirling disease caused by Myxosoma cerebralis (*Ghittino* 1962; *Hoffman et al.* 1962; *Uspenskaya* 1955). It can therefore be concluded that the disease described above is whirling disease.

This is believed to be the first incidence of whirling disease reported from Norway.

In two of the described cases the disease was introduced to the fish farms with fry from an infected hatchery, while in the first case the infection route is unknown. As no therapy is possible, the disease has to be controlled by sanitary means, including destruction of the diseased fish and disinfection of ponds, troughs and equipment.

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Three Myxosporeans Found in the Cranial and Branchial Tissues of Rainbow Trout in California

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Abstract.—Three myxosporeans were encountered in the cranial tissues of a California population of rainbow trout *Oncorhynchus mykiss* examined for the presence of *Myxobolus cerebralis*, the causative agent of whirling disease. Typical spores of *M. cerebralis* and a previously undescribed species of *Myxobolus* were found in the cranial tissues prepared by the pepsin HCl–trypsin digestion method. *Henneguya zschokkei* was also detected in digest preparations of cranial tissues, but was more numerous when branchial cartilage was included in the preparations. Microscopic examinations of tissues of individual rainbow trout showed occasional infections with both myxobolid species. *Myxobolus cerebralis* trophozoites and spores were found in the cranial and gill cartilage, and *Myxobolus* sp. was found in the brain and spinal cord. *Henneguya zschokkei* was also found within granulomas in the connective tissues below the gill arch. Both *M. cerebralis* and *H. zschokkei* were associated with a chronic inflammatory response in their respective tissues. In contrast, the *Myxobolus* sp. spores were found in pockets within the nervous tissues with no detectable host response. The spore measurements, calculated from fresh digests of infected tissues for the three myxosporeans ($N = 20$), for length × width × thickness in micrometers (SD) were 11.7 (0.6) without tails and 42.6 (5.2) with tails × 7.7 (0.8) × 7.0 (0.1) for *H. zschokkei*, 9.9 (0.4) × 8.4 (0.1) × 6.5 (0.3) for *M. cerebralis*, and 12.7 (0.7) × 10.5 (1.0) × 9.5 (0.8) for *Myxobolus* sp. Examined under scanning electron microscopy, the latter two species were morphologically similar although distinctive in size.

Myxosporeans are commonly encountered parasites in the tissues of wild and cultured fishes (Mitchell 1977). Among salmonids, several myxosporeans cause serious diseases. In North America, *Myxobolus cerebralis, Ceratomyxa shasta,* and PKX (the causative agent of proliferative kidney disease) are associated with morbidity or mortality (Wolf and Markiw 1985; Hedrick et al. 1986; Bartholomew et al. 1989). Vegetative or sporogonic stages of all three myxosporeans induce an intense inflammatory response, and with *C. shasta* and PKX, this may lead to organ dysfunction and death.

Fish health control programs in the western USA aimed at preventing the spread of important myxosporean diseases rely on detection and then containment or destruction of infected stocks (Hoffman 1970). With *M. cerebralis,* this requires accurate identification of the spore, particularly after active infections have ceased. The procedures commonly employed to detect spores rely on extraction of the spores from infected cranial or gill tissues with a regimen of pepsin HCl and trypsin (Markiw and Wolf 1974). This method removes most extraneous tissues and concentrates the spores into a small volume for microscopic examination. During examinations of several fish populations for evidence of *M. cerebralis,* several spore types have been encountered. In certain situations, the morphology and size of these *Myxobolus* spp. spores have led to confusion regarding their proper identification.

At least three *Myxobolus* spp. have been detected in cranial tissues of salmonids. *Myxobolus cerebralis* (Hofer, 1903), found in the cartilage or bone, has been detected in most species of salmonids in many European and North American countries, including those in several eastern and western U.S. states (Hoffman 1970, 1990; Wolf 1986). The parasite has been detected recently in additional western states including Oregon, Washington, Wyoming, Colorado, Utah, and Idaho (D. Anderson, U.S. Fish and Wildlife Service, personal communication).

Myxobolus neurobius (Schuberg and Schroder 1905) has been described from the nervous tissues of brown trout *Salmo trutta,* sockeye salmon *Oncorhynchus nerka,* coho salmon *O. kisutch,* round whitefish *Prosopium cylindraceum,* Arctic grayling *Thymallus arcticus,* Arctic char *Salvelinus alpinus,* and whitespotted char *S. leucomaenis* in the Soviet Union (Shul'man 1966; Konovalov 1971); in brown trout and Atlantic salmon *Salmo salar* in the UK (Kennedy 1974); lake whitefish *Coregonus clupeaformis,* round whitefish, Arctic grayling, lake trout *Salvelinus namaycush,* sockeye salmon, and coho salmon in Canada (Arthur 1975; Margolis and Arthur 1979); and sockeye salmon in Alaska (T. Meyers, Alaska Department of Fish and Game, personal communication).

A third myxobolid species, *M. kisutchi* (Yasutake and Wood 1957), has been found in the spinal cords of coho and chinook salmon in Washington and Oregon (Yasutake and Wood 1957; Wyatt 1978). In the following report, we describe an additional *Myxobolus* sp. and *Henneguya zschokkei* found in the cranial and branchial tissues of a single population of rainbow trout from northern California. These parasites were encountered in examinations for the presence of *M. cerebralis.*

Methods

Fish.—Tissues and spores observed in this study by light and electron microscopy were from rainbow trout from a private aquaculture facility in northern California. The fish were yearlings with an average weight of 70 g.

Spore isolation.—Fish were euthanized with 500 mg tricaine (MS 222)/L. The head, including all gill arches and the anterior spinal cord, were then removed, divided in half, and placed on ice. One-half of each head was placed into fixative for later histological examination, and the other was placed into a bag on ice. Portions of the head and gills from 30 fish were digested as five-fish pools by the pepsin HCl–trypsin method described by Markiw and Wolf (1974) and Amos (1985). Spores were concentrated by repeated cycles of centrifugation, including sedimentation through a dextrose column (Markiw and Wolf 1974). Spores were resuspended in 0.85% saline and placed onto glass slides.

Light and electron microscopy.—One-half of the heads from five of the fish used for the digestions were fixed in Bouin's solution, embedded in paraffin, sectioned, and stained with hematoxylin and eosin or Giemsa (Humason 1979). Ten measurements of each spore dimension were taken with a micrometer at 600× magnification (Lom and Arthur 1989). In addition, spore suspensions prepared by digestion were fixed in 2.5% glutaraldehyde, dehydrated in ethanol, critical-point-dried, sputter-coated with gold, and observed with a Philips SEM 501 scanning electron microscope.

Results

Myxobolus cerebralis

Three spore types were detected in the cranial and branchial tissues of the rainbow trout examined in this study. Two myxosporeans were classified as *Myxobolus* spp., one of which was identified as *M. cerebralis* based on its size and shape (Table 1). Trophozoites of *M. cerebralis* were associated with granulomatous lesions in the cartilage (Figure 1A). There were both multinucleated trophozoites (Figure 1B) and spores (Figure 1C, D) detected.

The two valves of *M. cerebralis* spores were thickened at the suture, and a prominent groove was visible in most spores (Figure 1E, F). A prominent, thickened ventral structure on either side of the suture (Figure 1F) and occasional mucous envelopes (not shown) were observed on the posterior aspect of the spores. In more mature spores, the apical openings for the polar filaments were visible on opposing anterior aspects of the two valves (Figure 1E). The spore measurements (N = 20), calculated from fresh digests of infected tissues, for length × width × thickness (SD) were 9.9 (0.4) × 8.4 (0.1) × 6.5 (0.3) μm (Table 1). Both polar capsules were of similar size with a length × width of about 5.4 × 3.3 μm. Spore measurements (N = 10) from tissue sections were 8.4 (0.8) × 7.7 (0.8) × 6.0 (0.7) μm (Table 1). Polar capsules were 3.5 × 2.0 μm.

Myxobolus sp.

Pockets of *Myxobolus* sp. spores were detected in the spinal cord in the absence of any detectable host response (Figure 2A, B). The spore concen-

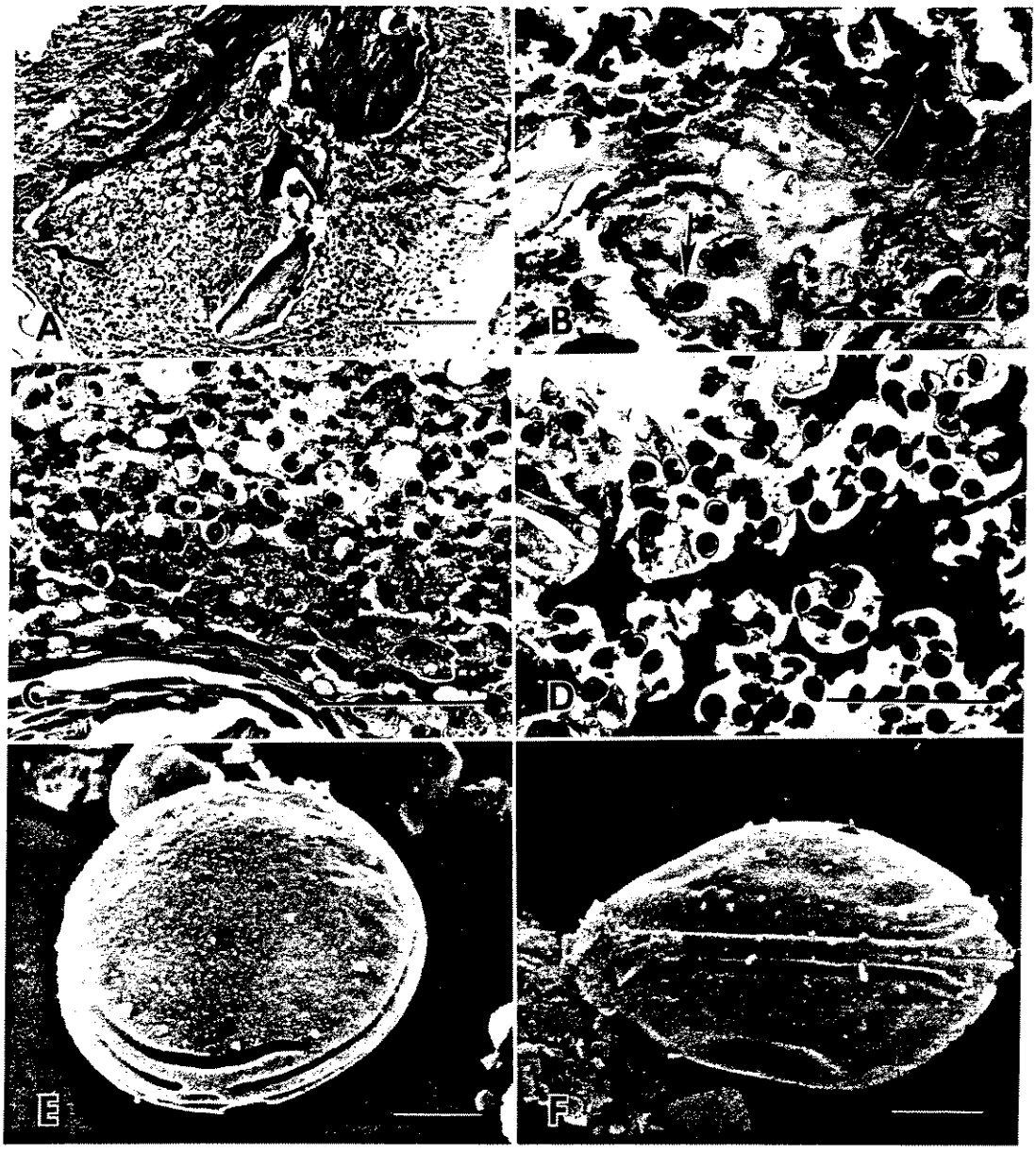

FIGURE 1.—*Myxobolus cerebralis* in the cranial cartilage of juvenile rainbow trout. (A–C) Tissue sections stained with hematoxylin and eosin. (A) Trophozoites inducing a chronic granulomatous response in cartilage with surrounding bone. Bar = 4 mm. (B) Multinucleated trophozoites. Bar = 0.5 mm. (C) Sporogonic stages and spores. Bar = 0.5 mm. (D) Spores surrounded by bone stained with Giemsa. Bar = 0.5 mm. (E and F) Scanning electron micrographs of spores from anterior and lateral perspectives, respectively. Bars = 2 μm.

trations were greatest in the anterior portions of the spinal cord, and fewer spores were found in the medulla oblongata. No well-formed cysts were detected, but instead the spores were lying freely within pansporoblasts between neurons (Figure 2B). Trophozoites were not detected. The *Myxobolus* sp. was found in three rainbow trout that also had active cartilage infections with *M. cerebralis*.

The spores of *Myxobolus* sp. were composed of two valves of similar shape to those of *M. cerebralis* except for their overall greater dimensions (Table 1). A prominent groove on the valve edge

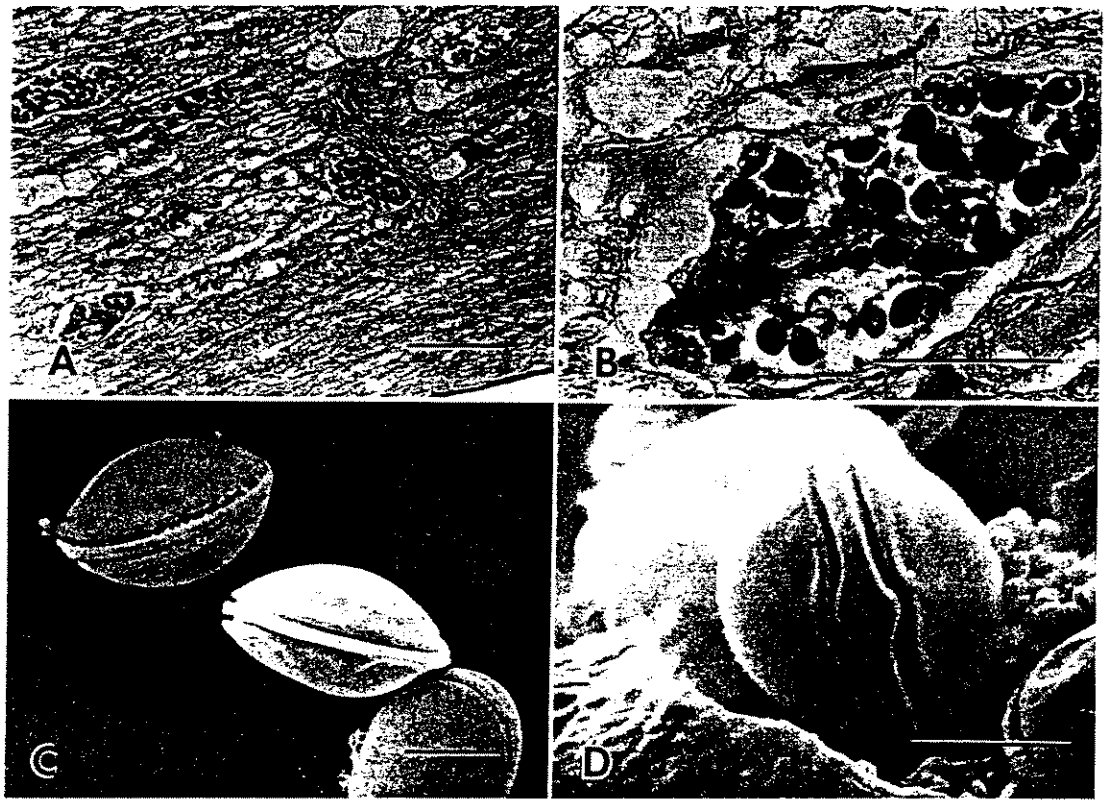

FIGURE 2.—*Myxobolus* sp. in the brain and spinal cord of juvenile rainbow trout. (A and B) Tissue sections stained with Giemsa. (A) Spores in the anterior spinal cord. Bar = 4 mm. (B) A small cyst with no host inflammatory response. Bar = 0.5 mm. (C and D) Scanning electron micrographs of spores from (C) dorsolateral and (D) ventral perspectives. Bars = 5 μm.

TABLE 1.—Mean dimensions (SD) of fresh and fixed spores of three myxosporeans found in the cranial and branchial tissues of rainbow trout. PC = length × width of polar capsules.

Species and type of spore	Dimension (μm)			
	Length	Width	Thickness	PC (μm)
Myxobolus cerebralis				
Fresh (N = 20)	9.9 (0.4)	8.4 (0.1)	6.5 (0.3)	5.4×3.3
Fixed (N = 10)	8.4 (0.8)	7.7 (0.8)	6.0 (0.7)	3.5×2.0
Myxobolus sp.				
Fresh (N = 20)	12.7 (0.7)	10.5 (1.0)	9.5 (0.8)	5.3–5.8×3.2
Fixed (N = 10)	11.2 (0.6)	9.5 (0.9)	7.8 (1.2)	4.5–4.7×2.3
Henneguya sp.				
Fresh (N = 20)	11.7 (0.6)[a] 42.6 (5.2)[b]	7.7 (0.8)	7.0 (0.5)	4.9×2.1
Fixed (N = 10)	9.9 (0.5)[a] 33.4[b,c]	7.4 (0.8)	5.5 (0.6)	3.7×2.8

[a] Length of spores without tails.
[b] Length of spores with tails.
[c] Standard deviation was not calculated because sufficient numbers of fully tailed spores were not found in fixed and sectioned tissues.

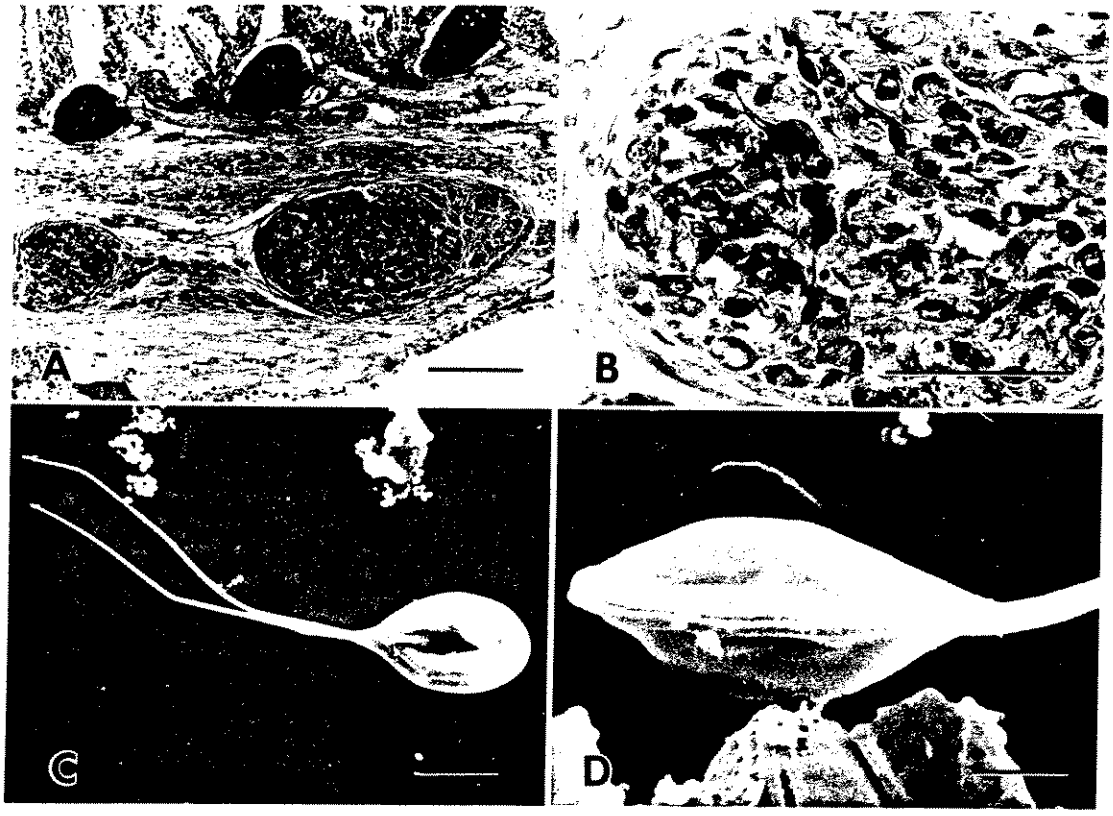

FIGURE 3.—*Henneguya* sp. in the connective tissue of the branchial arch of juvenile rainbow trout. **(A and B)** Tissue sections stained with hematoxylin and eosin. **(A)** Sporogonic stages and spores in a granuloma. Bar = 4 mm. **(B)** Tailed spores and paired polar capsules of spores within granuloma. Bar = 0.5 mm. **(C and D)** Scanning electron micrographs of spores from **(C)** dorsal and **(D)** lateral perspectives. Bars = 5 and 2.5 μm, respectively.

surrounding the suture, as in *M. cerebralis,* was also observed (Figure 2C, D). A thickened ventral aspect and mucous envelopes (not shown) on some spores were also detected (Figure 2C, D). Spore measurements (N = 20), calculated from fresh digests of infected tissues, length × width × thickness were 12.7 (0.7) × 10.5 (1.0) × 9.5 (0.8) μm (Table 1). Both polar capsules were of similar width (3.2 μm), but one was slightly longer than the other (5.3 versus 5.8 μm). Spore measurements (N = 10) from tissue sections were 11.2 (0.6) × 9.5 (0.9) × 7.8 (1.2) μm (Table 1). Polar capsules were 4.5–4.7 × 2.3 μm.

Henneguya sp.

Granulomas in the connective tissues underlying the gill arches were detected in several fish (Figure 3A). Layers of macrophages surrounding sporogonic stages, including spores, of a *Henneguya* sp. were also detected (Figure 3B). The *Henneguya* sp. was found in two fish that also had *M. cerebralis* infections. Both valves contained caudal extensions, giving the spore a tailed appearance (Figure 3C). The body of the spore was elliptical, and the valves were slightly thickened at the suture (Figure 3D). There were no prominent grooves near the edge of the valves as in the two myxobolid spores. The spore measurements (N = 20), calculated from fresh digests of infected tissues, for length × width × thickness (SD) were 11.7 (0.6) without tails and 42.6 (5.2) with tails × 7.7 (0.8) × 7.0 (0.5) μm. Both polar capsules were of similar size with a length × width of about 4.9 × 2.1 μm. Spore measurements (N = 10) from tissue sections were 9.9 (0.5) without tails and 33.4 with tails × 7.4 (0.8) × 5.5 (0.6) μm (Table 1). Polar capsules were 3.7 × 2.8 μm.

Discussion

Prespore stages of trophozoites were associated with the granulomatous lesions detected in trout with both *Henneguya zschokkei* and *M. cerebralis*

infections. Tissue sections from the cranium showed that trophozoites of *M. cerebralis* induced a chronic inflammatory response associated with digestion of the cartilaginous tissues (Figure 1A). This response is common in smaller, more susceptible salmonids and first appears at 30 d following exposure to the infective stages at water temperatures of 12°C (Halliday 1973). Onset is delayed and severity of whirling disease is lessened with lower temperatures or in larger fish (Halliday 1973, 1976; Hoffman and Byrne 1974; Hoffman 1976).

Chronic inflammation associated with extrasporogonic stages of myxosporeans is characteristic of the more pathogenic species (Lom 1987). This strong cellular response is presumably accompanied by a humoral reaction because circulating antibodies to the spore stages of *M. cerebralis* have been detected (Griffin and Davis 1978). Granulomatous lesions may resolve, but the entrapment of spores during ossification results in persistence of the resting stages of the parasite (Halliday 1973). *Myxobolus cerebralis* has now been found in salmonids from nearly all of the western USA and can be considered enzootic in several watersheds (Hoffman 1990; Anderson, personal communication). The effect of this disease on fish populations and the inability to eradicate the pathogen have resulted in limited control programs that stress management and restricted movements rather than stock destruction.

The larger myxobolid detected in our study seems to differ sufficiently from previously reported species to warrant further description. The spores can be found abundantly in certain rainbow trout populations in northern California, and their similar morphology and appearance in digest preparations has led to some confusion with *M. cerebralis*. In one case, destruction of fish harboring only the larger *Myxobolus* sp. occurred (our unpublished data). Confusion between the two spore types is understandable because they share microscopic details (Figures 1E, F and 2C, D). The shape of the valves, their characteristic thickenings, and grooves near the suture are seemingly identical between the two species (Figures 1E, F and 2C, D). These characteristics have been described for spores of *M. cerebralis* observed by scanning electron microscopy by Lom and Hoffman (1971), although the groove they observed near the suture was accentuated by what appeared to be more shrinkage of the valves than was observed in our study. As with both *Myxobolus* spp. we examined, Lom and Hoffman (1971) detected residual mucous envelopes on the posterior aspects of the spores even following pepsin HCl–trypsin treatments. The opposing openings, one on each valve at the anterior of the spore for extrusion of the polar capsules as reported for other *Myxobolus* spp. (Lom 1964), were detected in spores of *M. cerebralis* in our preparations (Figure 1E).

Although the two myxobolids we found were similar in morphological respects, their tissue tropism and corresponding host responses were remarkably different. In contrast to *M. cerebralis*, the larger myxobolid does not elicit a strong inflammatory response or accompanying tissue destruction, and no known detriment to the host has been associated with moderate to heavy infections (M. Willis, California Department of Fish and Game, personal communication). A similar response in coho salmon infected with *M. kisutchi* was previously reported (Yasutake and Wood 1957; Wyatt 1978). However, brain and spinal cord infections with a *Myxobolus* sp. similar to *M. kisutchi* in chinook salmon from Washington are believed to lead to progressive emaciation and death (J. Morrison, U.S. Fish and Wildlife Service, personal communication).

A third myxobolid found in nervous tissues is *M. neurobius* (Schuberg and Schroder 1905). However, both *M. neurobius* and *M. kisutchi* can be clearly separated from the *Myxobolus* sp. detected in our study by their shape and size, respectively. The smaller spore size of *M. kisutchi* in tissue sections (length × width × thickness) of 7.0–8.5 × 6.5–7.0 × 3.5–3.8 μm (Yasutake and Wood 1957; Wyatt 1978) distinguishes it from the *Myxobolus* sp. we observed (Table 1). Although the spores of *M. neurobius* are of similar size to the *Myxobolus* sp., they are characteristically pointed at the anterior end, giving the spore a pyriform shape (Shul'man 1966). In addition, the polar capsules of *M. neurobius* are significantly greater in length than are those of the *Myxobolus* sp. (7.0 versus 5.8 μm). Comparisons of the sizes of fresh and fixed spores between studies is difficult due to shrinkage during preparation and staining. However, our results indicate a fairly constant rate of shrinkage of the spore valves (about 15% or less). Assuming similar shrinkage with other observations of *M. kisutchi* (Yasutake and Wood 1957; Wyatt 1978), that spore would clearly be smaller than those of the *Myxobolus* sp. observed in the nervous tissue in our study (Table 1). We have examined fixed and stained spores from chinook salmon with *M. kisutchi* from

Washington, and their measurements correspond quite well with the measurements given in earlier descriptions (Yasutake and Wood 1957). Our conclusion is that the larger *Myxobolus* sp. is a third myxobolid that may be encountered in the nervous tissue of salmonids.

Pathological changes were not associated with infections of rainbow trout with the *Myxobolus* sp. in our study. In contrast, heavy infections of the nervous tissues with *M. neurobius* in sockeye salmon (Dana 1982) and of *M. kisutchi* in chinook salmon in Washington can cause clinical disease. These two parasites, when in great numbers, may therefore have substantial undetected effects on anadromous salmonid populations that have not been recognized with the *Myxobolus* sp. we observed.

The *Henneguya* sp. identified in digests originates from connective tissue cysts underneath the gill arches of infected rainbow trout (Figure 3A). This myxosporean presumably contaminates digest preparations as a result of the inability to remove all soft tissues from cartilage and bone used in the assay. The size of the spore, location in the connective tissue, and presence in salmonid fish are consistent with descriptions for *H. zschokkei* (Gurley, 1894; syn. *H. salminicola*: Ward, 1919) for Pacific salmonids from western North America, the USSR, and UK, as reported by Fish (1939) and Shul'man (1966). In North American salmonids, the cysts may be observed macroscopically in the musculature, rendering the flesh unsuitable for sale (Boyd and Tomlinson 1965).

Three myxosporeans have been reported from the cranial and branchial tissues of rainbow trout examined for the presence of *M. cerebralis*. We found that at least one population of rainbow trout may harbor all three myxosporeans and that a single fish may be the host for both myxobolid species simultaneously, or the fish may alternatively be coinfected with *M. cerebralis* and *H. zschokkei*. Although the severity of disease caused by *M. cerebralis* has been debated, heavy infections lead to moderate to severe lesions. The two other myxosporeans detected in our study were associated with minor or no microscopic lesions, suggesting that under conditions of light infections they are probably not serious pathogens of salmonid fish.

Acknowledgments

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EFFECTS OF WHIRLING DISEASE

Whirling disease, crippling and sometimes fatal, is caused by Myxosoma cerebralis, a cartilage-destroying sporozoan. If the fish is very young when infected, it may succumb. If the fish survives, the head--and the spinal column in a severe case--will be badly misshapen. The disease is spread from hatchery to hatchery by transfer of diseased fish.

The 2-year-old rainbow trout in the photograph shows the effects of whirling disease.

--GLENN L. HOFFMAN, Bureau of Sport Fisheries and Wildlife, Kearneysville, West Virginia.

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Hoffman, G.L. 1974. Disinfection of contaminated water by ultraviolet irradiation, with emphasis on whirling disease (*Myxosoma cerebralis*) and its effect on fish. Transactions of the American Fisheries Society. 3: 541-550.

Disinfection of Contaminated Water by Ultraviolet Irradiation, with Emphasis on Whirling Disease (*Myxosoma cerebralis*) and Its Effect on Fish

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ABSTRACT

Rainbow trout fry developed whirling disease upon exposure to water contaminated with *Myxosoma cerebralis*. When similarly contaminated water was irradiated with 2537 Angstrom units of ultraviolet light at dosages of 35,000, 43,000, and 112,000 microwatt sec/cm^2, infection of fry was prevented. The minimum effective dosage was not determined in the present experiments. High mortality and a 61% growth inhibition of survivors occurred in the infected fry.

Although some fish pathogens larger than 15 μm in diameter can be removed from water supplies by various methods of filtration, alternative methods of water treatment are necessary for the removal or destruction of pathogens smaller than 15 μm in diameter. Ultraviolet (UV) irradiation, which has been used for many years as a bactericide, appears promising as a possible means of destroying many of the smaller fish pathogens (Burrows and Combs 1968).

UV irradiation of water at 2537 Angstrom units (Å) wave length has proven effective and practical for the destruction of certain protozoans and is nontoxic to fish. Wave lengths below 2000 Å produce ozone which is toxic to fish, and wave lengths above 3000 Å have proven less effective (Giese 1967). The minimum effective exposure level of UV for most fish pathogens has not been fully determined.

Effective exposure level is apparently related to the size and transparency of the organism to UV. As little as 3620 microwatt sec/cm^2 (MWS) killed *Aeromonas salmonicida* (furunculosis) which is 0.5–1.0 μm wide (Normandeau 1968), but 1,717,200 MWS were needed to kill *Ichthyophthirius multifiliis* trophozoites (Vlasenko 1969) which are approximately 800 μm in diameter.

Whirling disease (WD) caused by the protozoan, *Myxosoma cerebralis*, is an exotic disease, having appeared in North America in 1957. It is a serious disease of cultured trout and salmon in Europe, South America, South Africa, and Great Britain as well as the U.S.A. (Hoffman 1970a).

The present study was designed to determine whether UV irradiation would prevent infection of rainbow trout exposed to water containing *M. cerebralis*. A dosage of 112,000 MWS was arbitrarily chosen for the initial experiment. Succeeding experiments tested 43,000 and 35,000 MWS in that order, in an attempt to ascertain the mininum effective dosage. Ultraviolet studies are also underway at the Benner Springs Fisheries Research Station, Bellefonte, Pennsylvania and the National Fish Hatchery, Tishomingo, Oklahoma.

Undocumented observations by American trout growers indicate that WD can cause high mortality of trout fry in earthen ponds. The other signs of WD (whirling, black tail, spinal curvature) are well documented. Schäperclaus (1931, 1954) mentions similar observations of mortality in Germany but cites no experimental data. Scolari (1954) observed 30% loss in the untreated group in his chemotherapy experiment.

Rydlo (1971) reported that control rainbow trout averaged 3.3 g, whereas those with WD averaged 1.8 g. Uspenskaya (1957) observed uninfected yearling rainbow trout averaging 64 g, whereas diseased fish weighed 8.5 g under hatchery conditions. The following year uninfected fish weighed 24 g and the diseased fish 17 g. In the present study, the initial ex-

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TABLE 1.—*Effect of UV on Fish Parasites and Certain Other Organisms for Comparison*

Organisms listed in increasing order of size	Size (μm)	Dosage used, or exposure time	Lamp type	Lamp wattage	No. of lamps	Wave length in Angstrom[b]	Water flow	Author's report of effectiveness	Reference
Aeromonas salmonicida	0.5 × 2	3,620 MWS	G.E. (G8Tg)	8	3	—	Static	99% inactivation	Normandeau 1968
Bacilus subtilis spores[a]	0.6 × 1.5	22,000 MWS	—	—	—	—	—	Min. lethal dosage	Nagy 1964
Sarcina lutea[a]	1.5	26,400 MWS	—	—	—	—	—	Min. lethal dosage	Nagy 1964
Saprolegnia zoospores	4–12 ?	35,000 MWS	BUV-15, tabletop expt.	—	1–3	2,537	Static	Effective	Vlasenko 1969
Saprolegnia zoospores	4–12 ?	—	MBU-3 (PRK-7)	35	?	3650–3663[b]	1 m³/hr (4 gpm)	Effective	Kokhanskaya 1970
Saprolegnia zoospores	4–12 ?	39,564 MWS	G.E. (G8T5)	8	3	—	—	Min. lethal dosage	Normandeau 1968
Costia necatrix	5–18	318,600 MWS	BUV-15, tabletop expt.	—	1–3	2,537	Static	Min. lethal dosage	Vlasenko 1969
Myxosoma cerebralis	6 × 10	35,000 and 112,000 MWS[c]	—	40	1	2,537	9.5 liter/min (2.5 gpm)	100% effective	Hoffman this paper
Ceratomyxa shasta	6 × 14	Hoffman est. 30,000 MWS ?	Westinghouse G36T-6L	40	2	—	37.9 liter/min (10 gpm)	Probably 100% effective	Bedell 1971
Ceratomyxa shasta	6 × 14	215,500 MWS[c]	Westinghouse G36T-L	40	2	—	9.6 liter/min (2.5 gpm)	100% effective	Sanders et al. 1972
Ceratomyxa shasta	6 × 14	360,000 MWS[c]	Westinghouse G36T-6L	40	8	—	6 liter/min (1.6 gpm)	98% effective	Sanders et al. 1972
Ceratomyxa shasta	6 × 14	37,000 MWS	—	40	2	—	37.9 liter/min (10 gpm)	Effective, no figures given	Burrows and Combs 1971[d]
Saprolegnia hyphae	8–24	10,000 MWS	G.E. (G8T5)	—	3	—	—	Min. lethal dosage	Normandeau 1968
Trichodina sp.	16 × 50	35,000 MWS	Tabletop with sensor	—	1	2,537	Static	Min. lethal dosage	Hoffman 1970b
Trichodina nigra	22 × 70	159,000 MWS	BUV-15, tabletop expt.	—	1–3	2,537	Static	Min. lethal dosage	Vlasenko 1969
Trichodina sp.	?	—	MBU-3 (PRK-7)	35	—	3650–3663[b]	1 m³/hr (4 gpm)	Effective	Kokhanskaya 1970
Trichodina sp.	?	2–3 min	DRSh-250	—	—	—	Static	Min. lethal dosage	Laptev 1967
Ichthyophthirius tomites	20 × 35	336,000 MWS	BUV-15, tabletop expt.	—	1–3	2,537	Static	Min. lethal dosage	Vlasenko 1967
Ichthyophthirius tomites	20 × 35	—	MBU-3 (PRK-7)	35	—	3650–3663[b]	1 m³/hr (4 gpm)	Effective	Kokhanskaya 1970

TABLE 1.—*(Continued)*

Organisms listed in increasing order of size	Size (μm)	Dosage used, or exposure time	Lamp type	Lamp wattage	No. of lamps	Wave length in Angstrom[b]	Water flow	Author's report of effectiveness	Reference
Ichthyophthirius tomites	20 × 35	100,000 MWS	Tabletop with sensor	—	1	2,537	Static	Min. lethal dosage	Hoffman 1970b
Cercariae	30 × 100 ?	2.5–3 min	PRK-7	—	—	—	Static	Min. lethal dosage	Laptev 1967
Nanophyetus cercariae	30 × 100 ?	37,000 MWS+	—	50 each	2	—	37.9 liter/min (10 gpm)	Killed 75%	Burrows 1971[d]
Chilodonella cyprini	35 × 70	1,008,400 MWS	BUV-15, tabletop expt.	—	1–3	2,537	Static	Min. lethal dosage	Vlasenko 1967
Chilodonella cyprini	35 × 70	2–3 min	DRSh-250	—	—	—	Static	Min. lethal dosage	Laptev 1967
Paramecium sp.	70–80	200,000 MWS	—	—	—	—	—	Min. lethal dosage	Nagy 1964
Paramecium sp.	70–80	6 min	DRSh-250	—	—	—	Static	Min. lethal dosage	Laptev 1967
Paramecium caudatum	180–300 long	24,000 MWS	—	—	—	2,537	—	Sensitization to heat	Giese 1967
Paramecium multimicronucleatum	200–300 μ long	194,000 MWS	—	—	—	2,537	—	50% immobilization	Giese 1967
Dactylogyrus sp.	60 × 20 × 300	—	MBU-3 (PRK-7)	35	—	3650–3663[b]	1 m³/hr (4 gpm)	Effective	Kokhanskaya 1970
Amoeba proteus	500–600 μ	21,600 MWS	—	—	—	2,537	—	Killed 50%, but not immediately	Mazia and Hirschfield *in* Giese 1967
Ichthyophthirius adult	800	1,717,200 MWS	BUV-15, tabletop expt.	—	1–3	2,537	Static	Min. lethal dosage	Vlasenko 1969
Pelomyxa illinoisensis	500–1000	50,000 MWS	—	—	—	2,537	—	100% death	Daniels *in* Giese 1967

[a] These two were included for comparison and because they are apparently more resistant to UV than other organisms of similar size.
[b] According to Giese (1967) 3,000–3,900 A is much less lethal than 2,000–3,000 A.
[c] It is probable that a lesser dosage will be effective.
[d] Burrows, R., personal communication, Salmon-Cultural Laboratory, 1440 Abernathy Road, Longview, Washington 98632.

periment, at 112,000 MWS UV treatment, provided an excellent opportunity to observe the effect of severe WD on rainbow trout.

REVIEW OF UV USAGE IN FISH CULTURE

Because particulate matter interferes with ultraviolet irradiation, water from streams and lakes, as well as re-use water must often be pre-treated before ultraviolet treatment. Therefore, this review will include some information on filtration work.

The minimum effective dosage of UV for most fish pathogens has not been fully determined. Some representative information of the effect on these and related organisms, is summarized in Table 1, which is intended as a guide for setting up experiments with fish pathogens.

The minimum safe dosage for treating human water supplies has been established at 16,500 MWS[2]. Huff et al. (1965) have described UV equipment and methods used to control human pathogens.

UV irradiation was tested for disease control at the Little White Salmon National Fish Hatchery, Cook, Washington by Burrows and Combs (1968). In the test installation 475 liters/min of Columbia River water contaminated with feral pathogens were passed through two 76-cm-diameter high-speed sand filters and then through an 18-lamp UV unit divided into three series of six 40-watt lamps each, with a capacity of 160 liters/min per series to give an estimated 37,000 MWS. The sand filter was designed to remove particles larger than 15 μm in diameter and would help to remove UV-opaque particles. Treated water was compared with regular Columbia River water using *Oncorhynchus kisutch* fingerlings in two 7.6-m circular tanks. In the raw water some fish were killed by columnaris disease and the survivors succumbed to *Ceratomyxa shasta* (Myxosporida). The fish in the treated water were normal. In preliminary experiments, Burrows and Combs (1968) also found that 75% of *Nanophyetus cercariae* were killed with the above procedure.

In an attempt to control *Ceratomyxa shasta*, UV equipment was tested at the Pelton Hatchery by the Oregon Fish Commission in 1967. A Steroline Model PVC-2 UV sterilizer (Steroline Systems Corporation, Santa Fe Springs, California)[3] was used, providing an estimated 215,500 MWS (probably excessive); *Salmo gairdneri* fingerlings were the test fish. With raw water, 20% of the fish died from *Ceratomyxa* infection. Only 1% died in the UV treated water and no *Ceratomyxa* was found. Similar results were obtained with coho salmon at the Bonneville Hatchery using a Model SWL-100 unit (Steritronics International, Orange, California)[3]. The dosage was estimated at 360,000 MWS (probably excessive); however, 2% of the coho salmon became infected with *Ceratomyxa shasta*, but this may have been due to an experiment accident (Sanders et al. 1972). Similar results were obtained by Bedell (1971) in California.

Rapid sand-gravel filtration followed by UV irradiation is being used to treat the lake water supply to the Craig Brook National Salmon Hatchery, Bucksport, Maine. Estimate of dosage was not reported. Since installation, parasites have been seen only once in the hatchery fish and this was probably due to contamination from fish brought in from elsewhere[4].

Clearwater River water is being used with filtration and UV irradiation at the Dworshak National Fish Hatchery, Ahsahka, Idaho. The UV system consists of sixteen 190-liter/min units, each consisting of six 7.6-cm chambers in serpentine arrangement, and each containing a UV lamp. The equipment is designed to produce 35,000 MWS and the lamps are discarded when they fade to 16,000. Because of a high flow rate, the initial dosage is probably less than 35,000 MWS. The water passes through a rapid 15-μm sand filter before UV treatment. The efficiency, as determined by bacterial reduction, stays at 99.5% during times of clear water but has dropped

[2] Ellner, Sidney, 1973. Personal communication, President, Ultraviolet Purification Systems, Inc. Scarsdale, New York.

[3] Mention of product names in this paper does not imply endorsement by the Bureau of Sport Fisheries and Wildlife.

[4] Dexter, Roger. 1972. Personal communication, Hatchery Biologist, Craigbrook National Fish Hatchery, East Orland, Maine 04401.

to 90% during 50 mg/liter turbidity. The effect on parasites has not been reported[5].

Overhead irradiation of flowing sea water reduced the coliform bacteria 99.96%; protozoa were not studied (Kelly 1961). Two types of equipment were used. The first consisted of two 30-watt germicidal lamps suspended under a reflector 15.6 cm over the water in a trough 91.4 cm long, 20.32 cm wide, and 15.2 cm high with a longitudinal center baffle 6.4 cm high. Water flow through the unit was 40 liters/min with an actual retention time of 45 sec. The second unit was similar, but larger, and consisted of thirteen 30-watt germicidal lamps 91.4 cm long in a reflector 14 cm over the water in a trough 208.3 cm long by 9 cm wide by 9.5 cm deep with water depth 1.9 cm. Water flow through the unit was 150 liters/min with an actual retention time of 15 sec. The amount of ultraviolet energy reaching the water at this flow rate was 57,600 MWS/cm^2.

Filtration followed by ultraviolet irradiation of sea water in a closed aquarium system was reported by Herald et al. (1962); it caused 98% reduction of bacteria. The UV system consisted of a 190 liter/min flow over four Westinghouse Sterilamps, each 50 watts and measuring 91 cm (36 in) long. The tubes discharged radiation in the 2537 Å region. The authors did not note the effect on protozoa.

An ultraviolet unit for continuous flow sea water was described by Culverhouse (1971)[6]; the system consisted of 228 liters/min flow over four General Electric G30T8 30-watt germicidal lamps 91 cm long, 2.54 cm in diameter. These lamps discharge radiation in the 2600 Å region. More than 90% of bacteria present were killed, but the effect on protozoa was not studied.

MATERIALS AND METHODS

Experiment 1 (112,000 MWS)

In the first experiment (112,000 MWS), water from tanks containing mud and water seeded with "aged" *M. cerebralis* spores (Hoffman and Putz 1971) was pumped to eight 150-liter experimental tanks. Spores were placed in mud-bottom aquaria and "aged" for at least 4 mo to ensure infectivity. Two tanks received untreated, contaminated water (controls). Two tanks received contaminated water which was passed through a cartridge-type filter (Micro-Wynd II D-PPPF, G. P. Dempler Company, Bridgeville, Pennsylvania)[7] having a 25-μm porosity. Two tanks received contaminated water that had passed through the above filter followed by exposure to UV irradiation at 112,000 MWS at 2537 Å in an Ellner Model EP-8 Water Purifier with built-in UV sensor[8]. Further specifications of this purifier at the recommended rate of 30.3 liters/min are: 2.5 liters/min per cm of lamp, 3,876 MWS/cm^2 output at 5 cm from lamp (G36T6) at 27 C, chamber size 10.16 cm diam and 76.2 cm length, retention time 17 sec, dosage 35,000 MWS/cm^2. Two tanks received contaminated unfiltered water exposed to 112,000 MWS in a second Model EP-8 Water Purifier. The water purifiers were designed to deliver 30.3 liters/min. Because only 9.5 liters/min of water were used, the reduced flow raised the UV exposure from the designed 35,000 to 112,000 MWS.

To determine flow rate for a different dosage for this equipment, the following formula was used: 35,000 MWS ÷ desired MWS × 30.3 = new flow rate in liters. To determine dosage for a different flow rate for this equipment, the following formula was used: 30.3 liters/min ÷ new flow rate × 35,000 = new dosage in MWS.

Before entering the tanks, the water contained 0.28 g/liter total hardness, 0.005 g/liter suspended solids, had a pH of 7.3, and was 12 C.

One hundred newly-hatched, commercially purchased rainbow trout fry from a single lot were placed in each of eight tanks receiving the above waters. The appropriate water was supplied continuously for 30 days, after which

[5] Wold, E. 1971. Personal communication, Dworshak National Fish Hatchery, Ahsahka, Idaho 83520.

[6] Culverhouse, B. J. 1971. An ultraviolet sterilizer for large volume marine aquariums. U. S. Tropical Atlantic Biological Laboratory, Miami, Florida 33149. (Manuscript in preparation).

[7] Mention of product names in this paper does not imply endorsement by the Bureau of Sport Fisheries and Wildlife.

[8] Ultraviolet Purification Systems, Inc., 109 Montgomery Ave., Scarsdale, N. Y. 10583.

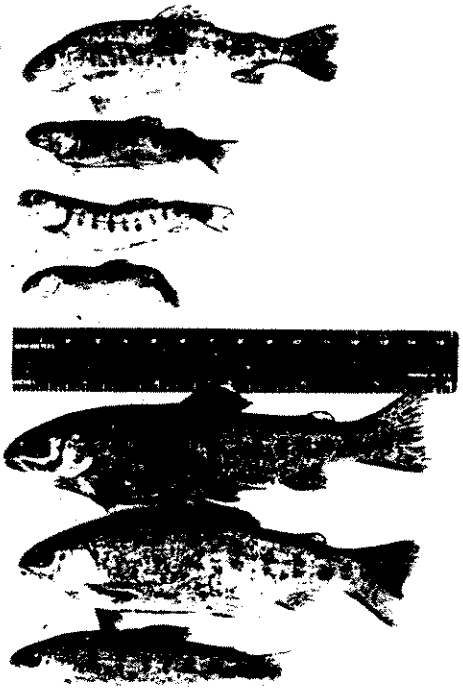

FIGURE 1.—Effects of whirling disease on rainbow trout. The upper four fish were selected randomly from a group receiving untreated *M. cerebralis*-contaminated water; note the smaller size, spinal curvature, and misshapen heads (the black tail coloration disappeared upon anesthetization). The lower three were selected randomly from a group receiving the same water, unfiltered but irradiated with 112,000 MWS of ultraviolet light.

spore-free spring water was supplied. Daily observations were made until the termination of the experiment.

At 57 days post infection (PI), 14 or more randomly selected fish from each tank were preserved in buffered 10% formalin for histological examination.

At 146 days PI, the remaining fish were counted, weighed, and returned to their tanks. At 6 mo, 20 fish from each tank were necropsied and examined for spores. The entire head was homogenized with a mortar and pestle with the aid of 380-μm sand, and 4 ml of water were added. One drop of the resultant suspension was placed on a microslide, a 22-mm-square coverslip was added, and the slide was examined at 430 × for the presence of spores.

TABLE 2.—*Effects of 112,000 MWS/cm² of ultraviolet irradiation and filtration, alone and in combination, on the infectivity of water containing* Myxosoma cerebralis *and incidence and effect of whirling disease on rainbow trout*

Treatment of contaminated water	No. and % of fish remaining at 146 days PI[d]	Average weight of fish at 146 days PI (g)	No. and % of fish containing spores at 180 days
Untreated	75 (37%)	2.9	7 of 7 (100%)
Filtered[a]	130 (65%)	5.9	15 of 15 (100%)
Filtered, followed by UV treatment[b]	170 (85%)	6.6	0 of 30 (0%)[c]
UV-treated[b] without filtration	128 (64%)	7.4	0 of 40 (0%)[c]

[a] Commercial filter cartridge with 25-μm porosity (loc. cit.).
[b] 112,000 MWS/cm².
[c] This sampling plus those found negative in histologic sections exceeds the 95% confidence level for 4% incidence.
[d] PI = post infection.

In all experiments the original plan was to necropsy only enough fish to determine if there were infected fish present. Therefore, the number of samples from the positive experiments is small. However, in those tests deemed negative larger numbers of samples were taken.

Experiment 2 (43,000 MWS)

Because the contaminated water contained very little particulate matter and the supply of contaminated water was limited, the experimental design was changed slightly for the second experiment (43,000 MWS): (1) two tanks received untreated water; (2) two tanks received similar water that was passed through a 25-μm filter (loc. cit.); and (3) four tanks received unfiltered water that passed through the Ellner EP-8 Water Purifiers at a flow rate resulting in an exposure of 43,000 MWS. Water contaminated with *M. cerebralis* was supplied 1–3 hr daily for 30 days and *M. cerebralis*-free water was supplied at all other times.

Experiment 3 (35,000 MWS)

During the third experiment (35,000 MWS) some silt was present in the water, so the experimental design was again changed slightly: (1) two tanks received untreated contaminated

TABLE 3.—*Effect of filtration and ultraviolet irradiation on* Myxosoma cerebralis *infection in rainbow trout*

	Untreated water	Filtered only	Filtered plus UV	UV-treated only
Experiment 1 (112,000 MWS)	100% incidence of severe[a] WD	100% incidence, moderate[b] WD	No WD No spores	No WD No spores
Experiment 2 (43,000 MWS)	100% incidence of severe[a] WD	90% incidence of mild[c] WD	Omitted	No WD No spores
Experiment 3 (35,000 MWS)	100% incidence moderate to severe[b] WD	Omitted	No WD No spores	Omitted

[a] Black tail, whirling, spinal curvature.
[b] Black tail, whirling.
[c] No black tail, only sporadic whirling.

water, and (2) two tanks received contaminated water passed through separate 25-μm filters and separate Ellner EP-8 Water Purifiers at a flow rate resulting in exposure to 35,000 MWS. Because of water flow limitations, each tank was drained and refilled with test water in the morning; the test water was allowed to remain 1 hr, and then a small trickle of spore-free water was supplied for several hours before the flow was returned to the normal rate. This procedure was repeated 28 times during the first 34 days of the experiment in order to expose the fish to the experimental water as long as possible.

RESULTS

Untreated Water

The untreated water contained infective *M. cerebralis*.

First experiment (112,000 MWS)—Control fish receiving untreated contaminated water were severely diseased as evidenced by signs of WD—whirling, black tails, spinal curvature (Fig. 1)—with 100% incidence of infection as determined by the presence of spores or trophozoites. At 57 days PI, trophozoites of *M. cerebralis* were seen in sections of all 23 specimens examined histologically. Many spores were found in all seven fish necropsied at 180 days. Severe whirling disease caused reduced weight gain and mortality; uninfected fish weighed 7.4 g and severely diseased fish weighed 2.9 g (Table 2).

Second experiment (43,000 MWS)—Five of the 100 fish from each of the two tanks receiving untreated water were taken at random at 4 mo and necropsied; all showed signs of whirling disease (black tail and whirling) and spores were present in all fish. The remaining fish were so severely diseased (black tail, whirling) that it did not seem necessary to necropsy more.

Third experiment (35,000 MWS)—Many fish died during the first 90 days of the experiment. Microscopic examination showed lesions typical of bacterial gill disease as well as whirling disease, and I could not determine to what extent each was involved in the mortality rate. The whirling disease signs were moderate to severe and spores were found in all 10 fish examined. Thirty (18.5%) of the 200 fish were alive at 171 days and averaged 6.9 g, whereas the uninfected fish averaged 8.5 g.

Filtered Water

Water from the same source as the untreated water was passed through a 25-μm cartridge filter (loc. cit.).

First experiment (112,000 MWS)—The fish receiving filtered water were moderately diseased (several with black tail and whirling). At 57 days, trophozoites were found in histological sections of 13 of 23 fish examined. Spores were found in all 15 fish (100% incidence) necropsied at 180 days (Table 3).

Second experiment (43,000 MWS)—Five fish from each of the two tanks receiving filtered water were taken at random at 4 mo and examined. At necropsy, spores were found in five from one tank and four from the other.

Third experiment (35,000 MWS)—Omitted.

Filtered Water Followed by UV Treatment

Water from the same source as the untreated water was passed through a 25-μm cartridge filter (loc. cit.) followed by UV treatment.

First experiment (112,000 MWS)—The fish

receiving filtered and UV-treated water showed no signs of disease (Fig. 1, Table 2). At 57 days PI, no trophozoites were seen in 21 fish examined. At 146 days the fish weighed slightly over twice as much as those showing signs of severe whirling disease. Thirty fish were necropsied and no spores were found. Sampling of 51 of the 170 surviving fish exceeds the 95% confidence level for 4% incidence.

Second experiment (43,000 MWS)—Omitted.

Third experiment (35,000 MWS)—No signs of whirling disease or gill disease were seen in fish in the two tanks receiving filtered UV-treated water. Ten of 10 fish from each of the two tanks were free of spores. This sampling of 20 of the 142 surviving fish exceeds the 95% confidence level for 10% incidence.

UV Treated Water

Water from the same source as the untreated water was passed through UV equipment (loc. cit.) at a flow rate and lamp wattage to produce the cited dosage.

First experiment (112,000 MWS)—The fish receiving unfiltered UV-treated water were also free from disease (Fig. 1). No trophozoites were seen in sections of 14 fish examined at 57 days PI. No spores could be found in 40 fish necropsied at 80 days; this exceeds sampling necessary for the 95% confidence level for 4% incidence; 128 of 200 fish (64%) remained. Surviving fish were 2.5 times as heavy as those with severe WD (Fig. 1, Table 2).

Second experiment (43,000 MWS)—No signs of whirling disease were seen in fish in the four tanks receiving UV-treated water. One hundred ninety-three of the remaining 280 fish were examined for spores at 4 mo; no fish were infected. This sampling exceeds the 95% confidence level for 0.5% incidence.

Third experiment (35,000 MWS)—Omitted.

DISCUSSION

Ultraviolet irradiation at a wavelength of 2537 Å, for 35,000, 43,000 and 112,000 microwatt sec/cm^2 (MWS) following filtration through 25-μm-pore cartridge-type filters killed or removed *Myxosoma cerebralis*. The omission of filtration at 43,000 MWS in the absence of visible particulate matter did not influence the results. Obviously, filters should be used when particulate matter is present. Fifteen-micron-pore filters plugged up too rapidly in the preliminary tests; 25-μm size was satisfactory with our contaminated water, which contains a moderate but undetermined amount of particulate matter. Apparently some of the 25-μm pores became partially plugged with detritus so that some of the 10-μm spores became trapped. With the relatively high dosages of UV used, the effect of filtration on subsequent UV irradiation could not be determined in the present experiments. However, because the filters reduced the infective load in the non-UV tests, filtration appears beneficial. Likewise in experiments with Millipore filters, Putz (1969) demonstrated that spores become trapped in 14-μm filter sizes. Further research on filtration and subsequent UV irradiation is needed.

The manufacturers of UV equipment state that water may contain suspended or dissolved material, transparent to the eye, but refractory to UV irradiation. This may be as important as visible suspended solids; at least one manufacturer, Ultraviolet Purifications Systems, Inc. (loc cit.)[9], is equipped to test sample water.

Commercially available equipment can be obtained that will deliver UV intensities ranging from 16,000 to 112,000 MWS, varied by flow regulation and monitored by built-in sensors. Less electricity per liter of pumped water is needed for the lowest intensity, but 16,000 MWS cannot be recommended until it has been tested and found effective; this work is in progress.

There has been little standardization in the reporting dosages used. Only the most helpful information is included in Table 1. The dosages given in Giese (1967) are listed in ergs; I converted to MWS for comparison. In some cases I was not able to determine the MWS or ergs dosages used and so listed the type of equipment used. Soviet scientists

[9] Mention of product names in this paper does not imply endorsement by the Bureau of Sport Fisheries and Wildlife.

were not able to furnish necessary data because of patent regulations. UV efficacy reports should include liters/min per cm of lamp, wavelength and output of lamp(s) at 5 cm, size of chamber, retention time in seconds, and MWS dosage. Such information would help avoid confusion that can occur in reporting only MWS/cm^2 since most equipment can not accurately measure MWS.

The present work and others have demonstrated that UV irradiation at 35,000 MWS will successfully disinfect waters containing *Aeromonas salmonicida*, *M. cerebralis*, *Saprolegnia* sp., small *Trichodina* sp. and probably other organisms of similar or smaller size (Table 1). Further testing is needed.

If the equipment can be installed in a gravity-flow system, the cost of the equipment is the only major expense. If water must be pumped, proper plumbing of the UV irradiators will result in the need for very little additional electricity. Most commercial units contain UV bulbs housed in a quartz cylinder around which the water flows. Great care must be taken to keep the cylinders clean; otherwise the effectiveness will be reduced. Some custom-built units consist of bulbs suspended over a thin sheet of flowing water; if splash is avoided, these units need little cleaning. UV bulbs tend to lose intensity with use, so it is very important to have reliable UV sensory equipment available, or permanently installed, to test them.

After many experiments with whirling disease in our laboratory (Hoffman and Putz 1969, 1971, unpublished) we have found that the intensity of whirling disease is directly related to the intensity of infection, i.e., few infective units in water produce subclinical infection, and many infective units cause severe whirling disease. The studies of the effect of filtration in the present experiments substantiate this observation. Although spores were found in the fish receiving filtered water, the fish showed fewer signs of disease than those receiving untreated water. It is apparent that some of the spores were removed and the intensity of infection was less, but total spore counts were not made.

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WHIRLING DISEASE (*Myxosoma cerebralis*): CONTROL WITH ULTRAVIOLET IRRADIATION AND EFFECT ON FISH

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Abstract: Water contaminated by *Myxosoma cerebralis* was disinfected with ultraviolet irradition to control whirling disease. Irradiation at 18,000 microwatt seconds/cm² (MWS/cm²) reduced infectivity of *M. cerebralis* by 31-86% and 27,650 MWS/cm² reduced infectivity by 86-100%, even in the presence of a small amount of silt.

INTRODUCTION

Since its introduction to North America about 1957, whirling disease (WD) of salmonids has spread to nine states.[2] With the cooperation of state and federal agencies and private fish culturists, the rate of spread of the disease has been reduced during the last six years. However, since the possibility for further spread continues to exist, better control and eradication methods are needed.

Ultraviolet (UV) irradiation was reported as a possible method of controlling WD in contaminated water. In the United States, 35,000 microwatt seconds (MWS)/cm² successfully disinfected water,[2,5] and in the U.S.S.R. UV also showed promise for WD control.[3] The present report concerns a continued search for the minimum effective dosage, which has not been determined.

MATERIALS AND METHODS

Two experiments were prepared as described by Hoffman.[2] Eight 150-liter stainless steel tanks each received 100 liters of contaminated water daily followed by running spring water as needed. The water in tanks 1 and 2 was irradiated with 18,000 MWS and the water in tanks 3 and 4 was passed through a 25-μm filter and treated with 27,650 MWS. Dosage was determined as in Hoffman (1974).[2] Water in tanks 5 and 6 was untreated; water in tanks 7 and 8 was passed through the same 25-μm filter used for tanks 3 and 4. Each tank was stocked with 100 two-week-old Nasuah strain rainbow trout (*Salmo gairdneri*).

In both sets of tests a small amount of visible silt which did not depress the UV-sensor needle was accidentally pumped through the equipment and into the tanks during the first four days of the test. The filter used for the 27,650 MWS series should have removed all particles larger than 25 μm.

Negative control fish were reared in spore-free spring water. Fish were examined for spores by the digest method.[4]

RESULTS AND CONCLUSIONS

Reduction of infectivity was 31% to 86% at 18,000 MWS and 86% to 100% at 27,650 MWS (Table 1). Negative control fish reared in spring water were free of WD and spores. I cannot account for the low survival rate (23%, tank 3) in one lot of fish receiving UV-treated water.

[1] Work performed at the Eastern Fish Disease Laboratory, Kearneysville, West Virginia.

TABLE 1. Effect of ultraviolet light on **Myxosoma cerebralis**, as indicated by the survival and weight gain of 2-week-old rainbow trout. Each tank was stocked with 100 fish; duration of exposure was 6 weeks.

Dosage and tank number	Clinical signs (percent)	Survival, number or percent	Spores		Average weight, grams ②
			Number fish sampled ①	Percent with spores	
18,000 MWS					
1	1	86	16	69	8.8
2	0	57	14	14	9.6
27,650 MWS					
3	0	23	18	0	8.0
4	0	92	22	14	8.5
Untreated positive controls					
5	100	48	14	100	4.1
6	100	58	15	100	4.2
7	100	23	18	100	3.1
8	100	79	20	100	4.9

① Number sampled exceeds the 95% confidence level for 10% incidence. (5.

② Difference in weight of fish in treated and untreated water was significantly different as determined by Chi Square ($P = 0.025$) in all samples.

At 4½ months post-exposure, fish receiving treated water were 1.8 to 2.6 times heavier than diseased controls; most of the controls had gross signs of WD (Table 1).

Since silt may have interfered with the effectiveness of UV in this experiment it would be advisable to repeat the study with silt-free water.

Until a lower dosage can be conclusively shown to control WD, 35,000 MWS should be considered the lowest effective level.[2] The marked reduction in both incidence and severity of infection observed in lots reared in water treated with successively higher dosages of UV supports the earlier determination that 35,000 MWS is adequate.

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Myxobolus cerebralis, a Worldwide Cause of Salmonid Whirling Disease[1]

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Abstract.—Salmonid whirling disease was discovered in Europe in 1893 and has since been spread around the world with shipments of cultured and wild fish. The causative agent is the protozoan parasite *Myxobolus cerebralis*, whose taxonomy and life cycle remain controversial. It causes lesions of salmonid skeletal tissues, particularly in the head. Diagnosis usually is straightforward, but can be complicated by the presence of other *Myxobolus* species and by other parasites that cause fish to whirl. The pathogen is difficult to eradicate, but its seriousness can be managed if fish are cultured in spore-free source water, in concrete raceways with strong water flows, or in ponds that are regularly disinfected, and if they are constantly monitored for the presence of spores. Fish can carry *M. cerebralis* spores and still be healthy. Such fish may be suitable for stocking into waters already containing *M. cerebralis*, and even for human consumption.

Recent findings of whirling disease in the western USA have prompted this brief review. Whirling disease is caused by the myxosporidan parasite *Myxobolus cerebralis* (previously known as *Myxosoma cerebralis*). Lom and Noble (1984) synonymized *Myxosoma* with *Myxobolus* because the only separating characteristic, the glycogen (iodinophilous) vacuole, is not always reliable taxonomically. However, this historically useful characteristic remains very helpful in identification of many species of the genus. Workers in the USSR have not accepted the synonymization of *Myxosoma*, and some of them have studied the vacuole rather intensively (Podlipaev and Schulman 1978; Schulman et al. 1978).

In this review I discuss the possible origin of *M. cerebralis*, its distribution in Europe, and its later recognition around the world, in addition to its biology, diagnosis, and control measures. Previous reviews include those of Hoffman (1970, 1976), Halliday (1976), Ghittino and Vigliani (1978), Wolf and Markiw (1985), and Wolf (1986).

Europe, 1893–1988

Apparently *M. cerebralis* evolved as a nonpathogenic parasite of brown trout *Salmo trutta* in central Europe and northern Asia (Hoffman 1970; Bogdanova 1972). It was not discovered until 1893, when Hofer reported whirling disease (Drehkrankheit) from rainbow trout *Oncorhynchus mykiss* (formerly *Salmo gairdneri*) in Germany that had been recently imported from the USA. Hofer (1893) described the parasite as *Myxobolus cerebralis*, but changed the name to *Myxobolus chondrophagus* in 1904. Plehn (1905) corrected the species name to *cerebralis*, following the International Code of Zoological Nomenclature. She introduced the new generic name *Lentospora* for species similar to *Myxobolus* but not possessing the iodinophilous vacuole, and then renamed the parasite *Lentospora cerebralis*. Some time later, Kudo (1933) reduced *Lentospora* to synonymy with *Myxosoma*, and the parasite's name became *Myxosoma cerebralis*. As mentioned earlier, the name was changed back to *Myxobolus cerebralis* by Lom and Noble in 1984. The Europeans have learned much about the biology, pathogenicity, and control of *M. cerebralis*. They have learned that in most instances, eradication is impossible, but that reduction in infection levels is possible, so fish can be raised that are not deformed by whirling disease (Plehn 1924—Germany; Schäperclaus 1931, 1954, 1979—Germany; Kocylowski and Miaczynski 1960—Poland; Bregnballe 1963—Denmark; Keiz 1964—West Germany; Rasmussen 1965—Denmark). European methods include (1) raising fingerlings to at least 6 cm in length in spore-free water, (2) converting earthen ponds to concrete raceways, and (3) draining, cleaning, and disinfecting earthen ponds that cannot be converted. I have seen the results of such practices in Germany and Denmark and recognize that apparently healthy fish can be raised where *M. cerebralis* cannot be eradicated.

[1] Invited paper given at the International Fish Health Conference of the Fish Health Section of the American Fisheries Society, Vancouver, British Columbia, Canada, July 19–21, 1988.

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World Distribution

Myxobolus cerebralis has probably been transferred worldwide in shipments of infected live and frozen trout and possibly as a contaminant with trout eggs (Hoffman et al. 1962; Bogdanova 1968; Elson 1969; Hewitt and Little 1972; Halliday 1976). Experimentally, it can be transmitted by fish-eating birds, but it is not known how long or how far it can be so carried (Taylor and Lott 1978). *Myxobolus cerebralis* has been found in Germany (1893), Italy (1954), the USSR (1955), the USA (1958), Bulgaria (1960), Sakhalin Island of the USSR (1960), Yugoslavia (1960), Sweden (1966), South Africa (1966), Scotland (1968), New Zealand (1971), Ecuador (1971), Colombia (1972), Lebanon (1973), Ireland (1974), Spain (1974), and England (1981). The published reports do not prove that *M. cerebralis* arrived in these countries at the reported times, but do indicate that it has been moved with fisheries activities. The signs of infection in fish culture are so spectacular that it is probable that *M. cerebralis* did not exist in most of these places earlier than reported (Hoffman 1970; Halliday 1976).

Because of greater awareness of whirling disease and the emergence of methods of certification, it is hoped that the spread of *M. cerebralis* worldwide will be slowed down and the effects diminished.

Situation in the USA

Whirling disease was first diagnosed in the USA in 1958 by S. F. Snieszko and G. L. Hoffman, assisted by Art Bradford of the State of Pennsylvania. The spectacular whirling and the black tails indicated Drehkrankheit (whirling disease) as described by Schäperclaus (1954) in his German text on fish diseases. Circumstantial evidence strongly suggested the origin of this disease to be from imported frozen European rainbow trout. At about the same time, whirling disease was found in Nevada (W. T. Yasutake, National Fishery Research Center, personal communication); again frozen European trout were suspected. Importation ceased, and no further new sites were found until 1961, when the disease was identified in Connecticut, where it was believed to have arrived in live rainbow trout. In 1965 it was found in a Virginia fish hatchery, and in 1966 in Massachusetts, California, and several more locations in Pennsylvania. During the next 3 years, whirling disease was found in New Jersey, again in Pennsylvania, in Ohio, in Michigan, again in Nevada, and in West Virginia. The spread continued: 1980—New Hampshire; 1984—New York; 1987—Oregon, Idaho, and Colorado (Holt et al. 1987; Horsch 1987; Barney et al. 1988; Hauck et al. 1988). Not all of these infections can be attributed to fish transfers.

The Fish Disease Subcommittee of the Colorado River Wildlife Council (Anderson and Goede 1988) held an emergency conference on whirling disease April 12–14, 1988, in Denver. There were 69 participants from state and federal agencies and from private trout farms. From their reports and the records listed above, we can surmise that whirling disease came to the USA in frozen table rainbow trout and that it has spread largely by transfer of live fish and by movement of infected fish in streams. Because *M. cerebralis* has been experimentally passed, alive, in the feces of fish-eating birds, it is possible that some otherwise unexplained transfers occurred in this fashion. There is no concrete evidence that *M. cerebralis* was transferred in the USA with trout eggs, but it remains possible that it could spread by contamination of eggs.

In the affected areas, facilities with earthen ponds usually contained heavily infected fish, whereas those with faster-flowing water in concrete raceways usually had unaffected or sometimes lightly infected fish.

The attitude toward *M. cerebralis* has changed in the USA. We have learned that condemnation and confiscation of infected fish do not eliminate whirling disease from fish hatcheries and trout streams, but control measures, as practiced in Europe and outlined later in this paper, help ameliorate the impact of the disease. Equally important and gratifying is evidence of better understanding between federal and state employees and commercial trout farmers. For example, J. W. Warren (U.S. Fish and Wildlife Service, personal communication) stated that his agency was able to help a commercial trout farmer sell his healthy-appearing but contaminated fish for stocking, without causing harm, in fishing waters where *M. cerebralis* already existed. In my opinion, this is a highly commendable attitude.

Life Cycle (Tubificid or Not Tubificid?)

The major aspects of the life cycle of *M. cerebralis* in rainbow trout tissues have long been established (Plehn 1924; Schäperclaus 1931, 1954; Uspenskaya 1957, 1978, 1979). In susceptible trout, the trophozoite parasite can first be found in the head cartilage 20 d after exposure (O'Grod-

nick 1978). The trophozoites stain well with Mallory–Heidenhain stain (Casson modification); color photographs of this material may be seen in Hoffman and Meyer (1974). Trophozoites of *M. cerebralis* grow, divide, and finally produce pansporoblasts, each of which produces two spores (Hoffman 1976; Příhoda 1978).

Some of the spores move, presumably by tissue pressures, and some are shed in feces and from the gills, but most are probably disseminated from dead infected fish. As long ago as 1931, Schäperclaus hypothesized that *M. cerebralis* might be carried by invertebrates such as snails, insect larvae, or crustaceans. It has been known since 1955 that spores are not infective when fresh; to be infective, they must be "aged" for about 3½ months (Uspenskaya 1955, 1978; Hoffman and Putz 1969). More recently, Wolf and Markiw (1984) demonstrated that *Tubifex* worms are the obligate intermediate hosts, where the parasite assumes the form of *Triactinomyxon* sp., a very dissimilar actinosporean sporozoan. Wolf and Markiw (1984) proposed the name *Triactinomyxon gyrosalmo* for the parasite. However, Corliss (1985) stated, "The organism's name, simply by application of the rule of priority, must remain *Myxobolus cerebralis* (Hofer, 1903) Kudo, 1933 (the older name for the identical species, with *Triactinomyxon gyrosalmo* following as a junior synonym." Both stages are thus correctly called *M. cerebralis,* one being called the triactinomyxon stage of *M. cerebralis.* In England, Hamilton and Canning (1987) reported "results—which do not support the actinosporean involvement in the life cycle." El-Matbouli and Hoffmann (1989) in Germany, support the *Triactinomyxon* connection, but Příhoda (1978), in Czechoslovakia, does not. Lom (1987), in a review written before the paper by El-Matbouli and Hoffmann (1989) stated,

> This surprising finding [*Triactinomyxon* connection] met with considerable misgivings. It is an unparalleled case of an organism alternating two different life cycles, each with its own asexual proliferation and sexual process occurring at different points in the cycle. If these findings were confirmed, they would radically alter our general concept of the life cycle and taxonomy of Myxosporea. If we accept the idea that each myxosporean has a matching actinosporean, representing the second half of its cycle, then the relatively few actinosporean species are clearly outnumbered by myxosporeans, and very markedly so in the marine environment. However, since that remarkable paper of Wolf and Markiw in 1984, no one else has confirmed their findings.

However, Markiw (1989) has found that the myxosporean and actinosporean stages cross-react in a direct fluorescent antibody test. It is obvious that more work must be done on the life cycles of *M. cerebralis* and other myxosporeans.

Diagnosis

Clinical signs, such as black tail, whirling, and skeletal deformities, are useful in diagnosing whirling disease in salmonids, but histological identification of typical spores of *M. cerebralis* in lesions in the skeletal tissues (Figure 1), particularly of the head, confirms the diagnosis (Plehn 1905; Schäperclaus 1931, 1979; Schulman 1966; Halliday, 1973; Ghittino and Vigliani 1978). The spores were originally described by Hofer (1904) and Plehn (1905); electron micrographic descriptions were provided by Lom and Hoffman (1971) and Lunger et al. (1975). Serologic verification of the presence of *M. cerebralis* (Markiw and Wolf 1978) has apparently been complicated by cross-reactions with other species of myxosporeans (Amandi et al. 1985; Horsch 1987; Markiw 1989); in addition, testing materials and equipment are not available in many laboratories. Hamilton and Canning (1988) devised an immunofluorescence method for identifying trophozoites of *M. cerebralis* in tissue sections. Bartholomew et al. (1989) developed immunodiagnostic methods for identifying trophozoites and spores of *Ceratomyxa shasta,* but some of their antibodies reacted with white blood cells and with myxosporeans of the genera *Myxobilatus* and *Sphaerospora.* Perhaps DNA analysis can be used in the near future to confirm myxosporean identifications. Other possible future aids in species identification might be the circumferential groove seen on the spore under scanning electron microscopy and the mucous envelope seen in India ink preparations (Lom and Hoffman 1971).

When time and circumstances permit, the use of caged rainbow trout fry as sentinel fish is one of the most reliable methods for establishing the presence of absence of *M. cerebralis* (Hnath 1970; Horsch 1987; N. Boustead, New Zealand Ministry of Agriculture, personal communication).

Sometimes confusing in the diagnosis of whirling disease is the finding of spores of other species in fresh preparations, including those prepared by the concentration methods of O'Grodnick (1975), Markiw and Wolf (1974), and others. This can sometimes be avoided by dissecting the fish carefully or by making histological sections to deter-

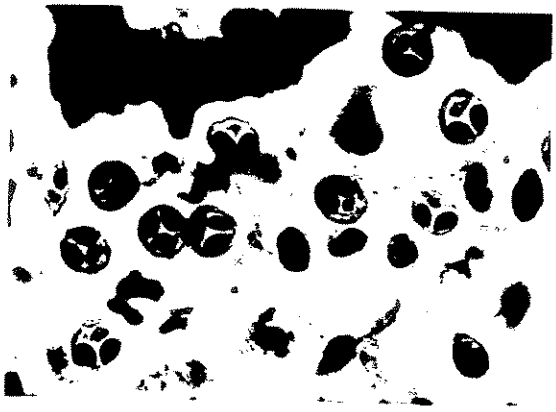

FIGURE 1.—Histologic section of rainbow trout showing spores of *M. cerebralis* in the cartilage. Stained with methylene blue. Courtesy of the late P. Ghittino, Torino, Italy.

mine which tissues are infected. *Myxobolus cerebralis* is almost always found in the skeletal parts and rarely in granulomas on the periphery of skeletal structures. Confusing species that have turned up in diagnostic work are

Myxobolus neurobius.—In central nervous system of trout, salmon, Arctic grayling *Thymallus arcticus,* and whitefish. Pear-shaped, larger than *M. cerebralis*; 10–13 μm long. Circumpolar distribution.

Myxobolus kisutchi.—In central nervous system of coho salmon *Oncorhynchus kisutch* and chinook salmon *Oncorhynchus tshawytscha,* possibly rainbow trout. Spore very similar to that of *M. cerebralis,* but with an iodinophilous vacuole; spores usually in clusters.

Myxobolus squamalis (*Myxosoma squamalis*).—In scales of rainbow trout and salmon. Very similar to *M. cerebralis,* but with two lateral ridges.

Myxobolus insidiosus.—In muscle of cutthroat trout *Oncorhynchus clarki* (formerly *Salmo clarki*), chinook salmon, and coho salmon. Pear-shaped, larger than *M. cerebralis*; 13–17 μm long.

Another disease that has been confused with whirling disease is vitamin C deficiency, which also causes black tail and spinal curvature. This is known to have occurred in the USA, Venezuela, and Portugal.

In addition, there are cartilage-infecting species of myxosporidans in other fishes:

Henneguya brachyura.—In cartilage of fin ray of pugnose shiner *Notropis anogenus*; Lake Erie, USA.

H. schizura.—In sclera of eye of northern pike *Esox lucius*; Germany and USA.

Myxobolus aeglefina.—Marine; in head cartilage of the cod *Gadus aferdeen.*

M. bilineatum.—Sometimes in cartilage of mummichog *Fundulus heteroclitus*; eastern USA.

M. cartilaginis.—In gill arches of bluegill *Lepomis macrochirus,* green sunfish *Lepomis cyanellus,* and largemouth bass *Micropterus salmoides*; West Virginia and Maryland, USA.

M. chondrophilus.—In gill rakers of the clupeid *Sardinella anchovina*; South America.

M. dentium.—Causes "loose-teeth" disease of muskellunge *Esox masquinongy*; North America.

M. ellipsoides.—In cartilage of the cyprinid *Leuciscus cephalus*; Russia and England.

M. eucaliae.—In skeleton of brook stickleback *Culaea inconstans*; Lake Michigan, USA.

M. gangulli.—Marine; India.

M. guyenoti.—Rarely in cartilage of European perch *Perca fluviatilis*; Switzerland.

M. hoffmani.—In cartilaginous sclera of eye of fathead minnow *Pimephales promelas*; North Dakota, USA.

M. hyborhynchi.—In mandibular bone of bluntnose minnow *Pimephales notatus*; Canada.

M. nuevoleonensis.—In cartilage and bone at bases of fins of shortfin molly *Poecilia mexicana* and guppy *Poecilia reticulata*; Northern Mexico.

M. petruschewskii.—In gill arch of the sculpin *Myoxocephalus* sp.; Bering Sea, USSR.

M. scleroperca.—In cartilaginous sclera of eye of yellow perch *Perca flavescens* and logperch *Percina caprodes*; Lakes Michigan and Erie, USA and Canada.

M. sp.—In cartilage and muscle of fathead minnow; Ohio, USA.

Sphaerospora platessae.—Marine; in sclera of eye of plaice *Pleuronectes platessa*; England.

In addition, there are two parasites that cause whirling of fish, but that are not found in cartilage:

Myxobolus drjagini.—In central nervous system and semicircular canals; causes whirling of silver carp *Hypophthalmichthys molitrix* in China (S.-R. Hsieh, Institute of Hydrobiology, Academia Sinica, Wuhan, China; unpublished).

Galactosomum sp.—Metacercariae in the braincase cause whirling of anchovies and cultured

yellowtail *Seriola lalandei* in Japan (Kimura and Endo 1979).

Control and Prevention

Control and prevention of whirling disease are well covered in rather recent review articles by Cordero del Campillo et al. (1975—Spain), Halliday (1976—Denmark), Hoffman (1976—USA), Ghittino and Vigliani (1978—Italy), and Wolf and Markiw (1985—USA). Extensive fish disease surveillance is essential.

Myxobolus cerebralis should not be transferred to fish culture facilities or other waters where it does not already exist. Persons receiving fish for such waters must insist that the fish be certified free of *M. cerebralis*. In the USA and many other countries, there are parasitologists and fish pathologists capable of determining the presence or absence of *M. cerebralis*. In cases of doubt, caged sentinel rainbow trout fry should be placed in waters where *M. cerebralis* may exist; spores should be found in the sentinel fish in about 4 months if *M. cerebralis* is present.

This method of prevention may sound easy to the novice, but to make it work there must be national as well as state or provincial regulations, not so much to restrict fish farmers as to help avoid the transfer of *M. cerebralis*. In the USA, e.g., as evidenced in the aforementioned Colorado River Wildlife Commission conference on whirling disease in May 1988, various states have fish disease laws that encourage monitoring of trout culture facilities for *M. cerebralis*. Many U.S. state laws prohibit the importation of trout with *M. cerebralis*, as do Canadian law and European and British regulations. Therefore, a system of certification is developing that surely will help prevent the uncontrolled spread of *M. cerebralis*.

In my opinion, no condemnation of fish stocks should be included in these regulations unless adequate indemnification is also provided.

Managing *M. cerebralis*

The construction of concrete raceways makes it possible to reduce infection levels of *M. cerebralis* to the point at which healthy-looking, but lightly infected, fish can be reared. For those who must use earthen ponds, the long-established European methods described by Schäperclaus (1931, 1954), Bregnballe (1963), and Christensen (1966), and now used by some U.S. facilities, should be employed as follows.

(1) All trout and salmon facilities using earthen ponds or surface water should be monitored regularly for the presence of *M. cerebralis* (not necessarily overt whirling disease).

(2) At facilities where *M. cerebralis* exists, all possible measures should be taken to prevent or reduce its impact. Concrete raceways with fast-flowing water should be constructed where feasible. Fry and fingerlings should be reared in known spore-free water as long as possible, but at least until they are 6 cm long. Springwater and well water are usually pathogen-free. Surface water can be disinfected by filtration followed by ultraviolet irradiation or ozone or chlorine treatment (Gerard and Tiret 1974; Hoffman 1974, 1975; Ghittino and Vigliani 1978; Williams et al. 1982; Horsch, 1987). Earthen rearing ponds or raceways should be drained annually, disinfected with quicklime (CaO), calcium cyanamide, or chlorine (calcium hypochlorite), and dried, if possible.

(3) Where proper control of *M. cerebralis* cannot be achieved, the trout grower should consider raising other species that are less prone to infection. O'Grodnick (1979) determined the susceptibilities of several species, here listed in order of decreasing susceptibility: rainbow trout, sockeye salmon *Oncorhynchus nerka*, brook trout *Salvelinus fontinalis*, chinook salmon, Atlantic salmon *Salmo salar*, brown trout, coho salmon. Lake trout *Salvelinus namaycush* and splake (hybrids between brook trout and lake trout) are refractory to infection by *M. cerebralis*.

Using the techniques outlined above, fish culturists do rear healthy fish, although often some fish carry spores. In my opinion, there is no reason why these fish should not be stocked in waters already containing *M. cerebralis* or used for human consumption. Such fish could be labelled "healthy, but may contain a few spores of *M. cerebralis*. Spores not visible, food quality excellent. Unused, uncooked parts must not be discarded in, or adjacent to, salmonid waters." This sort of warning should be put into a national fish disease law in the USA and perhaps in other countries. I believe such a national law is needed, and that it should include *M. cerebralis*. It should allow salmonids to be raised in the presence of *M. cerebralis*, but regulate the use of such fish. Such fish need not be confiscated if they are used appropriately. If there are condemnation procedures, they should be accompanied by appropriate indemnification.

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Fish Age as Related to Susceptibility to Myxosoma cerebralis, *Cause of Whirling Disease*

Rainbow trout up to 12 months old were experimentally infected with *Myxosoma cerebralis*. As determined by the presence of spores in exposed fish, individuals that were exposed at various ages from 2 weeks to 12 months became infected experimentally (see table). Except for one 8-month-old lot, the fish exposed to whirling disease when 4.5 months or older did not develop clinical signs of whirling disease.

***Age of rainbow trout and susceptibility to* Myxosoma cerebralis (*whirling disease*)**

Age of fish at time of exposure	Number of fish	Length of exposure	Clinical signs [1]	Presence of spores in one or more fish
2 weeks	100	0 (control)	0	0
2 weeks	50	2 weeks	+	+
4.5 months	200	2 months	0	+
6 months	25	6 weeks	0	+
8 months A	10	2 weeks	0	+
8 months B	many	8 months	+	+
11 months	5	8 days	0	+
12 months	4	2.5 months	0	+

[1] See Hoffman, Dunbar, and Bradford, 1969. Whirling disease of trouts caused by *Myxosoma cerebralis* in the United States. U.S. Fish and Wildlife Service, Special Scientific Report—Fisheries No. 427.

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STUDIES ON THE CONTROL OF WHIRLING DISEASE (*Myxosoma cerebralis*).

I. The Effects of Chemicals on Spores *in vitro*, and of Calcium Oxide as a Disinfectant in Simulated Ponds

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Abstract: Based on presumptive evidence of death (extrusion of polar filaments and disintegration of sporoplasm) 1.0%, 0.5%, and 0.25% calcium oxide or potassium hydroxide killed the spores of *Myxosoma cerebrallis in vitro*. Chlorine at 400 ppm destroyed 36% to 90% of the spores but 13% to 37% of those in the controls perished. Calcium hydroxide, ammonium chloride, sodium borate, potassium permanganate, Roccal (alkyl dimethylbenzylammonium chloride), and copper sulfate allowed survival of 38-96% of the spores, usually not much less than the rate of survival of the controls.

In simulated pond testing, quicklime at 380 grams or more per square meter (3360 lbs/acre) of pond bottom prevented whirling disease in rainbow trout *(Salmo gairdneri)*.

INTRODUCTION

Whirling disease, caused by *Myxosoma cerebralis* (Protozoa: Myxosporida), has caused great concern among trout culturists ever since it first became evident in Germany about 1900.[2,7] Control and eradication of the disease involves cleaning and disinfecting the ponds. Schäperclaus,[8] Tack,[11] and Ghittino[1] used calcium cyanamide ($CaCN_2$), and Schäperclaus[8] also recommended quicklime (CaO) for disinfection. In addition, a noncontaminated water supply must be provided and, where possible, concrete raceways should be built. Whirling disease does not exist in properly designed raceways supplied with spore-free water.

Although widely used in Europe, neither calcium cyanamide nor calcium oxide has been tested under laboratory conditions. Calcium cyanamide is a rich source of nitrogen and its effect on natural waters should be considered. Calcium oxide does not cause significant pollution when used in drained trout ponds. Quicklime and fresh hydrated lime also have been used to control ectoparasites in ponds.[5,10] Fresh calcium oxide is also bactericidal; a 1% aqueous solution of calcium oxide in 20% chicken feces killed *Staphylococcus aureus* in 90 min.[6]

The spore wall of *M. cerebralis* appears to be very resistant and it has long been assumed that only rather drastic disinfection procedures would kill the spores.

Hoffman and Putz[4] found in preliminary experiments that the following chemicals and concentrations would kill the spores by releasing the polar filaments, destroying the sporoplasm, or causing disintegration of the spore valves: calcium hydroxide, 0.5% and 2%; available chlorine (as sodium hypochlorite) 1600 ppm; Roccal (alkyl dimethylbenzylammonium chloride), 200 and 800 ppm active ingredient.

The work reported consists of (1) a search for better or additional disinfectants by observing the treated spores microscopically, and (2) a test of calcium oxide against *M. cerebralis* under simulated pond conditions.

MATERIALS AND METHODS

1. In vitro effects of disinfectants.

To prepare the spore concentrate, the heads of 8-10 month old infected fish were cut off and as much soft tissue as possible removed under a dissection microscope. The remaining parts were then cut into small pieces and macerated with a small mortar and pestle. The resulting mass was diluted and strained and the cleaned material allowed to sediment in the refrigerator. Although satisfactory suspensions in which the spores could be counted were prepared, they were never free of fish tissue. A fresh batch was prepared for the work represented by each table. Spore preparations were tested in small vials at room temperature (*ca* 22 C). About 0.75 ml of disinfectant dissolved in water was added to an equal amount of spore concentrate. Untreated spores in similar vials served as control. After the spores and test materials were placed in loosely stoppered vials they were observed at intervals of 2 to 4 days for 2 weeks or more. From 10 to 183 spores (average 69) were counted for each observation. Numbers were dependent on availability. Death of a spore was assumed when the polar filaments were extruded, the sporoplasm disintegrated, or the spore wall opened.

The chemicals tested were ammonium chloride (NH_4Cl) 0.1%; calcium hypochlorite ($CaClO)_2$) 200 and 400 ppm chlorine; sodium borate ($Na_2B_4O_7.10\ H_2O$) 0.1%; ammonium carbonate 0.1%; potassium hydroxide (KOH) 0.01%, 0.1%, and 1.0% calcium hydroxide ($Ca(OH)_2$) 0.125%, 0.25%, and 0.5%: potassium permanganate ($KMnO_4$) 0.01%, 0.1%, and 1.0%; calcium oxide 0.25%, 0.5%, and 1.0%; Roccal (alkyl dimethylbenzylammonium chloride) 50, 100, and 200 ppm of active compound; and copper sulfate ($CuSO_4.5H_2O$) 0.1%, and 0.5%. These chemicals were selected because they have been used as disinfectants for bacteria, coccidia, nematodes, or fish parasites. Inadvertently; calcium cyanamide, which has been used for whirling disease control, was omitted.

2. Testing quicklime in simulated ponds.

To each of four 340-liter fiberglass tanks were added 20 liters of mud (about 3 cm deep) from a source contaminated with *M. cerebralis*. The entire lot of mud was thoroughly mixed prior to placement in the tanks. While the mud was still wet, three of the tanks were treated with quicklime.[1] We added 240 grams of calcium oxide to one tank bottom (6180 sq cm) equivalent to approximately 380 grams per square meter (3360 lbs per acre). In addition, 300 (475 grams per square meter) and 3400 (5380 grams per square meter) grams of quicklime were used in other tanks. The fourth tank served as control. After 2 weeks, each tank was supplied with about 1800 ml per minute of 12 C disease-free spring water and 100 two-week-old rainbow trout (*Salmo gairdneri*) were added to each tank. Control fish were kept in standard facilities with the same water supply and they did not become infected. The fish were fed commercial crumbles and pellets *ad libitum*. Six months later, samples were netted randomly, and the fish measured and examined for *M. cerebralis* spores as in Hoffman, Snieszko, and Wolf.[3]

RESULTS

1. Microscopic effect of chemicals on spores.

Of the chemicals tested, only calcium oxide and potassium hydroxide, each at 1.0%, 0.5%, and 0.25% concentration, produced 100% mortality in spores under test-tube conditions. Other chemicals produced less severe alterations in spores and presumably a lower kill. Tables 1-4 present the data obtained in the experiments.

[1] Labelled "Washington High Calcium Chemical Hydrated Lime" containing 73.7% calcium oxide and supplied by Standard Lime and Refractories Company, Martin Marietta, Baltimore, Maryland.

TABLE 1. Effect of various chemicals on the spores of **Myxosoma cerebralis (in vitro)**, trial 1.

Disinfectant	Percentage "killed" 2 days	4 days	8 days	14 days
1. Control	00.0	14.3	13.0	16.3
2. NH_4Cl (0.1%)	00.0	12.0	16.3	38.3
3. *$Ca(ClO)_2$ (400 ppm)	100.0	100.0	100.0	90.0
4. *$Ca(ClO)_2$ (200 ppm)	28.6	61.9	42.2	40.8
5. $Na_2B_4O_7\ 10H_2O$ (0.1%)	32.4	19.6	35.8	34.3
6. $(NH_4)_2CO_3$ (0.1%)	6.45	4.00	21.6	27.3
7. KOH (.01%)	18.2	26.7	20.6	39.2
8. KOH (0.1%)	25.0	11.8	17.5	30.3
9. KOH (1.0%)	100.0	100.0	100.0	100.0
10. $Ca(OH)_2$ (0.125%)	29.4	45.6	37.0	22.8
11. $Ca(OH)_2$ (0.25%)	26.7	33.3	22.5	36.9
12. $Ca(OH)_2$ (0.5%)	29.3	24.6	35.4	33.9

*Parts active chlorine. Subsequent experimentation produced insignificant destruction of spores.

TABLE 2. Effect of various chemicals on the spores of **Myxosoma cerebralis (in vitro)**, trial 2.

Disinfectant	Percentage "killed" 2 days	4 days	6 days	8 days	10 days	14 days
1. Control I	18.2	12.5	20.0	17.4	15.7	37.2
2. KOH (1.0%)	100.0	100.0	100.0	100.0	100.0	100.0
3. $Ca(ClO)_2$ (400 ppm)	40.0	43.8	42.8	33.3	20.0	33.3
4. $Ca(ClO)_2$ (200 ppm)	5.0	45.5	14.3	13.0	36.9	33.3
5. $Ca(OH)_2$ (0.5%)	35.7	55.0	41.7	38.5	18.7	61.5
6. $Ca(OH)_2$ (0.25%)	17.7	53.0	33.8	27.3	40.0	6.66
7. NH_4Cl (0.1%)	18.7	35.0	16.1	27.8	38.1	30.0
8. $Na_2B_4O_7.10H_2O$ (0.1%)	8.33	53.8	16.7	13.6	23.6	17.9
9. Control II	8.34	14.8	17.9	26.1	17.9	22.2

TABLE 3. Effect of various chemicals on the spores of **Myxosoma cerebralis (in vitro)**, trial 3.

Disinfectant	Percentage "killed" 2 days	6 days	14 days
1. Control I	12.5	18.5	13.7
2. $KMnO_4$ (1.0%)	20.5	4.17	25.3
3. $KMnO_4$ (0.1%)	10.2	16.0	24.5
4. $KMnO_4$ (0.01%)	14.5	14.3	15.6
5. $KMnO_4$ (1.0%)	21.4	25.9	26.7
6. $KMnO_4$ (0.1%)	20.8	25.0	26.7
7. $KMnO_4$ (0.01%)	12.3	15.9	17.7
8. CaO (1.0%)	100.0	100.0	100.0
9. CaO (0.5%)	100.0	100.0	100.0
10. CaO (0.25%)	80.3	100.0	100.0
11. Cl ($Ca(ClO)_2$ (400 ppm)	21.4	25.7	36.4
12. Cl (200 ppm)	19.3	15.0	28.6
13. Control II	6.94	19.6	19.3

TABLE 4. Effect of various chemicals on the spores of **Myxosoma cerebralis (in vitro)**, trial 4.

Disinfectant	Percentage "killed" 2 days	4 days	8 days	14 days
1. Control	5.00	8.33	7.55	9.6
2. Roccal (200 ppm)	7.14	8.05	11.6	11.9
3. Roccal (100 ppm)	7.27	6.05	0.00	6.66
4. Roccal (50 ppm)	12.5	17.3	18.8	10.9
5. KOH (0.5%)	100.0	100.0	100.0	100.0
6. $CuSO_4$ (0.5%)	17.6	4.00	10.0	13.3
7. $CuSO_4$ (0.1%)	0.00	8.33	0.00	5.72

2. Effect of quicklime in simulated ponds.

Our results show that fish remained uninfected in those tanks treated with quicklime and that fish were significantly larger than the control fish (Table 5). It must be noted that the mud in the aquaria was not stirred after the fish were added; therefore, it is not known if the disinfection was complete to the bottom.

(see addendum)

TABLE 5. Effect of quicklime on whirling disease in simulated ponds.

Treatment	No. of fish measured	Size of fish, cm	Number infected	Degree of infection
Control	26	7.6 (4.5-11.4)	6 of 10	mild
3400 grams of CaO	26	10.5 (8.0-12.3)	0 of 26	none
300 grams of CaO	25	10.8 (7.8-12.6)	0 of 25	none
240 grams of CaO[1]	26	10.7 (7.8-13.1)	0 of 26	none

[1] Approximately 380 grams/sq meter (3360 lbs/acre).

DISCUSSION

These results verify that, although difficult, the chemical disinfection of *M. cerebralis* is possible. Ghittino[2] stated that calcium cyanamide is more effective than calcium oxide in Italy. Some German workers[3] prefer calcium oxide because it does not have the undesirable nutrient fertilizing effect of calcium cyanamide. Calcium oxide reacts with carbon dioxide in air and is converted to calcite; therefore, one should make certain the chemical is fresh when used. Because of the importance of whirling disease in the United States, further testing of disinfectants would be highly desirable. Of greatest importance is the need to thoroughly test the potential disinfecting ability of chlorine-bearing compounds such as calcium and sodium hypochlorite.

In the present experiments some of the lower kills at 8, 10, and 14 days were probably due to complete chemical destruction of killed spores.

Because the tissue residue was not completely removed, the data given cannot be considered exact but rather as an indication of the probable range of effectiveness for the chemicals. These chemicals should be tested with fish, but time permitted only the examination of calcium oxide.

[2] Ghittino, P. 1970. Personal communication. Institute Zooprofilattico Sperimentale, Via Bologna 148, 10154 Torino, Italy.

[3] Wiesner, E. R. 1968. Personal communication. Oberfishereirat bei der Regierung von Schwaben in Augsburg, West Germany.

ADDENDUM

Six months after the completion of Results 2 (above), the calcium oxide treated mud and the control mud were stirred thoroughly. Newly hatched rainbow trout were added as before. Five months later when the fish were examined, the controls displayed signs of whirling disease, and spores were present; the fish in the treated tanks were apparently normal and no spores were found in them.

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Control of whirling disease (*Myxosoma cerebralis*): use of methylene blue staining as a possible indicator of effect of heat on spores

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Methylene blue staining (0·08%) was used to determine efficiency of heat treatment in killing spores of *Myxosoma cerebralis*. Nearly all spores heated at 90° C for 10 min and 70° C for 100 min became stained giving presumptive evidence that they were killed.

I. INTRODUCTION

Heat might disinfect tanks and equipment contaminated with the causative agent of whirling disease (*Myxosoma cerebralis*). Heat-treatment of food products such as smoked or canned salmon could destroy spores of *M. cerebralis*.

Intra-vital stains for *M. cerebralis* were ineffective, hence we tested methylene blue as a stain indicator of death. Preliminary testing indicated that the heat-killed spores (80° C, 10 min) of *M. cerebralis* became stained with methylene blue, whereas most of the spores in unheated samples did not become stained. The purpose of this study was to determine the minimum heat necessary to kill the spores as demonstrated presumptively by staining with methylene blue.

II. MATERIALS AND METHODS

The spore sample was obtained from rainbow trout (*Salmo gairdneri*) experimentally infected 7 months previously at 12° C. Only the fish heads were used; the soft tissue was removed with forceps and the remaining skeletal parts cut into small pieces. The pieces were pulverized with mortar and pestle and strained through a 250 μm-mesh screen. The filtrate was diluted 50 : 50 with water, stored 16 h at 6° C, and the supernatant was discarded. This procedure was repeated until the supernatant was clear, leaving a few millilitres of spore concentrate. The concentrate was diluted with water so that one could easily count microscopically 100 or more spores. For heat exposure, 0·15 ml samples of spore concentrate were placed in small tubes and heated in a water bath. Controls were maintained at room temperature.

Staining was accomplished by adding 18 μl of 0·25% aqueous methylene blue to 37 μl of spore concentrate on a microscope slide; final concentration of methylene blue was 0·08%. Glass medicine droppers were drawn to deliver the above amounts in one drop, respectively.

When more stain was used, the mount was too dark for study. The slides were examined at least 5 min after preparation, but no longer than 30 min. If left overnight, almost all spores, heated or not, became stained; presumably they were killed by the stain. In each trial, 100 spores were observed with the results expressed as percentage of stained spores.

III. RESULTS

Heat-treated spores became stained quickly, but sometimes lightly in the 50 or 60° C preparations (Table I). Those in the 80 and 90° C tests stained quickly and intensely. All spores were stained after heating at 90° C for 10 min and we presume they were killed. At 80° C for 10 min all spores were stained except in one of five trials when 88% were stained. At 70° C for 10 min, an average of 60% were stained in contrast to an average of 22% for the controls. At 60° C for 10 min an average of 34% were stained, not much more than the controls (Table I). After 70° C for 100 min nearly all spores became stained (Table II).

TABLE I. Effect of heat for 10 min on the spores of *Myxosoma cerebralis* as determined by methylene blue staining, expressed in percentage stained; 100 spores were counted in each case

Temperature (° C)	Trial					
	1	2	3	4	5	Average
Control	0	8	15	23	26	22
50	10	15	32	19	42	24
60	30	8	36	34	60	34
70	87	26	60	48	80	60
80	100	100	100	100	88	98
90	100	100	100	100	100	100

TABLE II. Effect of prolonged heat at 70° C on spores of *Myxosoma cerebralis* as determined by methylene blue staining, expressed in percentage stained; 100 spores were counted in each case

Exposure in minutes at 70° C	Trial			
	1	2	3	4
Control	23	26	29	26
10	69	—	—	—
20	63	—	—	—
30	85	72	—	—
40	82	84	79	—
50	—	63	—	—
60	—	70	92	87
80	—	—	91	84
100	—	—	—	99

IV. DISCUSSION AND CONCLUSIONS

Hoffman & Putz (1969) and Hoffman & Hoffman (1972) reported presumptive microscopic evidence of the death of spores of *Myxosoma cerebralis* using high concentrations of certain chemicals. A more reliable method for determining viability of spores was needed. In preliminary tests we found that neutral red and trypan blue were not satisfactory vital stains for *M. cerebralis*. Presumably the spore wall and polar capsule plugs of normal spores resist the entrance of many chemicals. However, it is apparent that methylene blue will enter the heat-treated spores and, until tested with susceptible fish, we may assume that the spores were no longer viable. The present study demonstrates a method of staining presumably killed spores, as well as the altering of the spores with heat.

We presume that since 100% of the spores treated at 90° C for 10 min do stain with methylene blue that they are killed. This, of course, should be verified with a live fish bioassay method. It is possible that the small percentage of stained spores in the controls and lower temperature tests represent young spores in which the polar capsule plugs have not become completely hardened. Hoffman & Putz (1969) reported that heat caused the distortion of spores; such was not seen in this study except some polar filament extrusion.

This work was inspired by Dr Pietro Ghittino, Torino, Italy, who uses methylene blue in his diagnostic work for whirling disease.

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HOST SUSCEPTIBILITY AND THE EFFECT OF AGING, FREEZING, HEAT, AND CHEMICALS ON SPORES OF *MYXOSOMA CEREBRALIS*

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FOR MANY YEARS WHIRLING DISEASE, caused by Myxosoma cerebralis, has been a threat to trout and salmon culture. The disease has been found in brook trout (Salvelinus fontinalis), rainbow trout (Salmo gairdneri), brown trout (S. trutta), Atlantic salmon (S. salar), and grayling (Thymallus sp.) in Europe and the United States (Schäperclaus, 1931; Hoffman et al., 1962; Johannson, 1966; Uspenskaya, 1957; Volf, 1957), and in chum (Oncorhynchus keta), pink (O. gorbuscha), seema (O. masu), Koundja (Salvelinus leucomaenis), and malma salmon (S. malma) on the island of Sakhalin (USSR) in the Sea of Japan (Bogdanova, 1960). In spite of the high incidence of the disease in some areas, little is known about the biology of the organism or of its sensitivity to heat or to chemicals.

This paper will report on the susceptibility of other important North American salmonids to the disease; it will present results of preliminary experiments on the effect of aging, freezing, heat, and disinfectants on the spores of the organism; and it will record the first experimental transmission of the disease in the United States.

Materials and Methods

Rainbow trout, 6 to 8 months old, that had spontaneously acquired whirling disease and that had many spores in their tissues, were used as source material for the experimental infection studies.

Fiberglass tanks and stainless-steel troughs were used for the infection studies with aged or frozen spores. About 7.5 centimeters of spore-free pond mud was added to each tank, and cut-up infected heads and running spring water (at 12.5° C.) were supplied. The frozen heads were kept at -20° C. for periods of 3 days to 9 months.

All salmonids were raised from eggs obtained from a hatchery that had no history of whirling disease. For the experiments on host susceptibility, trout and salmon fry were placed in live-boxes in the stream receiving the effluent from the laboratory containing fish that had whirling disease. Control fish were raised in the same water before it was exposed to infected and diseased trout.

Results and Discussion

Host Susceptibility.--The coho salmon (Oncorhynchus kisutch), chinook salmon (O. tshawytscha), and lake trout (Salvelinus namaycush) were exposed to the disease in live-boxes receiving water in which diseased fish were held. All three species became infected, showing typical symptoms of whirling disease (which was verified by demonstrating the spores). This proved that whirling disease is a threat to the fisheries involving any of the above-named salmonids.

The brown trout may not show symptoms but may be an important carrier of the disease. Volf (1957) reported serious

losses of brown trout fingerlings as well as losses of rainbow trout, brook trout, and grayling.

Aging.--Many preliminary attempts to infect trout fry with fresh spores failed, although the fry could be infected as early as 2 days after hatching by placing them in live-boxes in water where the disease was well established (Putz and Hoffman, 1966). Uspenskaya[1] succeeded in infecting fish by placing aged (4-month-old) spores in their stomachs.

In the present study, nine aquaria were set up and spores were added on 0 day, 3 days, 113 days, 4 months, and 6 months before the fry were added. In only two aquaria did the fish become infected. In one of those aquaria the spores had been added 4 months before the fish. In the second of those aquaria, the spores had been added when the fry were introduced, but the fish did not show symptoms until 7 months later--an indication that these spores were about 4 months old when the fish became infected. The symptoms appeared at 71 days. The disease was verified by demonstration of the presence of spores at 94 days post-exposure.

These experiments indicate that the spores are not infective when first removed from the fish, but they are infective when they have been "aged" in cool running water for 4 to 5 months. We do not know what factors, living or dead, present in the water or mud, may exert an influence upon the infectivity of aged spores.

Freezing.--Table trout (mostly rainbow) are commonly supplied as yearling fish and preserved by freezing at about -20° C. It has been recommended that the fish reach the consumer in less than 2 months. Therefore, it is desirable to know whether the spores of Myxosoma cerebralis survive 2 months at -20° C.

Six aquaria were set up and supplied with spores frozen for 3 days, 18 days, and 9 months. Fry were added on 0 day, 113 days, and 4 months later, all at 12° C.

Whirling disease developed in only the one aquarium in which the spores were frozen 18 days and aged 4 months. Details were as follows: On August 1, 1967, 11 frozen infected heads were added to this trough, and 200 rainbow trout fry were added to it on December 5, 1967 (4 months). The mud was stirred gently about once a week. Three whirling fish were first seen on February 16, 1968 (73 days). By March 11, there were many whirlers. Spores were found in three of six fish examined on March 11 (3 months). This proved that the spores survive freezing at -20° C. for at least 18 days.

We hope to be able to find out whether spores will survive 2 months or longer. Until then, we must treat frozen infected fish with great caution. It is very probable that Myxosoma cerebralis reached the United States in frozen table trout. We hope that proper precautions will prevent further spreading.

Heat.--As in some facilities it might be possible to apply heat as a disinfecting agent, spores were exposed to heat in 0.85 percent saline in 50-milliliter beakers and observed microscopically. Ten minutes at 60°, 80°, and 100° C. caused distortion and probable death of spores, whereas spores held at 40° C. and room temperature showed no change.

We assume that 60° to 100° C. for 10 minutes--perhaps less--will kill the spores of M. cerebralis.

Selected Chemicals.--The spores were observed microscopically for 2 weeks after at least 24-hour exposure to chemicals. Disintegration of the sporoplasm and/or the polar filaments was interpreted as death of the spore.

The following were effective in causing distortion and probable death of spores: Calcium hydroxide, 0.5 percent and 2 percent; available chlorine (as sodium hypochlorite), 1,600 p.p.m.; Roccal (alkyl dimethylbenzylammonium chloride), 200 and 800 p.p.m. (active ingredient). Chlorine at 200 p.p.m. gave variable results.

[1]A. V. Uspenskaya, of the Institute of Cytology, Academy of Sciences of the USSR. Pr. Mavliha 32, Leningrad, USSR. Personal communication, 1963.

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EFFECT OF FREEZING AND AGING ON THE SPORES OF *MYXOSOMA CEREBRALIS*, THE CAUSATIVE AGENT OF SALMONID WHIRLING DISEASE

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EVER SINCE WHIRLING DISEASE was reported by Schaperclaus (1931) to have reappeared in a pond that had been drained and frozen, it has been assumed that the spores of Myxosoma cerebralis survive freezing. In 1969 we reported that rainbow trout (Salmo gairdneri) fry became infected when exposed to spores that had been frozen for 18 days at -20° C. and then aged for 4 months in spring water in an aquarium containing mud. The results reported here show that spores can survive freezing much longer.

Materials and Methods

Aging

The aquariums used were as follows: Five 340-liter fiberglass tanks (four of them containing 3 to 5 inches of mud, and one without mud); four 150-liter stainless steel tanks (one containing mud, and three without); and four 75-liter glass aquariums without mud.

Fourteen to twenty macerated infected trout heads were added to each aquarium, and about 600 milliliters per minute of spring water at 12° C. was supplied to each aquarium. The spores were allowed to "age" from 0 to 6 months. After this aging, the water flow was increased to about 1,800 milliliters per minute and about 100 3- to 10-week-old rainbow trout were placed in each aquarium.

After 5 months, these fish were examined. Trout in only 4 of the 13 lots had become infected. The remaining fish in all aquariums were discarded, and new fry were added to eight of the aquariums containing the original spores with or without mud. These fish were examined 4.5 months later. (See table 1.)

Freezing

The aquariums used were as follows: Eleven 340-liter fiberglass aquariums (nine of them containing mud, and two without); two 150-liter stainless steel tanks with mud.

Spores, water, and fish were supplied as for the aging experiments. The spore-bearing heads had been frozen from 0.1 to 32 months at -20° C.

After 5 months, these fish were examined. Only 2 of the 13 lots had become infected. The remaining trout were discarded, and new fry were added to seven aquariums. These latter fish were examined 4.5 months later. (See table 1.)

Control

Fry from the original lot were held in similar facilities to serve as a control for both the aging and the freezing experiments.

Results

None of the control fish showed symptoms of whirling disease, and examination of the fish revealed no spores.

TABLE 1.--Results of attempts to infect rainbow trout fry with Myxosoma cerebralis in aquariums

Experiment No.	Type of aquariums	3-5 inches of mud added	Spores (months) frozen	Spores (months) "aged"	Age of fish at start (weeks)	Onset of symptoms	Results of 1st run	Results of 2d run[1]
			Aging Experiment					
66-16B	150-liter steel tank.	No	0	4	3	0	0	0
67-1A	150-liter steel tank.	Yes	0	4	4	3.5 mo.	+	+
68-14	150-liter steel tank.	No	0	2	3	0	0	0
67-6(1)	340-liter fiberglass.	Yes	0	3	10	([2])	+	([3])
67-6(3)	340-liter fiberglass.	Yes	0	3.5	3	2.5 mo.	+	+
67-6(4)	340-liter fiberglass.	Yes	0	3.5	3	3.5 mo.	+	([3])
68-11	340-liter fiberglass.	Yes	0	6	3	0	0	0
68-13	340-liter fiberglass.	No	0	2.5	3	0	0	0
68-14	150-liter steel tank.	No	0	2.5	3	0	0	([3])
69-5A	75-liter glass.	No	0	0	10	0	0	0
69-6	75-liter glass.	No	0	3.5	3	0	0	0
69-7	75-liter glass.	No	0	3.5	3	0	0	-
69-8	75-liter glass.	No	0	3.5	3	0	0	-

(See footnotes at end of table.)

TABLE 1.--Results of attempts to infect rainbow trout fry with Myxosoma cerebralis in aquariums--Continued

Experiment No.	Type of aquariums	3-5 inches of mud added	Spores (months) frozen	Spores (months) "aged"	Age of fish at start (weeks)	Onset of symptoms	Results of 1st run	Results of 2d run[1]
			Freezing Experiment					
66-16A	150-liter steel tank.	Yes	9	4.5	([2])	0	0	([3])
67-1B	150-liter steel tank.	Yes	.5	4	([2])	2.5 mo.	+	+
67-6(2)	340-liter fiberglass.	Yes	.1	0	([2])	0	0	([3])
67-6(5)	340-liter fiberglass.	Yes	.1	3.5	([2])	0	0	([3])
67-6(6)	340-liter fiberglass.	Yes	2	2.5	([2])	0	0	([3])
68-5	340-liter fiberglass.	Yes	2	6	3	0	+	+
68-6	340-liter fiberglass.	Yes	2	6	3	0	0	0
68-7	340-liter fiberglass.	Yes	2	6	3	0	0	+
68-8	340-liter fiberglass.	Yes	11	6	3	0	0	0
68-9	340-liter fiberglass.	Yes	11	6	3	0	0	0
68-10	340-liter fiberglass.	Yes	11	6	3	0	0	0
69-1	340-liter fiberglass.	No	16	4.5	3	0	0	([3])
69-2	340-liter fiberglass.	No	32	5.5	3	0	0	([3])

[1] A second lot of fry was added after removing the first lot.
[2] Not observed or not recorded.
[3] Second run omitted.

Table 1 summarizes the results of these experiments.

No fish in the 10 aquariums without mud became infected.

Fish became infected in two tanks containing mud and spores which had been frozen 2 months. In one of these tanks, one lightly infected fish was found in the first lot of fish and spores were found in the fish of the second lot. There were no symptoms, and spores were not numerous. In the second of these tanks, no infected fish were found in the first lot, but a few spores were found in the fish of the second lot. There were no symptoms.

Fish became infected in five other aquariums that contained mud and spores aged 3.5 to 4 months.

Apparently neither aquarium size nor tank material affected results, since fish became infected in 150-liter steel tanks as well as in 340-liter fiberglass tanks.

Discussion

Spores of Myxosoma cerebralis survive for at least 2 months at -20° C., and it is very probable that they survive much longer. Our negative results are inconclusive because it is very difficult to infect fish experimentally, as evidenced by our 18 negative experiments and the failure of others with other Myxosporidea.

Aging of previously frozen spores for about 4 months is probably necessary for infectivity. Longer aging may not destroy all spores, but our results are inconclusive because the first lots of fish may have shed spores which became infective for the second lots. We have underway experiments in which we shall try to demonstrate how long spores remain viable in spring water at 12° C.

The mechanics of infection with any of the hundreds of species of histozoic Myxosporidea have never been clearly demonstrated. Uspenskaya[1] reported that she had infected rainbow trout fingerlings by pipetting 4-month-aged spores directly into the stomach. In 1969 we reported that we had infected rainbow trout by placing them in aquariums into which mud and spores had been placed 4 months previously. Schafer (1968) was able to infect trout with Ceratomyxa shasta by holding experimental fish in a contaminated lake and by injecting infected material into the visceral cavity, but trout placed in tanks containing 5-month-aged spores did not become infected. In the present experiments fish became infected only in tanks containing mud and spores; but this does not prove conclusively that the mud and all the resultant organisms are necessary, because nine lots with mud and spores were negative. Schaperclaus (1931) theorized that the spores may be carried temporarily by small invertebrates which are later eaten by trout fry. However, because we (Putz and Hoffman, 1966) were able to infect 2-day-old sac fry (pre-feeding stage), it may be that the spores do not have to be eaten.

It is possible--but not proved--that fish become infected, not only through the intestinal tract, but also through the gills or otherwise.

[1] A. V. Uspenskaya, of the Institute of Cytology, Academy of Sciences of the USSR, Pr. Mavliha 32, Leningrad, USSR. Personal communication, 1963.

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A case history of whirling disease in a drainage system: Battle Creek drainage of the upper Sacramento River basin, California, USA

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Abstract. Whirling disease was diagnosed in steelhead trout, *Salmo gairdneri* Richardson, during 1985 at Coleman National Fish Hatchery in the Battle Creek drainage of the upper Sacramento River basin, California, USA. Early confirmation of the aetiologic agent, *Myxobolus cerebralis* Hofer, 1903, was difficult. However, later investigation confirmed the identity of the agent and led to a management decision to destroy the 1985 brood year of steelhead trout at the hatchery. Sentinel fish and collections of feral trout were used to survey Battle Creek watershed to determine the source of infectivity. Feral rainbow trout, *Salmo gairdneri* Richardson, from South Fork Battle Creek were found to be infected with *M. cerebralis* and other infected trout were later found at two commercial rainbow trout hatcheries. No reinfection with *M. cerebralis* has been detected at the Coleman hatchery since ozonation was begun in early 1986.

Introduction

Whirling disease, caused by the myxosporean *Myxobolus cerebralis* Hofer, 1903 (Lom & Noble 1984), was first detected in California, USA, in 1966 at a trout farm on Garrapata Creek (Fig. 1) near Monterey (Hoffman 1970). In 1968, it was diagnosed at a Nevada Department of Wildlife trout hatchery on the Truckee River, which drains the California east slope of the Sierra Nevada mountains (Hoffman 1970). *Myxobolus cerebralis* has since been found in 14 streams and lakes in northern Nevada (Pat Coffin, Nevada Department of Wildlife, personal communication) and is now known to occur in at least 48 streams and lakes in California ('known distribution of whirling disease in California, October 1985' California Department of Fish and Game, unpublished report).

The 1984 detection of *M. cerebralis* in fingerling, yearling and adult rainbow trout, *Salmo gairdneri* Richardson, and yearling golden trout, *Salmo aguabonita* Jordan, at Mt Whitney State Fish Hatchery (SFH) (D. Manzer, California Department of Fish and Game, personal communication) prompted California authorities to determine the statewide host and geographic ranges of the parasite.

In mid-February 1985, myxosporean spores of 12–15 μm diameter were found in material taken from heads of yearling rainbow trout sampled at the Darrah Springs SFH (Fig. 2). The trout were immediately destroyed; however, later examination of the spores recovered from the fish showed that they were *M. kisutchi* located in nervous tissue rather than in cartilage and bone.

Darrah Springs SFH is on Battle Creek, a major tributary to the Sacramento River,

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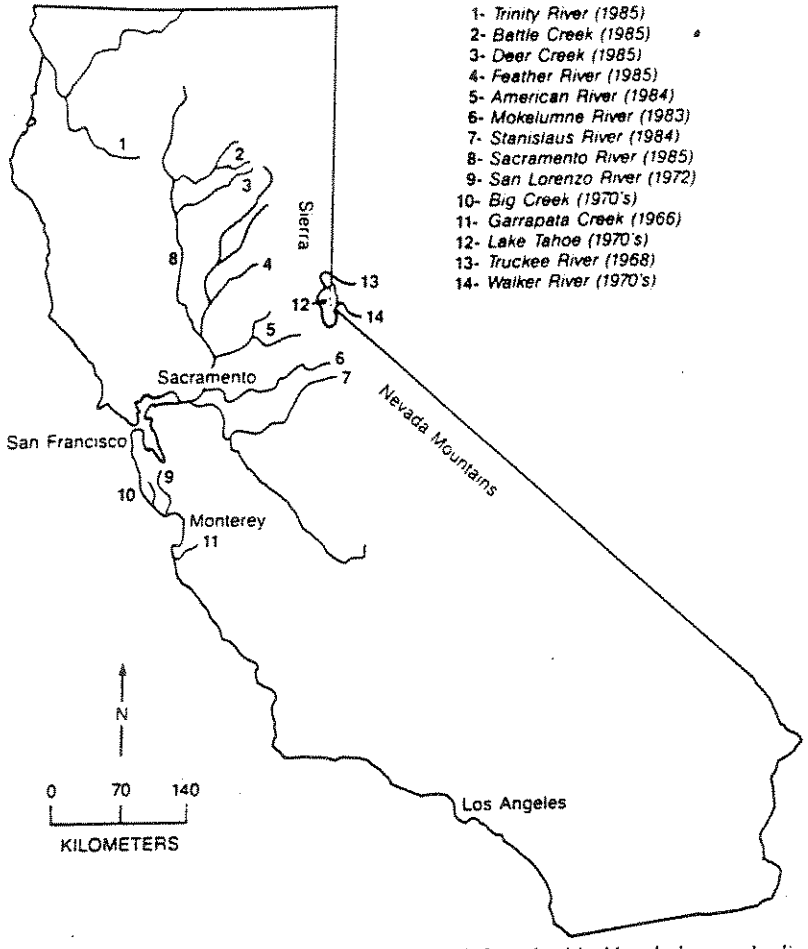

Figure 1. Major waterways in California from which trout, infected with *Myxobolus cerebralis*, have been collected through 1986.

16 km upstream from Coleman National Fish Hatchery (NFH). Effluent from this hatchery drains into the Coleman Canal, a major water source for Coleman NFH (Fig. 2).

The emergency disease eradication project at Darrah Springs SFH justified an extensive monitoring programme at Coleman, which was begun in late-February 1985. The purpose of the programme was to determine if *M. cerebralis* has been established in the Coleman Canal, and if infections were present in yearling steelhead trout, *Salmo gairdneri* Richardson, and parr of fall chinook salmon, *Oncorhynchus tshawytscha* (Walbaum).

Materials and methods

Sampling fish populations for M. cerebralis

Sample collections for the detection of *M. cerebralis* from steelhead yearlings of brood year 1984 (BY84) and fall chinook salmon smolts (BY84) began at Coleman NFH in

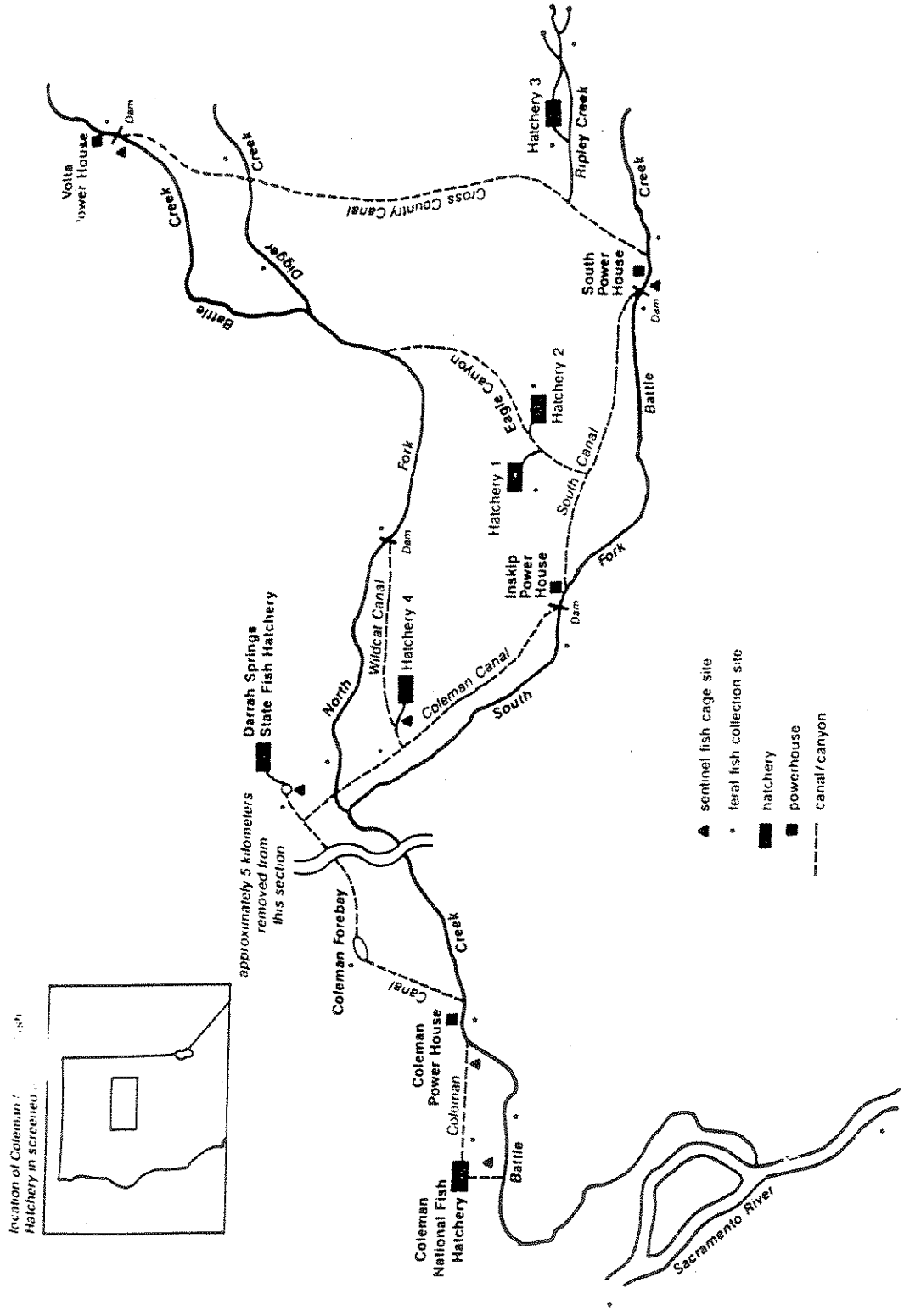

Figure 2. Sampling locations for *Myxobolus cerebralis*, 1985 and 1986.

mid-February 1985. Collections of steelhead parr (BY85) and fall chinook salmon parr (BY85) were continued in June 1985. Collections were made by netting 3-month-old fish from the upper, middle and lower end of each raceway. Fish showing clinical signs of whirling disease were collected. Sample size ranged from 30 to 150 fish. Fish from each sample were pooled in groups of 30. After *M. cerebralis* was confirmed in a sample, additional samples were pooled in groups of five or examined individually to measure prevalence of infection and to enumerate spores.

In March 1985 and thereafter, rainbow trout of various ages were collected from Coleman Canal by electrofishing. Three collections were made at the entrance of the canal to the hatchery. Collections were also made of fish trapped on intake screens.

Beginning in October 1984, eight samples were taken from commercial trout hatcheries located on Battle Creek or Coleman Canal. Ten to 30 fish of various sizes were obtained at each hatchery. Depending on fish size, samples were processed in pools of 5–15. Four commercial hatcheries were under investigation in this survey, of which three were owned and operated by the same trout farm (Hatcheries 1–3, Fig. 2). The fourth (Hatchery 4, Fig. 2) was owned by an independent operator who obtained eggs from the aforementioned trout farm.

Collecting of rainbow and steelhead trout began in mid-September 1985, from 12 locations on Battle Creek and two from the Sacramento River. Sites were selected on the basis of proximity to fish-culture activity, water diversions and hatchery stocking programmes. Samples were collected by electrofishing and by angling. Fish were pooled in groups of five; occasionally single fish were examined. Additional collections from North Fork Battle Creek were made in August 1986.

Sampling for M. cerebralis *with sentinel fish*

Fry of cutthroat trout *Salmo clarki* Richardson, and rainbow trout were used as sentinel fish in live cages at four sites on Battle Creek and two in hatchery effluent streams that drain into Battle Creek or Coleman Canal (Fig. 2). Each cage held 50–100 fish. Cages were left in place for 2 weeks; the fish were then transferred to and held in pathogen-free water for 1·5 months, until a pump failed and the fish had to be supplied with untreated creek water. Half of the fish were sacrificed at this time and examined histologically for lesions of whirling disease. The rest were maintained in untreated water and samples were examined by the plankton centrifuge harvest method of O'Grodnick (1975) at 1-month intervals until all fish had been used.

Control populations consisted of two species of trout held in live cages in untreated water at Coleman NFH. Two additional control groups were started at Coleman NFH in March 1986. A total of 500 steelhead trout fry and 100 fall chinook salmon fingerlings were placed in troughs supplied with untreated creek water and held for 5 months until August 1986. Each control population was sampled at 1-month intervals and examined histologically for the presence of *M. cerebralis* trophozoites.

Sample processing and diagnosis

Pooled or individual fish were processed and examined by the methods of O'Grodnick (1975) and Wolf & Markiw (1985). If no spores were detected after up to three drops of

the resultant concentrate had been examined for 30 min, the material was further purified by trypsinization (Markiw & Wolf 1980) and examined for an additional 30 min.

Diagnosis was based on the presence or absence of *M. cerebralis* spores or trophozoites, either in tissue concentrates after 1 h of microscopic examination or in histologic tissue sections of the head. Confirmatory diagnosis was made by using the fluorescent antibody technique (FAT) of Markiw & Wolf (1978) on spore concentrates. Estimates of spore numbers in each concentrate were made with a haemocytometer.

Results

Coleman NFH survey

About 75% of the BY84 steelhead trout were found to harbour spores of *M. kisutchi*. Of the spores recovered from these fish, about 5% were morphologically similar to spores of *M. cerebralis* and also lacked iodinophilous vacuoles commonly seen in *M. kisutchi*. Histologic examination revealed spores of this type in lesions in cranial cartilage. These spores were presumptively diagnosed as *M. cerebralis* but FAT confirmation of the spores was inconclusive. The trout were released in March 1985.

In July 1985, nine coded-wire tagged juvenile steelhead (Coleman NFH stock, BY84), collected from the Sacramento River, were found to harbour spores identified by FAT as *M. cerebralis*.

Evidence of *M. cerebralis* was not found in fall chinook salmon (BY84, BY85, BY86) that were examined by the plankton centrifuge harvest method of O'Grodnick (1975) or by histologic section.

In June 1985, *M. cerebralis* was confirmed by FAT in one steelhead fingerling (BY85) at Coleman NFH that had shown clinical signs of whirling disease. Infected fish were then found in all raceways. Examination of individual fish indicated that the average prevalence rate was 75%.

In October 1985, a gross examination of 50000 steelhead trout from one raceway yielded 12 fish (0·01%) with clinical signs suggestive of whirling disease. However, no spores or trophozoites of *M. cerebralis* were recovered from six of these trout.

Battle Creek collections

Eight collection sites, in addition to Coleman NFH, yielded normal-looking rainbow trout harbouring spores of *M. cerebralis* (Table 1). All sites from which trout infected with *M. cerebralis* were collected in September 1985, were downstream from the confluence of Cross Country Canal and South Fork Battle Creek (Fig. 2). In October 1985, trout were examined from Ripley Creek spring, which drains into Cross Country Canal; spores 10–12 μm in diameter, indistinguishable except in size from *M. cerebralis*, were recovered. However, attempts to identify these spores by FAT were inconclusive. In November 1985, Cross Country Canal was drained, and nearly 1500 rainbow trout and brown trout, *Salmo trutta* L., were salvaged by California Department of Fish and Game personnel and stocked into the North Fork Battle Creek below Volta Power House (Fig. 2). The rainbow trout were found to harbour spores of *M. cerebralis* but the brown trout were not

Table 1. Results of rainbow trout* examinations for the occurrence of *Myxobolus cerebralis* during 1985

Location	Collection date	No. of fish	Results†
Darrah Springs State Hatchery	5 Sept.	10	−
North Fork Battle Creek at Volta Power House	6 Sept.	30	−
	22 Nov.	39	+
North Fork Battle Creek at Wildcat Road	6 Sept.	30	−
North Fork Battle Creek at Wildcat Dam	6 Sept.	33	−
Digger Creek at Manton Road	6 Sept.	30	−
Digger Creek at Bristol Benton Canal	5 Sept.	52	−
South Fork Battle Creek at South Power House	6 Sept.	62	+
	20 Sept.	121	+
Battle Creek at Coleman NFH	5 Sept.	27	+
Battle Creek at Coleman Power House	5 Sept.	5	+
Coleman Canal at Coleman NFH	15 Sept.	60	+
South Fork Battle Creek below South Power House	20 Sept.	60	+
Cross Country Canal at Ripley Creek	1 Oct.	30	−
South Fork Battle Creek at Inskip Power House	20 Sept.	25	+
Sacramento River above Battle Creek	15 Sept.	100	−
Sacramento River below Battle Creek‡	22 July	9	+

*All trout sampled except in Sacramento River below Battle Creek were 0–2 years old.
†Minus sign (−) indicates the absence of *M. cerebralis* by plankton centrifuge harvest; + indicates *M. cerebralis* present.
‡Coded-wire-tagged BY84 Coleman steelhead trout.

infected. Samples taken from North Fork before the stocking of trout salvaged from Cross Country Canal showed no evidence of *M. cerebralis*.

Commercial hatchery survey

In May 1986, commercial trout hatcheries 1 and 2 (Fig. 2) in Battle Creek drainage, each with its own water supply, were found to have trout with *M. cerebralis*. Samples taken during October 1984 had shown no evidence of *M. cerebralis*.

Flooding during February 1986 caused the Eagle Canyon Canal to overflow into both of the hatcheries with infected fish. This canal originates in the North Fork Battle Creek below Volta Power House (Fig. 2), where known infected trout were planted in October 1985. Infectivity thus might have been introduced to these hatcheries by the flooding.

Hatchery 3 (Fig. 2) yielded trout with *M. kisutchi* but there was no evidence of *M. cerebralis*. The water supply for this hatchery is Ripley Creek spring, from which trout harbouring spores intermediate in size between *M. cerebralis* and *M. kisutchi* were collected.

No myxosporeans were detected in trout from Hatchery 4.

Sentinel fish

Sentinel fish held for 2 weeks in six live cages in various locations in Battle Creek drainage (Fig. 2) failed to contract whirling disease. Control fish maintained in untreated

creek water at Coleman NFH remained uninfected until late April 1986 (spores were first recovered from steelhead and rainbow trout in early August 1986). The infection was light (<50000 spores per five-fish pool) when compared with the previous infection of steelhead trout in August 1985 (294000 average number of spores per five-fish pool).

O'Grodnick (1979) recovered an average of early 3·7 million *M. cerebralis* spores from five-fish pools of rainbow trout during four stream exposure studies in Pennsylvania. Recoveries from feral rainbow trout from Battle Creek and hatchery steelhead trout from ·man NFH were less than 10% of that number (301000 spores from five-fish pools of ·ow trout; 294000 spores from five-fish pools of steelhead). This moderate level of infectivity in Battle Creek could account for the chinook salmon remaining uninfected, as O'Grodnick (1979) demonstrated a 27-fold greater spore population in rainbow trout than in chinook salmon that received equal exposure to infection.

Discussion

Clinical signs of disease at Coleman NFH attributed solely to infection with *M. cerebralis* ·e lacking. Mortalities among steelhead trout could not be attributed to *M. cerebralis*. ·udging by the results of this investigation, the occurrence of *M. cerebralis* probably ·egan as a localized infection in rainbow trout in the vicinity of South Fork Battle Creek and Cross Country Canal. It then spread downstream, infecting steelhead trout (BY84 and BY85) at Coleman NFH. Activities of the California Department of Fish and Game spread the infection to the North Fork. The occurrence in 1986 of *M. cerebralis* at the two commercial hatcheries in the North Fork drainage suggested that the parasite had established itself there. Collections made in late August 1986 from North Fork Battle Creek below Volta Power House yielded young-of-the-year rainbow trout with very light infections with *M. cerebralis*, confirming the presence of infectivity in that stream.

The release of steelhead trout (BY84) from Coleman NFH (later confirmed to be ·ected with *M. cerebralis*) might contribute to the spread of *M. cerebralis* to other drainages in the Sacramento River basin. Better methods for early detection of low level infection are needed.

Control Measures

Drastic measures were taken at Coleman NFH to prevent further spread of infection. After 8 months of quarantine and observation, all infected or suspect steelhead trout ·BY85) were destroyed in February 1986, and sent to a rendering plant. All adult ·elhead mortalities and spawned fish were destroyed by incineration or burial. Because ·f practical limitations, eradication measures were not attempted in the extensive Battle Creek drainage.

In an attempt to continue production of steelhead trout at Coleman NFH, well water was used to incubate eggs after iodophor disinfection. Fry were then moved to a recirculating water system supplied only with well water, and were held in this system until the completion of an ozone water treatment system to disinfect Battle Creek water. Ozonation began in April 1986. All steelhead production (BY86) has been maintained in

this system. Periodic shutdowns of the ozonation units have occurred, but no evidence of infection with *M. cerebralis* has been detected either by parasitological assay of fresh tissue or by examination of histologic sections. Steelhead trout will be released as smolts provided they remain free from infection. Chinook salmon will be reared in untreated water and also released as smolts if they, too, remain uninfected.

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The phylum Myxozoa has been considered to comprise two classes, Myxosporea Bütchli, 1881 (primarily of fishes) and Actinosporea Noble in Levine et al., 1980 (primarily of aquatic oligochaetes). About 10 years ago it was demonstrated that the life cycle of *Myxobolus cerebralis* Hofer, 1903 (Myxobolidae: Platysporina) of salmonid fishes requires transformation of the myxosporean into an actinosporean stage in the oligochaete worm *Tubifex tubifex* (Tubificidae), and that the stage infective to fish is the actinosporean spore. This type of two-host life cycle has now been demonstrated or strongly implicated for 14 myxosporean species, belonging to 6 genera in 4 families. In light of these findings, the taxonomy of the Myxozoa is revised. We propose the following: suppression of the newer class Actinosporea and the order Actinomyxidia Štolc, 1899; and suppression of all families in the Actinosporea except Tetractinomyxidae. This family and its one genus, *Tetractinomyxon* Ikeda, 1912, are transferred to the order Multivalvulida Shulman, 1959 (Myxosporea). We also propose that actinosporean generic names be treated as collective-group names, thus they do not compete in priority with myxosporean generic names. *Triactinomyxon dubium* Granata, 1924 and *Triactinomyxon gyrosalmo* Wolf and Markiw, 1984 are suppressed as junior synonyms of *Myxobolus cerebralis*. The myxosporean stage of no other previously named actinosporean has been identified. Other actinosporean species are therefore retained as species inquirendae until their myxosporean stages are identified. A revised description of the phylum Myxozoa is provided that includes our proposed taxonomic and nomenclatural changes.

Isolation of *Myxosoma cerebralis* (Whirling Disease) Spores from Infected Fish by use of a Physical Separation Technique

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KOZEL, T. R., M. LOTT, AND R. TAYLOR. 1980. Isolation of *Myxosoma cerebralis* (whirling disease) spores from infected fish by use of a physical separation technique. Can. J. Fish. Aquat. Sci. 37: 1032–1035.

Immiscible, liquid, two-phase systems are produced when aqueous solutions of dextran and of polyethylene glycol are mixed above certain concentrations. This phase system was used to isolate *Myxosoma cerebralis* spores from infected fish tissue. *Myxosoma cerebralis* partitioned into the polyethylene-glycol-rich upper phase, whereas the tissue debris partitioned into the dextran-rich lower phase. *Myxosoma cerebralis* spores partitioned into the upper phase, regardless of the electrostatic potential in the phase system, indicating that isolation of *M. cerebralis* was unrelated to surface charge, which suggests that relative to fish tissue, the surface of the spore is highly hydrophobic. The technique is useful for detection of small numbers of spores from populations of infected fish; however, the greatest value of the technique is the isolation of spores for biochemical and immunologic studies that are unmodified by the relatively harsh techniques usually used.

Key words: Myxosoma cerebralis spores, *Myxosoma* spore isolation techniques, polymer aqueous two-phase systems

KOZEL, T. R., M. LOTT, AND R. TAYLOR. 1980. Isolation of *Myxosoma cerebralis* (whirling disease) spores from infected fish by use of a physical separation technique. Can. J. Fish. Aquat. Sci. 37: 1032–1035.

Quand des solutions aqueuses de dextran et de polyéthylène glycol sont mélangés à des concentrations dépassant un certain niveau, il en résulte des systèmes à deux phases liquides non miscibles. Un tel système a été utilisé pour isoler des spores de *Myxosoma cerebralis* de tissus de poissons infectés. *Myxosoma cerebralis* se répartit dans la phase supérieure riche en polyéthylène glycol, alors que les débris tissulaires se répartissent dans la phase inférieure riche en dextran. Les spores de *M. cerebralis* se répartissent dans la phase supérieure, sans égard au potentiel électrostatique du système de la phase, ce qui indique que la séparation de *M. cerebralis* dans le système n'est pas liée à la charge superficielle. Ces résultats laissent croire que la surface de la spore est fortement hydrophobe par rapport au tissu du poisson. Cette méthode est utile pour la détection de petits nombres de spores chez des populations de poissons infectés; cependant, elle a le plus de valeur dans la séparation de spores en vue d'études biochimiques et immunologiques qui ne sont pas modifiées par les techniques relativement brutales généralement utilisées dans l'isolation de spores de *M. cerebralis*.

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WHIRLING disease caused by *Myxosoma cerebralis* may be tentatively diagnosed in severely affected salmonids by clinical signs such as tail chasing, black tail, and skeletal deformities. Unfortunately, most fish have subclinical infections, yet such fish are quite capable of transmitting the infection. As a consequence, several microscopic procedures have been developed to identify the trophozoite form of the organism on microscopic examination of thin cartilage sections; however, conventional techniques require demonstration of spores in wet mount preparations of head cartilage. *Myxosoma cerebralis* is identified by its size ($8.8 \times 8.8\ \mu m$), demonstration of the absence of an iodinophilic vacuole, and by the location of the spore in the host. These criteria still cause confusion in differentiating *M. cerebralis* from certain other *Myxosoma* and *Myxobolus* species.

Various serologic techniques offer a promising new approach to diagnosis of whirling disease (Griffin and Davis 1978; Markiw and Wolf 1978; Pauley 1974). Direct assays for protozoal antigen could identify the organism in infected tissue. Alternatively, assays for antibody to *M. cerebralis* could be used to screen serum from fish believed to have a subclinical infection. Such assays will require purified preparations of spores as well as specific, high-titered antiserum against the spores.

Unfortunately, existing techniques for purification of *M. cerebralis* spores are not entirely suitable for prepa-

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ration of antigens for use in immunologic studies. Extraction of spores from infected fish by use of trypsin (Landolt 1973) or trypsin and pepsin (Markiw and Wolf 1974) may produce spores with an antigenic composition that has been altered by the proteolytic action of the enzymes. Use of ether extraction (Landolt 1973) in combination with enzymatic digestion could further accelerate degradation of spore antigens.

The objective of our study was to develop a physical separation technique for isolation of *M. cerebralis* spores that are unmodified by the harsh enzymatic or ether extraction procedures used previously and are uncontaminated by tissue debris. This paper describes an effective isolation technique that utilizes an aqueous polymer two-phase system.

Materials and methods — Rainbow trout, *Salmo gairdneri*, fingerlings were infected by exposure for five or more months to *M. cerebralis* contaminated mud. Spores obtained by grinding of head cartilage were identified as *M. cerebralis* by size and absence of an iodinophilic vacuole.

The head cartilage and spinal columns of the fish were minced with a pair of scissors and then ground in a mortar with a pestle. This material was further homogenized at room temperature in a Waring Blendor for 1–3 min, filtered through a double layer of gauze, and centrifuged at 10 000 × *g* for 20 min at 4°C. The supernatant was poured off, and the pellet was resuspended in saline (0.15 mol/L NaCl) and centrifuged at 700 × *g* for 30 min. This pellet was used for isolation of spores in the phase system.

The two-phase system was prepared from aqueous stock solutions of 20% (w/w) polyethylene glycol (PEG) 4000 (J. T. Baker Chemical Co.), 20% (w/w) polyethylene glycol (PEG) 6000 (J. T. Baker Chemical Co.), and 20% (w/w) dextran T 500 (Pharmacia Fine Chemicals, Uppsala, Sweden). All stock solutions were stored at 4°C.

Isolation of spores by phase system partition using dextran-PEG 4000 was achieved by mixing 1.0 mL infected debris, 3.5 mL 20% dextran, 3.5 mL 20% PEG 4000, and 2.0 mL saline (0.15 mol/L NaCl). Phase system partition using dextran-PEG 6000 was achieved by mixing 1.0 mL infected debris, 2.93 mL 20% dextran, 2.25 mL 20% PEG 6000, and 3.82 mL saline. The final phase system of dextran-PEG 4000 contained 7% dextran and 7% PEG 4000. The final phase system of dextran-PEG 6000 contained 5.86% dextran and 4.50% PEG 6000. The tubes were mixed by inversion 30 times. The phase system was then allowed to separate for 30 min at room temperature. After separation, the top phase contained the spores. This phase was drawn off, diluted with an equal volume of saline, and the spores were collected by centrifugation at 800 × *g* for 10 min. The spore pellet was washed one time with saline. The pellet of spores was resuspended in 1 mL saline, and recycled through the phase system a second time. Spore suspensions were counted in a hemocytometer.

A series of aqueous two-polymer phase systems having different ionic compositions and different separation properties were prepared as described by Walter (1975). Briefly, phase systems with the following compositions were prepared: (a) 5% (w/w) dextran and 4% (w/w) PEG 6000 containing 0.11 mol/L sodium phosphate buffer, pH 6.8; (b) same polymer concentrations but 0.09 mol/L sodium phosphate buffer and 0.03 mol/L NaCl; (c) same polymer concentrations but 0.06 mol/L sodium phosphate buffer and 0.075 mol/L NaCl; (d) same polymer concentrations but 0.03 mol/L sodium phosphate buffer and 0.12 mol/L NaCl; and (e) same polymer concentrations but 0.01 mol/L sodium phosphate buffer and 0.15 mol/L NaCl. All phase systems were prepared to contain 2 mL of top phase and 2 mL of the bottom phase. Partitioning of spores between the phases was determined by hemocytometer counts. Data is reported as the percentage of spores added that partitioned into the upper phase.

Bulk phase potential differences were measured by a modification of the techniques described by Reitherman et al. (1973). Electrodes were Pasteur pipettes filled with 1% agar containing 3 mol/L KCl. A Beckman R411 Dynograph was used to measure the electrical potentials. Data is reported as the mean of at least five determinations.

Results — Both the dextran-PEG 4000 system and the dextran-PEG 6000 system produced spore preparations that were largely free of tissue contamination as shown by microscopic examination. Spores partitioned into the PEG-rich upper phase, whereas tissue debris partitioned into the interface and dextran-rich lower phase. Tissue contamination could be further reduced by repeated cycling of isolated spores in additional phase systems.

An experiment was done to determine what physical–chemical property of the myxosoma spore permits isolation in a phase system. Reitherman et al. (1973) have shown that a phosphate buffer system produces a measurable electrostatic potential difference between the phases whereas NaCl produces no potential difference between the phases. Therefore, partitioning of myxosoma spores was studied in the presence of various concentrations of phosphate buffer and NaCl. Equal concentrations of spores were added to each of the phase systems described in Materials and methods. After 20 min incubation at room temperature, the percentage of spores in the upper phase was determined and compared with the measured potential difference between the phases. The results (Fig. 1) showed that essentially all spores were partitioned into the upper phase, regardless of the concentration of phosphate buffer in the phase system. Thus, isolation of *M. cerebralis* spores in an aqueous two-polymer phase system is due to surface properties of the spore other than surface charge.

Discussion — Liquid polymer phase systems have been used successfully to isolate a wide variety of biological products such as proteins (Albertsson 1965b), nucleic acids (Albertsson 1965a), viruses (Philipson et al. 1960), bacteria (Stendahl et al. 1973), chloroplasts (Albertsson and Baltscheffsky 1963), and mammalian cells (Brunette et al. 1968). The properties and uses of liquid polymer systems have been reviewed extensively (Albertsson 1965b). Briefly, a system is prepared containing a two-phase system such as the water–ether separation used previously (Landolt 1973) for isolation of *M. cerebralis* spores. If particles are added to such a system, the particles distribute according to their surface properties. The factors which

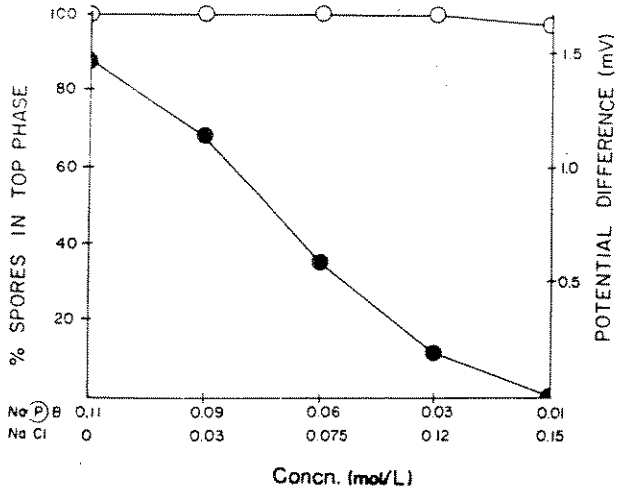

FIG. 1. Partitioning of *M. cerebralis* spores into the upper phase of a system containing 5% (w/w) dextran and 4% (w/w) PEG 6000 at different sodium chloride and sodium phosphate concentrations (○). The electrostatic potential difference (mV) between the two phases is shown at each salt concentration (●).

effect partition include particle size and conformation, hydrophobicity, and surface charge.

Some ions, notably phosphate, partition unequally between the phases, producing an electrostatic potential difference between the phases (Reitherman et al. 1973). Partitioning of cells in such systems is due predominantly to cell surface charge (Reitherman et al. 1973; Walter et al. 1976). *Myxosoma cerebralis* spores partitioned almost entirely into the upper phase, regardless of the electrostatic potential in the phase system, indicating that isolation of *M. cerebralis* in the phase system was unrelated to surface charge. The basis for partitioning of cells in an uncharged system is not fully known, but hydrophobicity is one likely possibility. Walter et al. (1976) have reported that partitioning in uncharged systems is closely related to surface lipid concentration, and Albertsson (1965b) reports that the PEG phase is more hydrophobic than the dextran phase. These results are in good agreement with an earlier report by Landolt (1973) that *M. cerebralis* spores partition into the hydrophobic ether phase of an ether–water extraction system.

An uncharged phase system is recommended for isolation of *M. cerebralis* spores from infected fish. Spores partition into the upper phase regardless of the concentration of phosphate buffer; however, introduction of an electrostatic potential into the system increases the likelihood that contaminating fish tissue will also partition into the upper phase. Thus, we recommend that a phase system consisting of 5.86% dextran and 4.5% PEG 6000 be used for routine isolation of *M. cerebralis* spores from infected tissue. We recommend the PEG 6000 system because this polymer has received the most extensive study with regard to induction of a potential difference by phosphate ions (Albertsson 1965b; Reitherman et al. 1973; Walter 1975; Walter et al. 1976).

We have successfully used the liquid polymer phase technique for detection of small numbers of spores in populations of infected fish; however, the mechanical disruption of infected tissue may be a less efficient means for releasing spores than previously described enzymatic procedures (Landolt 1973; Markiw and Wolf 1974). The principal advantage of the liquid polymer phase technique is the fact that isolation is based solely upon physical surface properties. The isolated spores are unaltered by harsh isolation techniques and are thus quite suitable for biochemical and immunological analysis. Continuing studies in our laboratory are directed toward appropriate immunochemical studies of *M. cerebralis* because these may permit identification of infected tissue.

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Myxosoma cerebralis: Isolation and Concentration from Fish Skeletal Elements — Trypsin Digestion Method

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LANDOLT, M. L. 1973. *Myxosoma cerebralis*: isolation and concentration from fish skeletal elements — trypsin digestion method. J. Fish. Res. Board Can. 30: 1713–1716.

An improved method for detection of *Myxosoma cerebralis* spores is described. Spores were freed from skeletal tissue by trypsin digestion and ether was used for purification of spore suspension. As compared to mechanical grinding, this procedure resulted in a sixfold increase in observable spores.

LANDOLT, M. L. 1973. *Myxosoma cerebralis:* isolation and concentration from fish skeletal elements — trypsin digestion method. J. Fish. Res. Board Can. 30: 1713–1716.

Nous décrivons une méthode améliorée pour la détection des spores de *Myxostoma cerebralis*. Les spores sont dégagées du tissu squelettique par digestion tryptique, et on utilise l'éther pour purifier la suspension de spores. Comparée au broyage mécanique, cette méthode produit une augmentation de six fois du nombre de spores observables.

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WHIRLING disease, caused by the histozoic parasite *Myxosoma cerebralis* (Protozoa: Myxosporidea), is a disfiguring and often fatal disease of salmonid fishes (Hoffman et al. 1962). The trophozoites localize in the cartilage of young fish and undergo sporulation (Hoffman et al. 1962). The mature spores then become trapped as the skeleton ossifies, to be released primarily when the fish dies and decays, and secondarily to be shed with fecal products (Hoffman et al. 1962).

While heavy infections of whirling disease are easily diagnosed by identifying the spores in minced skeletal tissue (Hoffman 1970; Lom and Hoffman 1971; MacLean 1971), light infections or carrier states with low numbers of spores are difficult to detect with present methods. For this reason, a method for obtaining pure concentrations of spores has been sought that would facilitate the visible detection of the parasite.

The most important defense mechanisms against invasion by pathogens in fishes are nonspecific factors such as the body covering and the *p*H of intestinal fluid (Lom 1969). Fish do, however, also possess specific internal inflammatory responses (Finn and Nielson 1971), and immunoglobulins (Summerfelt 1966; Snieszko 1970), and immunization of fish against certain bacteria has been well documented (Klontz and Anderson 1970). An immune response to protozoa is well known in the higher vertebrates (Garnham et al. 1963), and evidence has been presented for a host response by white catfish (*Ictalurus catus*) to the ciliate *Ichthyophthirius multifiliis* (Bauer 1959; Beckert and Allison 1964). No immunological work has been done with myxosporideans; however, Coccidia evoke a specific immune response in domestic animals (Horton-Smith et al. 1963). Salmonids may well produce antibodies on exposure to *M. cerebralis*. A pure spore suspension will be required for the serological detection of exposed fish.

Since spores of this parasite are embedded singly throughout the bone and cartilage, and seldom in discrete granulomas, the preparation of pure spore suspensions is difficult.

MacLean (1971) outlined a procedure for detecting spores. Prasher et al. (1971) described a second procedure which yielded concentrations of up to 70,417 spores/ml. Neither procedure, however, gave pure suspensions of spores. Preliminary work at this laboratory indicated that concentration by sedimentation, density gradient, filtration, or sonication is not satisfactory for obtaining pure preparations of spores. The need for chemical digestion of the skeletal elements was apparent; two enzymes, pepsin and trypsin, proved to be the most effective for releasing spores from cartilage. Rydlo (1971) described the use of pepsin but expressed his results qualitatively rather than quantitatively. Markiw

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TABLE 1. Increase in total number and concentration of *Myxosoma cerebralis* spores at each step of digestion procedure, based on the mean values obtained from 20 fish.

Treatment	Total no. spores per lot	Increase over initial count	Concn spores/ml	Increase over initial concn
Decalcification (initial count)	325,000	–	9,365	–
Trypsin, 12 hr	612,000	1.88 fold	61,200	6.53 fold
Trypsin, 24 hr	1,525,000	4.69 fold	152,500	16.28 fold
Acidification	1,750,000	5.38 fold	175,000	18.69 fold
Etherization	1,340,000	4.12 fold	134,000	14.31 fold
Final washing	1,840,000	5.66 fold	184,000	19.68 fold
Pelletting	1,840,000	5.66 fold	101,200,000	10,800.00 fold

(personal communication) described the use of pepsin followed by trypsin, with well quantified results.

The purpose of this paper is to describe the use of trypsin to implement release and allow concentration and purification of spores. This technique results in a new method of detecting spores, and it provides a source of antigen which will permit the exploration of immunological–serological procedures for a more rapid and sensitive detection of carrier or subclinically infected fish.

Methods

Rainbow trout (*Salmo gairdneri*) ranging in size up to 15 cm in fork length were killed and immediately placed in 45 C water for 10 min to soften the flesh (MacLean 1971). The skeletal elements, with the exception of the fin rays, were then removed, weighed and placed in Decal®, a commercial decalcifying agent. Since spores of *M. cerebralis* did not survive at a *p*H lower than 2.0 it was necessary to raise the *p*H of Decal® to 2.0–2.5 with 0.1 N NaOH. For purely cartilaginous tissue 30 min was sufficient, but with increasingly bony tissue the time required to decalcify the tissue increased to as much as 12 hr. The softened tissue, after being washed in distilled water, was ground in a mortar and pestle or pushed through a garlic press in order to expose maximal surface area to the digestion fluid. The ground tissue was diluted 1:10 with water and duplicate spore counts made with a hemacytometer.

The digestion fluid consisted of 2.0% (w/v) solution of trypsin in Hanks' Balanced Salt Solution. The *p*H of the solution was adjusted to 8.0–8.5 with 0.1 N NaOH and buffered with Tris® at *p*H 8.0. It was found that spore survival was very low at *p*H 9.0 or higher. To avoid bacterial growth, potassium buffered penicillin G (10,000 IU/ml) and streptomycin sulfate (10,000 μg/ml) were added to the digestion fluid. Fresh trypsin was prepared for each day's use and clarified by pressure filtration through a Millipore microfiber prefilter.

The minced, decalcified tissue was placed in the trypsin in a ratio of one part tissue to 100 parts digestion fluid, and stirred constantly at 37 C for 18–24 hr. After digestion, the solution was cloudy and contained considerable tissue debris.

The material was centrifuged at 800 g for 10 min at 25 C and the excess fluid decanted leaving 10–20 ml for easy handling. Using 0.1 N HCl, the *p*H of the solution was gradually lowered to 3.0. As the *p*H approached 4.0 the dissolved proteins were precipitated in a flocculent form. If the drop in *p*H were too sudden the internal anatomy of the spores was converted to a homogeneous mass. To this turbid solution an equal volume of ether was added. The ether: spore suspension ratio could not exceed 1:1 because at low *p*H ether is more soluble and the spores became homogeneous internally. The ether was thoroughly mixed with the solution and allowed to stand. As the ether rose to the top, all of the proteinaceous precipitate, whose buoyant density may have been altered, was carried up with it, leaving a clear, pure spore suspension in the bottom of the container. This clear suspension was immediately withdrawn, centrifuged at 800 *g* for 10 min at 25 C and the solution was decanted. The spores were resuspended in distilled water and centrifuged (800 *g*, 10 min 25 C) twice, after which the spores were resuspended in Hanks' BSS and stored at 4 or –20 C.

Davis 1925 and Hoffman et al. 1962 speculated that the number of spores formed in any given fish is fixed and that some spores are shed as the fish ages. Young fish, therefore, were considered preferable to mature fish both for their greater ease in decalcification and higher ratio of spores per gram of tissue. Only fish that were 1-year-old or younger were used in this series of tests.

This process takes 18–24 hr for its completion; however, only a few hours of the biologist's time are actually required. This method also diminishes the amount of time that has previously been spent in the tedious process of microscopically looking for spores.

Results

To compare the efficiency of recovery of spores using mechanical means and using trypsin digestion, two lots of 10 fish were employed. All of these fish were the same age, had been exposed to *M. cerebralis*

simultaneously, and maintained in the same aquarium. In the first trial, each of the 10 fish was processed separately and duplicate spore counts were made after each step of the procedure. The initial count represented the number of detectable spores following mechanical disruption of the tissue. In the second trial, the skeletal tissues from all 10 fish were pooled and then divided into 10 subsamples of equal weight in an attempt to minimize differing degrees of infection among the fish. Again, duplicate spore counts were made after each step of the digestion procedure. The combined mean values from these two trials are presented in Table 1. After tryptic digestion and ether separation, approximately a sixfold increase over the initial total number of recoverable spores was achieved. Centrifugation of the spore suspension yielded a packed cell volume of 1,840,000 spores in 0.18 ml. Since the initial concentration was 9,365 spores per ml, the final concentration of 101,200,000 spores per ml represented about an 11,000-fold increase in concentration (spores per milliliter).

Since it is not possible to naturally infect fish with a known number of spores, 100,000 spores were added to each of 10 uninfected skeletons prior to mincing of the tissue. Obviously, this was an artificial situation and could not be compared with infected material; however, this was intended only as a control measure to help estimate spore loss during the procedure. At the termination of the digestion procedure, a mean number of 89,600 spores were recovered from each fish and represented approximately a 90% recovery. This indicated that with careful handling and a minimal number of transfers there should theoretically be little loss of available spores.

At the termination of the purification process the spores were judged to be alive and undamaged in that the sporoplasm, polar capsules, and polar filaments were intact. At this time the mucopolysaccharide envelope could also be demonstrated after staining with India ink. The same conditions prevailed after repeated freezing and thawing of the spore suspension.

Discussion

The described procedure provides a new method for the detection of *M. cerebralis* infections. This process has two potential uses. First of all, it is especially valuable in cases in which there are low numbers of spores that might escape detection by purely mechanical means. By increasing the total number of recoverable spores by a factor of six, the hatchery biologist who is called upon to certify certain lots of fish to be free of whirling disease consequently increases his chances of recognizing low-grade infections. Secondly, a potential source of antigen has been made available. Because the mucus envelope is intact, because the spore wall is not visibly disrupted, and because the internal anatomy is not visibly altered after digestion and purification, hopefully, the antigenic determinants on the surface of the spore as well as those contained within the spore are intact and serological detection of whirling disease will soon be implemented.

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Myxosporea: a New Look at Long-known Parasites of Fish

J. Lom

With the identification of Myxosporea as agents of swimbladder inflammation (SBI) in carp, and proliferative kidney disease (PKD) in salmonids, this group of over 1000 protistan species has been receiving renewed attention. Almost all species are parasites of fish, and some can cause serious losses in commercial fish farms. Yet adequate control measures cannot be planned without clarification of basic features of the parasites' life cycles and modes of transmission. In this article, Jiří Lom reviews progress in understanding these biological puzzles.

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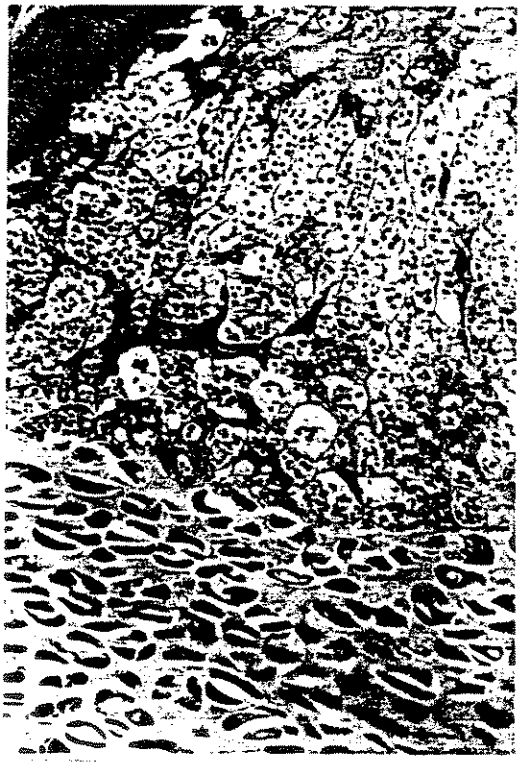
Myxosporea have been known since the early 19th century. Now about 1200 species are recognized, in 46 genera. They constitute a class of the protistan phylum Myxozoa and are mostly parasites of fish[1,2]. Histozoic species live in tissues, either in intercellular spaces, in blood vessels or within the cells, while coelozoic species infect cavities – mainly of the gallbladder and swimbladder. In both cases the trophic (vegetative) stages grow in the form of small or large plasmodia. In histozoic species the plasmodia are encased by a layer of host fibroblasts and when full of mature spores, they appear as cyst-like formations up to several millimetres in size.

Within the plasmodia, in tightly fitting vacuoles, are numerous separate generative cells. These cells produce multicellular spores, either by direct division or in a special formation known as pansporoblast. The spore consists of a shell composed of two or more specialized cells, containing an infective sporoplasm, and nematocyst-like polar capsules – refractile in live spores – containing a coiled, extrudable polar filament. Thus spores and trophozoites are both multicellular, with germinal cells separated within a somatic plasmodium. This, and the functional specialization of these cells, are features that surpass the simple unicellular level of most protistans. That is why myxosporea have sometimes been separated from the kingdom Protista as kin or degenerate descendants of multicellular organisms[3,4], but this view is no longer accepted.

The pathogenic potential of Myxosporea was recognized at the beginning of this century (Box 1). However most of the early studies described new species and their morphology. About 70 papers were devoted to myxosporean life cycles in the fish hosts – often proposing very complicated interpretations of life cycles, including alleged gamete formation. In 1944, Noble[5] summarized the main problems which

Fig. 1. Typical myxosporean spores: fresh spores of *Myxobolus endovasus* from a North American freshwater fish, *Ictiobus cyprinellus* (× 2100).



I. Dyková

Fig. 2. Trophozoites of *Myxobolus cerebralis* digesting the head cartilage of rainbow trout, *Salmo gairdneri* (× 344).

The Myxosporea life cycle debate still rages. Wolf and Markiw provided evidence implying that when the spores of Myxobolus cerebralis are ingested by an intermediate host, they develop into a Triactinomyxon – a member of the Actinosporea, a completely different class within the Myxosporea. This creature produces different spores within the intermediate host and it is only these spores which appear able to initiate infection in the fish. If confirmed, these findings would indicate an unprecedented life cycle system – with each organism alternating two life cycles each with its own sexual and asexual stages. But if each myxosporean has a corresponding actinosporean form, one difficulty becomes immediately obvious – currently the myxosporeans greatly outnumber the actinosporeans, particularly in the marine environment.

arose from controversial interpretations of myxosporean sexuality and the way in which the pansporoblast is produced. Much later, electron microscopy revealed that pansporoblasts were formed by the union of two generative cells[6] (Box 2), and photometry[7] appeared to confirm that autogamy – fusion of nuclei in the sporoplasm – is a primitive, and the only, sexual process in myxosporea. The known facts fitted a simple life cycle (Box 2).

This life cycle diagram is based on two assumptions: (1) transmission through spores is direct; and (2) the sporoplasm, once released from the spore, reaches without delay the final site of infection where it grows and gives rise to the sporogonic plasmodium. Both assumptions have recently been challenged (see below).

Transmission of Myxosporea

As early as 1895, Thélohan[8] reported observations on the hatching of myxosporean spores in the digestive tract, but most attempts at experimental infection by perorally administered spores have failed. Of the few successes, most concern *Myxobolus* (= *Myxosoma*) *cerebralis*, using spores that were left aging in water for at least four months[7]. In *Ceratomyxa shasta*, a serious pathogen of salmonids in North America, even this method failed[9] and led some authors to suspect the presence of some unspecified infective agent other than the spore in the contaminated water[10].

The failures of experimental infection might be explained by the results of Wolf and Markiw[11,12]. Supported by what seem to be meticulously conceived and fool-proof experiments, they reported that the spores of *Myxobolus cerebralis*, when ingested by an intermediate host (the oligochaete worm *Tubifex tubifex*), developed into a *Triactinomyxon* – an organism belonging to another class of the phylum Myxozoa, the Actinosporea. This creature produces quite different spores in *Tubifex* and only these spores, when released from the worm and ingested by the trout, can initiate a *M. cerebralis* infection in the fish.

This surprising finding met with considerable misgivings. It is an unparalleled case of an organism alternating two different life cycles, each with its own asexual proliferation and sexual process occurring at different points in the cycle. If these findings were confirmed, they would radically alter our general concept of the life cycle and taxonomy of Myxosporea. If we accept the idea that each myxosporean has a matching actinosporean representing the second half of its cycle, then the relatively few actinosporean species are clearly outnumbered by myxosporeans, and very markedly so in the marine environment. However, since that remarkable paper by Wolf and Markiw[13] in 1981, no-one else has confirmed their findings.

Extrasporogonic Phase of the Life Cycle

In 1976, G. Csaba, a fish pathologist in Hungary, reported[14] a pioneering finding of a curious organism in the blood of fingerlings of common carp, a fish whose blood has been often studied in Europe during many decades. Csaba found amoeboid organisms, exhibiting a constant twitching or rotating on-the-spot movement. Giemsa staining revealed a cell with up to eight inner cells. It was difficult to assign them to any protozoan group and therefore Csaba called them unidentified extracellular protozoans[14]. Subsequent epizootiological and ultrastructural analyses have indicated[15] that these C-protozoa (for Csaba) or UBOs (for unidentified blood organisms) might represent a proliferative phase of the life cycle of *Sphaerospora renicola*, a common myxosporean parasite of carp in intensive cultures. Small sporogonic pseudoplasmodia of this parasite live in the renal tubules of carp. Soon afterwards, these UBOs were found in many other countries, and in other fish species harbouring other species of *Sphaerospora*[16].

This discovery prompted studies on extrasporogonic stages which were not involved in sporogenesis but were destined to increase the number of parasites in the host. They probably occur in the host both before and during the sporogonic stages, which is why the term extrasporogonic is to be preferred.

Box 1. Pathogenic Potential of Myxosporeans

Myxosporean infections are for the most part relatively harmless, with insignificant or moderate host reactions. However, heavy infection may cause serious damage, either by numerous large cyst-like histozoic trophozoites, or by lytic action[32] of the trophozoite mass pervading the muscle tissue accompanied by various types of regressive changes – especially inflammatory and granulomatous tissue responses to the parasites.

The first serious myxosporean disease described was the whirling disease of cultured salmonid fingerlings caused by *Myxobolus cerebralis*[33,39]. This disease destroys the cartilage (Fig. 2) and causes movement disorders, and frequently leads to host death. *M. cerebralis* is still a serious problem in trout hatcheries, and we now know several other serious myxosporeoses both in freshwater and in marine fish cultures where high densities of fish exposed to various stress factors can provide conditions favourable for the spread of infection. It is chiefly the fry and fingerlings that are susceptible to myxosporean epizootics. For example, juvenile salmonids in North America frequently suffer from infection with *Ceratomyxa shasta*[35], while juvenile carp in European intensive cultures suffer from gill infection with *Sphaerospora molnári*[36], from kidney and swimbladder infection with *S. renicola*[37,38], from brain infection with *Myxobolus encephalicus*[39], and from fin infection with *Thelohanellus nikolskyi*[40].

To date there is no efficient medication against fish myxosporeoses, and we urgently require clarification of the parasite transmission cycles in order to plan appropriate preventive measures.

The stages of the second extrasporogonic cycle of *S. renicola* (Box 3) pervade the tissue of the swimbladder wall of small carp fry. They give rise to swimbladder inflammation (the stages are called SBI stages) which is a serious disease in 2–4 month old fry that can cause high mortality or growth retardation. When the SBI stages are injected into uninfected fry, they give rise to sporogonic plasmodia[17] confirming their relationship with *S. renicola*. A similar swimbladder cycle has been found in tench infected with *Sphaerospora* (W. Körting, unpublished) and may be expected in other hosts.

Intracellular Development and Host Cell Hypertrophy

Intracellular development is no exception in the life cycle of myxosporeans. Most species of the order Multivalvulida[18] live intracellularly in myocytes (eg. the *Kudoa* and *Unicapsula* genera). Myxosporean genera infecting the urinary tract (eg. *Chloromyxum*) have intracellular stages in the epithelial cells, and even in the genus *Myxobolus*, where most species live in intercellular spaces, some species are intracellular (eg. *M. cyprini* in myocytes).

Sphaerospora renicola has one or more types of extrasporogonic development, represented by intracellular stages in renal tubules. The infected cells are greatly hypertrophic, and fuse together to form a syncytium resembling a nodule full of parasites. These stages reach a high level of structural complexity, but are then spontaneously degraded, become nonviable and are eventually eliminated by the host tissue reaction. Thus they seem to represent a 'blind alley' in the development of *S. renicola*. These stages have been mistaken for trophozoites of another kidney carp myxosporean, a view still held by some[19].

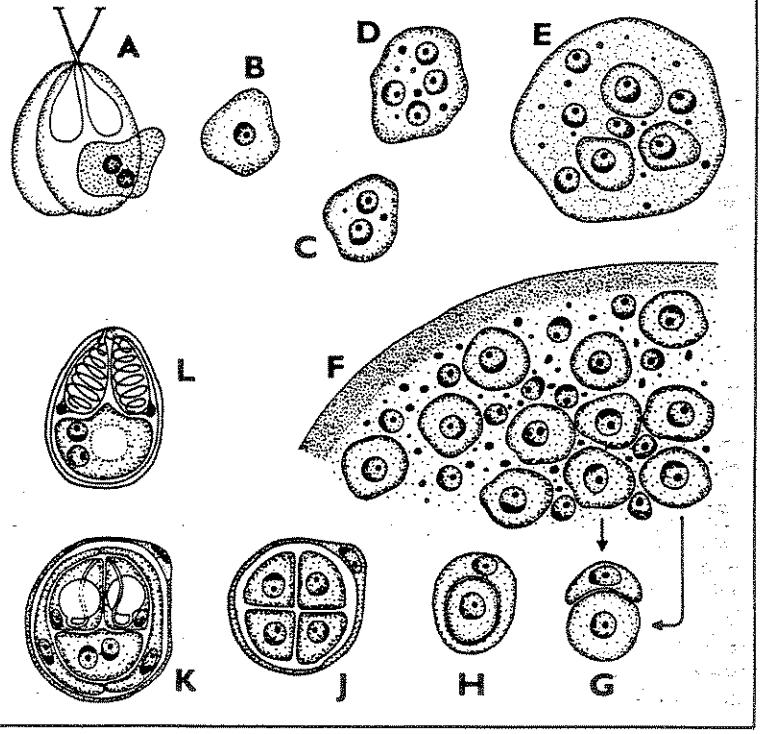
Intracellular existence and extrasporogonic development are combined in some stages of *Myxidium lieberkühni*. This species, found perhaps in all specimens of pike (*Esox lucius*), forms large polysporic plasmodia in the urinary bladder and small trophozoites are found even in renal tubules. In the renal corpuscles, nodules up to 1 mm long can be found, representing greatly hypertrophic Bowman capsule cells filled with numerous parasites. Earlier, some authors[20] considered these nodules to be a separate, rather enigmatic parasite called *Nephrocystidium pickii*. The parasite stages in the nodules consist of a primary cell containing secondary, sometimes even tertiary cells. Most nodules are destroyed by the tissue reaction, but some may rupture to release parasites into the tubular lumen where they may develop into sporogonic plasmodia. The hypertrophic host cell together with its parasites forms a physiological unit similar to that found in cells infected with the microsporidian *Glugea*[21] and known as a xenoparasitic complex or xenoma.

Hypertrophy of the host cell can also be induced by contact with the myxosporean, without actual infection of the cell. Such

Box 2. A Conventional Idea of a Myxosporean Life Cycle

An interpretation of the myxosporean life cycle, which was widely accepted until the late 1970s, can be exemplified by the histozoic genus *Myxobolus* which forms large sporogonic (spore-forming) trophozoites. A spore hatches in the digestive tract of the fish (A), polar filaments extrude and the amoeboid sporoplasm escapes to complete a primitive sexual process of autogamy in which the two haploid nuclei fuse to give rise to synkaryon (B). If there are two uninucleate sporoplasms (as in *Sphaerospora*) they fuse together to produce a zygote – the only uninucleate stage in the myxosporean life cycle. This stage is supposed to reach – in an unknown way – the final site of infection where it grows to become a sporogonic plasmodium. The nucleus divides (C,D,E) until generative cells appear within the multinucleate plasmodium in addition to the many nuclei belonging to the plasmodium itself (vegetative nuclei). Finally a huge plasmodium is formed, a sector of which is shown in F. In other genera, the plasmodium may be small with just one vegetative nucleus (pseudoplasmodium) that produces only one or two spores. Coelozoic plasmodia generally divide by cleaving into two or more parts (plasmotomy) or by producing outer or inner multinucleate buds. In *Myxobolus*, two generative cells unite, one becoming the pericyte enveloping the inner one, which becomes the sporogonic cell (G,H) thus giving rise to a pansporoblast (J), a special spore-producing structure. The pericyte remains a mere envelope, while the sporogonic cell divides to produce the number of cells necessary to complete two sporoblasts (valvogenic cells produce shell valves, capsulogenic cells produce polar capsules and a sporoplasmic cell) which then mature (K) to become spores (L). As the sporoblast is formed, its cells are supposed to undergo a one-step meiosis to reach the haploid state[16].

In pseudoplasmodia of *Sphaerospora*, and also in some large plasmodia such as *Kudoa*, spores are produced without pansporoblasts by direct division of generative cells.



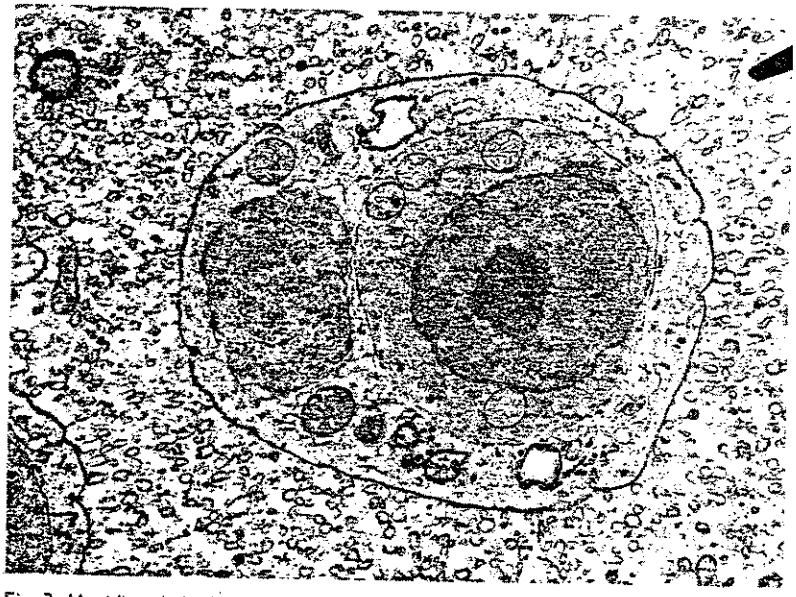


Fig. 3. *Myxidium lieberkühni*: a primary cell enveloping a secondary cell (corresponding to A in Box 3). This is a characteristic of myxosporean cells, that all stages consist of enveloping (primary) cells containing enveloped (secondary) ones.

'contact hypertrophy' has been observed in *Thelohanellus pyriformis* infection of tench branchial arteries when a huge hypertrophic endothelial cell becomes wedged in the interior of a sporogonic plasmodium. This is probably to enlarge the host–parasite interface in order to improve nutrient uptake by the parasite[22].

Proliferative Kidney Disease of Salmonids

A very special case of extrasporogonic developmental stage is the 'PKX cell' – the agent of proliferative kidney disease (PKD) of salmonids. This disease often inflicts heavy mortalities in rainbow trout fingerlings and is thus of great concern for trout fisheries. The protozoan found in the kidney of diseased fish was first thought to be an amoeba[23]. Later, haplosporean affinities were suggested[24] because of the presence of small dense bodies in its cytoplasm which were reminiscent of haplosporosomes – inclusions typical of haplosporeans. More recently, comprehensive evidence has emerged that the PKX is a myxosporean[25].

Even a small myxosporean cell complex (UBO stage or PKX) can now be distinguished from other organisms parasitic in the fish host. Invariably, there is a primary cell with one or a number of secondary ones, and perhaps with tertiary cells. This is a feature known in myxosporeans and haplosporeans such as *Marteilia*. However, myxosporean secondary (and generative) cells have a characteristic bundle of microtubules close to the nucleus and lack a centriole. Secondary cells also have numerous free ribosomes in their cytoplasm and pseudopodia-like cell extensions.

The primary PKX cells contain secondary and tertiary cells and are found among interstitial cells of the kidney, and in blood vessels and other organs. They provoke a disproportionately acute host reaction manifested by interstitial hyperplasia and granulomatous interstitial nephritis. These cells migrate into the lumen of the renal tubules, where the primary cell is shed from the secondary cells which then develop into a sporogonic stage producing sporoblasts. These sporoblasts are similar to those of *Sphaerospora* and *Parvicapsula*, but in salmonids they never develop into a mature spore[25]. The heavy host reaction, as well as the failure to complete the spore development, lend support to the idea that PKX is a myxosporean parasite of some other fish living in the water in which the juvenile salmonids were released. Salmonids are susceptible to infection, but – as nonspecific hosts – do not provide conditions necessary for spore production.

Developmental Stages of Myxosporea in Nerve Axons

Among the Myxosporea, extrasporogonic stages dispersed in tissues, with the absence of conspicuous refractile spores, easily escape attention. Rare observations of these stages include the organisms found in the skin of rainbow trout[26] or in more unusual sites such as the axons of several species of fish[27] and an amphibian[28]. The axonal stages contained numerous secondary cells within a large primary cell (plasmodium), greatly distending the myelin sheath. It is not clear how the parasite gets into the axon, nor whether the axonal stages are just early proliferative stages or if they later transform into sporogonic plasmodia. In bullheads (*Cottus gobio*), sporogonic plasmodia of a new *Myxobolus* species do develop within the enlarged axons (J. Lom, S. W. Feist, I. Dyková and T. Kepr, unpublished), but in other species the true nature of axonal stages is still to be solved.

How Does a Myxosporean Infection end?

Infection with coelozoic species of Myxosporea, where the plasmodia constantly divide and produce spores, may last a long time although the exact duration has not been determined experimentally. The small plasmodia of *Sphaerospora* in kidney tubules exhibit seasonal variation in spore production.

In histozoic plasmodia, spore production is synchronous and eventually the plasmodium becomes an envelope full of spores. If there is a natural outlet, as in plas-

Whirling disease – Myxobolus cerebralis *invades host cartilagenous tissues, including those of the brain, and digests the host's cartilage; this leads to movement disorders and may eventually lead to death.*

Proliferative kidney disease – *in salmonids, the myxosporean extrasporogonic 'PKX cell' stage of* Myxidium lieberkuhni *can initiate a disproportionately acute host reaction in the lumen of the renal tubule and consequently cause extensive damage to kidney tissue.*

Swimbladder inflammation – *the stages of the second extrasporogonic cycle of* Sphaerospora renicola *invade the swimbladder wall of small carp fry, thus initiating a host inflammatory immune response, which can prove debilitatory or fatal, particularly in two to four month old fry.*

modia sited in the gills, skin or intestine, then the spores may be released from the envelope and the tissue lesion is repaired. At other sites, once the spores are mature the plasmodium is attacked by the host tissue reaction and is destroyed through granuloma formation. Usually the spores are ingested by macrophages and transported into melano-macrophage centres in the kidney, spleen and liver where they are completely digested. Thus it seems that transmission of some histozoic species depends on death of the host and its decomposition (or perhaps ingestion by other animals) to release the spores.

The full life cycle and mode of transmission of myxosporeans clearly requires clarification before adequate measures to control species pathogenic to fish can be applied. In particular, the Wolf–Markiw hypothesis on the transmission of *M. cerebralis* requires further study. If it is true, does it apply to other myxosporeans? Also, is the extrasporogonic development outside the final site of infection a general rule for the group, or does it apply only to genera such as *Sphaerospora* and *Myxidium*? One thing is clear: in view of the irregularities displayed by the vegetative stages of certain species, the events taking place in some genera need not necessarily apply to all.

Myxosporeans are generally well adapted to their hosts so that young sporogonic stages, before spore maturation, evoke practically no tissue reaction[29]. But the SBI stages and the PKX myxosporean both produce a heavy host reaction causing serious disease. In the case of PKX, this may be because salmonids represent an 'unusual' host, while in the case of SBI this may reflect recent adaptation of this stage to life in the swimbladder.

Yet the origin of myxosporeans is a perennial puzzle. Speculation on their phylogenetic origin must take into account the similarities between the polar capsule and the nematocysts of the coelenterates[6,30,31] – which seems impossible to interpret in terms of convergence. Did myxosporeans evolve from some protistans, or could they be descendants of coelenterates degraded by parasitism? Parasitic coelenterates are known in fish, for example *Polypodium hydriforme* in sturgeons has very primitive larval stages.

Box 3. Stages of the Extrasporogonic (Proliferative) Parts of the Myxosporean Cycle

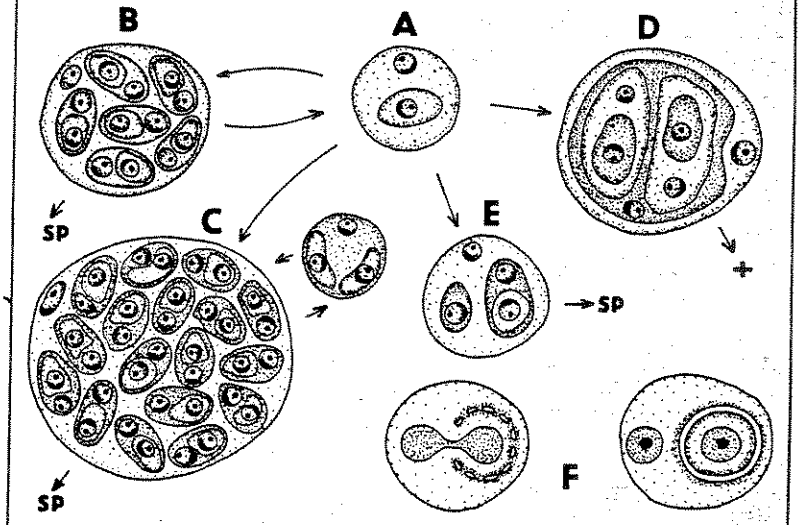
The initial stage of the extrasporogonic cycle is always a primary cell containing a secondary cell (A) (see Fig. 3) and situated within a tightly fitting, membrane-bound vacuole. This is also the type of cell that is produced by the released sporoplasm. The primary cell is homologous to the plasmodium or pseudoplasmodium of the sporogonic phase (see Box 2) and the secondary cell is homologous to the generative cell.

In the diagram, A–B shows the bloodstream cycle in *Sphaerospora*: the primary cell grows, secondary cells divide to produce six and more cells, which, by endogenous division, produce tertiary cells. The primary cell eventually disintegrates, releasing the secondary cells which may begin the cycle again or may pass through the A–C or A–D sequence or into the sporogonic (spore-forming) phase (SP) (see Box 2).

C represents the SBI stage in the cycle of *S. renicola* (the stage responsible for swim bladder inflammation (SBI) in carp). It produces primary cells with up to 80 secondary cells each with one or two tertiary cells. AD is the intracellular, 'blind alley' development in *S. renicola*. The primary cell develops into a highly complex but nonviable unit.

A–E represents production of the PKX stages responsible for proliferative kidney disease (PKD) in salmonids or intracellular stages of *Myxidium lieberkühni*. The cycle may be repeated, or the cells may be destroyed by host tissue reaction or may give rise to sporogony (SP).

F illustrates endogenous division in myxosporean primary (or secondary and tertiary) cell. Vesicles of the endoplasmic reticulum surround one of the daughter nuclei to produce the plasmalemma of the secondary cell and the vacuolar membrane of the primary cell. This is one of the most important characteristics of myxosporeans – that all their stages consist of enveloping (primary) cells containing enveloped (secondary) ones.



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MORPHOLOGY OF THE SPORES OF *MYXOSOMA CEREBRALIS* (HOFER, 1903) AND *M. CARTILAGINIS* (HOFFMAN, PUTZ, AND DUNBAR, 1965)*

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ABSTRACT: The spore of *Myxosoma cerebralis* is redescribed and compared with that of a close relative, *M. cartilaginis*. *M. cerebralis* is smaller, with greater variability in size and shape. It possesses a mucous envelope whereas *M. cartilaginis* does not. The surface architecture of *M. cerebralis*, as seen with the scanning electron microscope, differs in possessing a conspicuous circumsutural groove, prominent polar filament pores, and a mucous envelope.

Since a unanimous decision on the correct generic status of *M. cerebralis* has not been reached, we continue in this paper to call this species *Myxosoma cerebralis*, in spite of the views of Walliker (1968) and Lom (1969), who do not consider the absence of the iodinophilous vacuole as adequate reason to warrant generic separation of *Myxobolus* and *Myxosoma* and consider the latter a junior synonym of the former. *Myxosoma cerebralis*, one of the most notorious myxosporidean species, is the causative agent of whirling disease of salmonid fish. This parasite, discovered first in Germany, has been subsequently found in other European countries, the United States, far eastern USSR territories, and even South Africa (Hoffman, 1970). According to Shulman (1966) and Hoffman, Dunbar, and Bradford (1969) it has thus far been found in the following species: *Oncorhynchus gorbuscha*, *O. keta*, *O. kisutch*, *O. masu*, *O. tshawytscha*, *Salmo gairdneri*, *S. salar*, *S. trutta*, *Salvelinus fontinalis*, *S. leucomaenis*, *S. malma*, *S. namaycush*, and *Thymallus thymallus*. Quite recently, whirling disease has been reported to have spread through additional areas of the United States (Hnath, 1970; Yasutake, 1970).

While Shulman (1966) supposes that *M. cerebralis* has long had an holarctic distribution, occurring in naturally infected water reservoirs or streams from which it is able to spread and infect cultured salmonids, it is generally assumed that the recent appearance of *M. cerebralis* infections in North America is due to the importation of infected fishes from Europe. The final solution of this problem would require, among other things, a complex comparative analysis of *M. cerebralis* from European, Asian, and American sources, including a detailed morphological evaluation. Such a morphological analysis would rely largely on the morphology of the spores and similar comparisons should also be made for other myxosporidean species. Curiously enough, no accurate description of spores of *M. cerebralis* is offered in the literature.

Kudo (1920), in his now classical monograph, used spore drawings made by Plehn (1904). These rather simple drawings show a certain degree of spore variability, and depict the spores as having a protruding and narrow sutural valve. The more recent and authoritative monograph of Shulman (1966) again uses the drawings of Plehn, in addition to more recent but schematic drawings of the spores. In other publications on *M. cerebralis*, the spores are also represented very schematically. Everyone seems somehow to take the definition of

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M. cerebralis for granted, and yet it may be very desirable, for practical reasons, to know the degree of differences, biological as well as morphological, among the populations of *M. cerebralis* from individual species of salmonid fishes and its difference from populations in other possible hosts. Ramirez Medina (1962), e.g., reported *M. cerebralis* also from *Tinca tinca*, *Gobio gobio*, *Esox lucius*, and *Perca fluviatilis*; Volf (pers. comm., 1965) found an allegedly different strain (or species?) of *M. cerebralis* in *Thymallus thymallus*. An exact knowledge of the spore would therefore be very useful for purposes of definitive determination.

A well-founded differentiation of *M. cerebralis* from other cartilage- and bone-invading species of *Myxosoma* such as *M. hoffmani*, *M. dentium*, and *M. cartilaginis* would also be useful. In the case of the last species, the authors themselves point out its close resemblance to *M. cerebralis*, claiming that the spore differs mainly in being larger.

The purpose of this communication is to give an accurate redescription of the spore of *M. cerebralis* and to point out how the spore could be used for differential diagnosis, using as an example the cartilage-invading *M. cartilaginis*.

MATERIALS AND METHODS

Only fresh (not preserved) spores were used for our observations, since fixation causes changes in size and structure of the spores (Kudo, 1921). The spores of *M. cerebralis* were obtained from trout fingerlings (*Salmo gairdneri*) artificially infected at the Eastern Fish Disease Laboratory. The fish and infections were about 5 months of age. Spores of *M. cartilaginis* were retrieved from moderate infections in bluegills (*Lepomis macrochirus*) from rearing ponds at the Eastern Fish Disease Laboratory. No special methods were necessary because large masses of spores were obtained almost completely free of other particles.

It is difficult to separate the spores of *M. cerebralis* from fish tissue. Halved fish heads were digested in aqueous pepsin–HCl solution (pepsin 0.5%, HCl 0.5%) at 39 C for 2 hr. The softened tissue was removed under the dissecting microscope with forceps and the remaining skeletal parts macerated with a mortar and pestle. The macerated material was screened through 224-, 154-, and 70-μ mesh screens to remove tissue and the spores were allowed to settle out at 6 C. These spores were not visibly different from those not so treated.

In addition to the direct observation of the above spores, photomicrographs were made by employing the technique described in an earlier paper (Lom, 1969). To reveal the presence of mucous envelopes we used the simple India ink technique (Lom and Vavra, 1963).

For scanning electron microscopy, the spores of *M. cerebralis* were prepared essentially according to the technique of Marszalek and Small (1969). The fresh spores were further separated from the tissue debris by means of pipettes and micropipettes, and a drop of the suspension with concentrated spores was placed on a microscope slide. The spores settled and adhered slightly to the glass surface, allowing for further washing by adding and withdrawing distilled water by micropipettes. After several such washing procedures, spores were covered by Parducz fixative (1 part of saturated $HgCl_2$ to 6 parts of 2% osmic acid) for about 6 min, after which the spores were thoroughly washed on the slide with distilled water.

Drops of spore suspension of about the size of a pinhead were placed on the supporting specimen stubs, several drops to each specimen stub. Some of the stubs were coated thinly with a toluene extract of the glue from the transparent double-coated tape (3M Corporation) for better adhesion and to minimize the static charge of the specimen. The spores were then allowed to settle in the drops and were quickly frozen by plunging the stubs into liquid nitrogen. The subsequent sublimation was performed overnight in a tissue lyophilizer. After a thorough gold coating the stubs were examined with a Cambridge Stereoscan Mark III electron microscope operated at 10 and 20 kv accelerated voltage. Our attempts at preparing sections for electron microscopy were unsuccessful.

RESULTS

(a) *Myxosoma cerebralis* (Hofer, 1930). In frontal view (perpendicular to the sutural plane) the spores are mostly broadly oval, sometimes more elongated, rarely completely circular, and exceptionally broader than long (Fig. 1). Although it does not show in the photomicrographs, the spores are often asymmetrical. In side view they are broadly lenticular, both shell valves being considerably vaulted, sometimes one more than the other. The dimensions of 40 spores taken from 5-, 8-, and 11-month infections were: length 8.7 (7.4 to 9.7) μ, width 8.2 (7 to 10) μ, thickness 6.3 (6.2 to 7.4) μ. The spore wall could not be measured accurately but appeared to be about 0.25 μ thick. When viewed from the polar capsule end, three very faint striations (grooves?) were seen on some specimens in bright light microscopy.

The oviform polar capsules measured 5.1 (5 to 6) by 3.2 (3 to 3.5) μ. Rarely, one polar capsule is slightly larger than the other. As a rule, there is no true intercapsular appendix; in

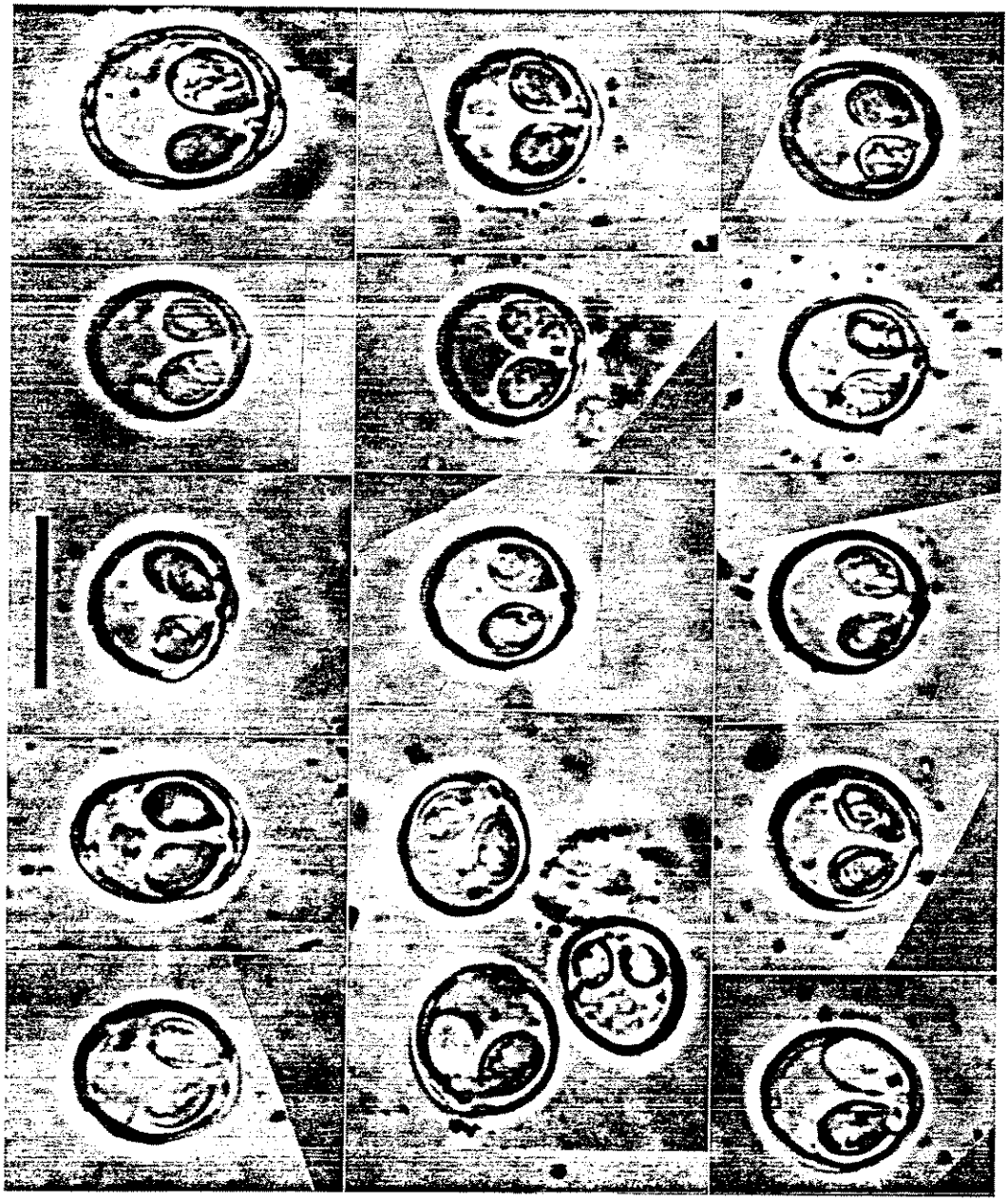


FIGURE 1. Variation in shape of fresh *Myxosoma cerebralis* spores from rainbow trout fingerlings. The most typical spores are in the right column—spores are oval, almost circular in outline with equal polar capsules. The left column shows rather aberrant spores, either by shape or by unequal size of polar capsules. × 3,000; the line indicates 10 μ.

most cases the border of the shell valves at the anterior end is only slightly thickened on the inner face, forming a ledge separating the discharging canals of the polar capsules. The hypothetical extensions of the longitudinal axis of the capsules always cross anteriorly but the converging pointed anterior ends of the capsules sometimes just overlap in the front view, sometimes lying more or less apart. The filament usually makes five or six coils, rather

loose and irregular, inside the capsule; it is not very distinct in this species.

In front view, the posterior border of the spore does not exhibit any distinct markings, but the inner outline of the posterior border is generally uneven. India ink reveals a typical mucous envelope around the posterior end of the spore (Figs. 2–4). One of us (J.L.) observed this mucous envelope on *M. cerebralis* sometime ago in France on spores not treated with pepsin digest solution; it is evidently a constant character of fresh spores. After a period of storage in the refrigerator, the mucous coat diminishes a little, but persists for months. The sporoplasm with two nuclei fills the rest of the spore space.

Observed in the scanning electron microscope, the spores of *M. cerebralis* are characterized by the presence of a deep ridge running parallel to the sutural border. Our experience with scanning electron microscope observations of myxosporidean spores is limited, but we did not observe such a deep furrow in spores of several other species. The furrow circumvents the thickened part of the sutural border of the anterior end of the spore. The sutural border of the valve bears at that spot an opening which is presumably the mouth of the discharging canal of the polar filament (Figs. 6, 7). The openings seen in Figure 7 are obviously an anomaly, or one opening split into two; in *Myxosoma* and *Myxobolus*, each of the two valves bears the opening for the filament of one of the polar capsules (Lom, 1964). Here again, an open, gaping discharging canal in the shell valve border is different from the spores of other myxosporidean species observed thus far.

The shell valves of the spore are shrunk a little in most of the spores, more posteriorly than anteriorly, where the rigid polar capsules give support to the spore wall. The degree of shrinkage in individual spores varies, but as a rule the majority of all observed spores always reveals some degree of shrinkage.

Posteriorly, the surface of the spore is covered by an irregular network of intertwined strands, which represent constituents of the mucous envelope. Some of the thin mucous strands are also seen to cross the circumsutural furrow (Figs. 6, 7). In some of the spores, the shell valves are clearly asymmetrical (Fig. 5).

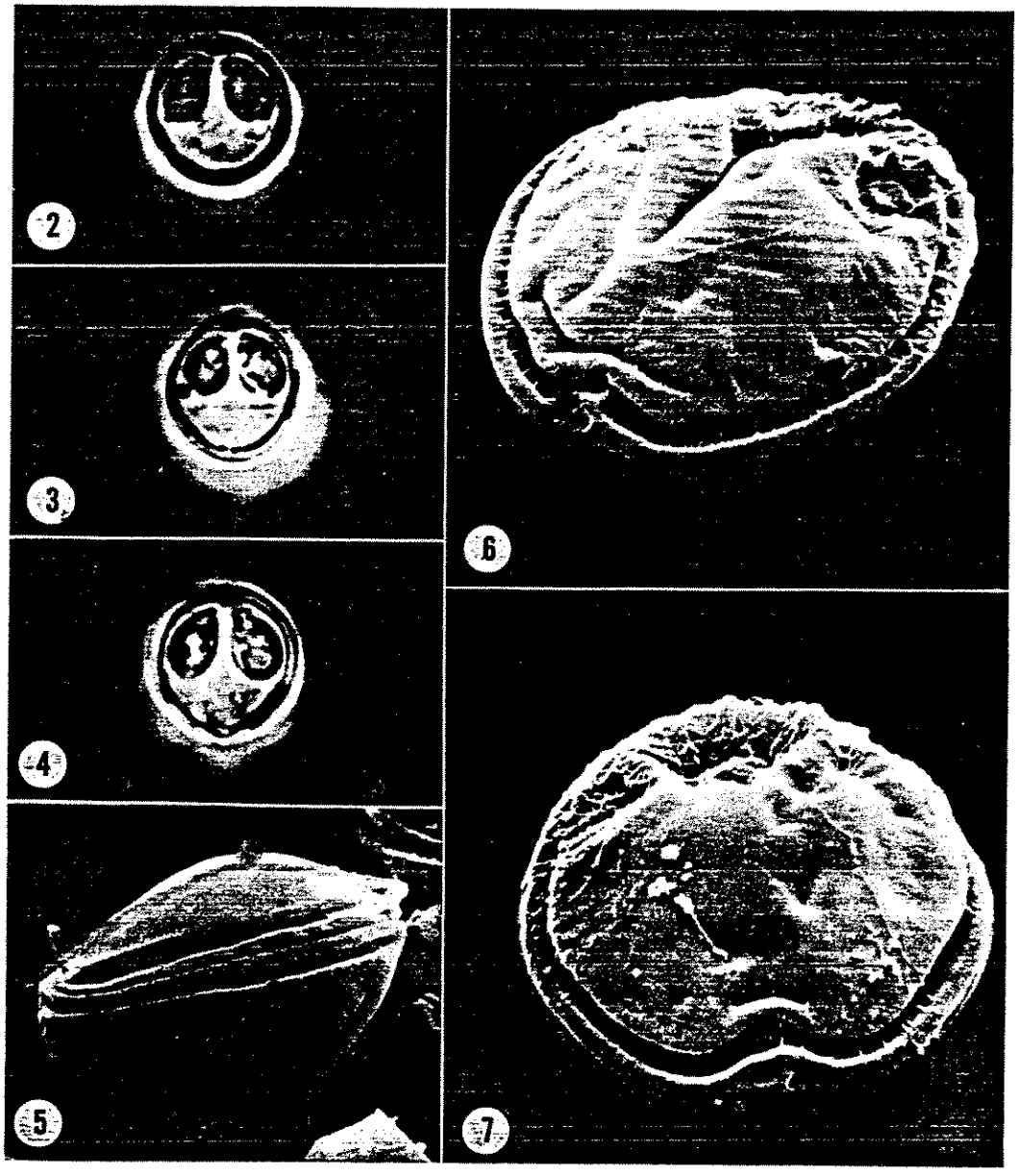
b) *Myxosoma cartilaginis* (Hoffman, Putz, and Dunbar, 1965). In frontal view (Fig. 8) the spore is oval, of a very regular shape and a quite uniform size—11.25 (10.7 to 11.7) by 9.5 (9 to 10) μ. The variation is thus less than in *M. cerebralis*. The present measurements are only slightly larger than those given in the original description by Hoffman et al. (1965). There are six to nine, usually eight, rather indistinct sutural markings at the posterior border of the spore. Anteriorly, there is a triangular thickening of the spore wall border. In side view, the spore is broadly lenticular. The two always equal polar capsules converge less than in *M. cerebralis*, forming a smaller angle, and their pointed anterior ends never overlap in front view. The size of the capsules averages 5.75 by 3.5 μ, varying very little. There are usually seven coils of polar filament in the capsule, the threads being tightly but not very neatly arranged.

There is no mucous envelope around the spore, and no iodinophilous vacuole in the binucleate sporoplasm filling the rest of the space within the spore.

In the scanning electron microscope, the spores reveal a smooth surface without any trace of mucous strands, and there is no furrow parallel to the sutural line of the spore (Figs. 9, 10). In most cases, the surface of the spore is rather shrunken into furrows and ridges, and only a small number of spores seems to have resisted shrinkage.

CONCLUSIONS

We intentionally refrained from presenting drawn pictures of spores, which, though being easier to reproduce than photographs, represent a certain risk of schematization, do not accurately illustrate how the spores look in the microscope, and are less suitable for demonstration of variability. Our material clearly indicates the differences between the two species and we hope the same scrutiny will be done with other *Myxosoma* species. In the case of *M. cerebralis*, these initial observations, performed on material from typical cases of whirling disease, should be followed by a study of variation and distinctions of various strains from different hosts and areas of distribution. Combined with a computerized, statistical evaluation of quantitative data, it should yield



FIGURES 2–4. Fresh spores of *M. cerebralis* in India ink to show the mucous envelope unpenetrated by the suspended India ink particles. The mucous coat is especially thick on the posterior half of the spore. These spores were recovered from a rapid digestion sample. × 3,000.

FIGURES 5–7. *M. cerebralis* spores as seen in the scanning electron microscope. 5. The spore wall reveals almost no shrinkage; one of the shell valves is more vaulted than the other. The furrow along the sutural line is quite distinct. × 9,500. 6. The spore in an upper anterior view. Notice the characteristic furrow parallel to the sutural line, the fine mucous strands concentrated on the posterior half of the spore, and the seemingly open canal for filament discharge in the thick sutural border of the shell valve at the left. × 11,000. 7. Same as in Figure 6; the opening of the canal for filament discharge seems to be split into two.

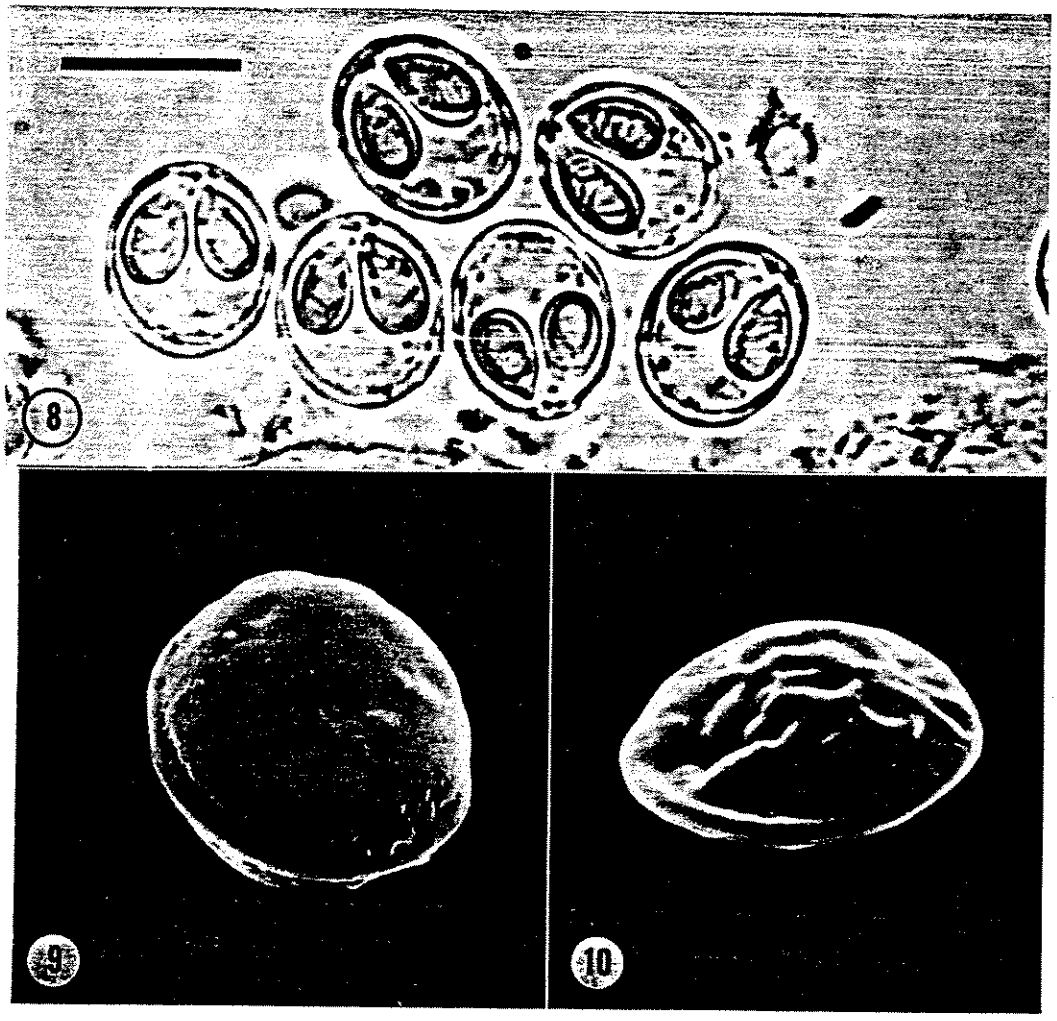


FIGURE 8. Fresh spores of *M. cartilaginis* from the bluegill. Note uniform appearance of this group of spores selected at random. × 2,800; the line indicates 10 μ.

FIGURES 9, 10. *M. cartilaginis* spores seen in scanning electron microscope. **9.** With smooth shell. **10.** With the usual, somewhat shrunken appearance of the shell. In both cases, note absence of furrow along sutural line as well as lack of mucous strands on the surface.

a solid base for species and strain distinctiveness in *Myxosoma* and *Myxobolus*.

We would like to avoid giving the impression of mistaking a single developmental stage, the spore, for the whole organism with all its developmental changes and stages. The importance of the spore shape and structure is due to a well-known lack of really distinct characters of the trophozoite, both on the light microscope and on the electron microscope level.

Although the spores of both species here reported were not treated in exactly the same manner, we feel that the differences cited will prove to be valid. The study of *M. cerebralis* spores is not possible without using a concentrating technique. There is no indication that the mucous envelope of *M. cerebralis* is produced during pepsin digestion because we saw it previously on spores not so treated. The mucous envelope has also been observed on other Myxosporidea not treated with pepsin solution (Lom and Vavra, 1963). The spore

wall of *M. cerebralis* is so tough that it is difficult to believe that pepsin solution would produce the spectacular furrow.

In addition to light microscopy to observe the shape and inner structure of the spore, we used scanning electron microscopy to bring out the surface patterns. Thus far, no data exist on this subject in Myxosporidea, but the ultrafine outer sculpturing and surface patterns revealed by this technique may be of great interest for taxonomy. Sutural markings, ridges, and folds can sometimes be seen on fresh spores of *Myxosoma* and *Myxobolus*; it is probable that this was accentuated by our preparation methods, resulting in shrinkage of the spore wall (Figs. 6, 7, 10). In fresh spores, such folds may be made more distinct by vital staining and, in fact, they do not represent artifacts. The smooth-shelled spores (Figs. 5, 9) seem on the other hand always to represent a small part of the sample, and appear sometimes even more swollen than natural; the possibility is not excluded that they are rather immature spores.

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Detection of *Myxobolus* (*Myxosoma*) *cerebralis* in Salmonid Fishes in Oregon

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Abstract.—*Myxobolus* (*Myxosoma*) *cerebralis*, the etiological agent of whirling disease, was detected in salmonid fish populations in northeastern Oregon. This is the first record of *M. cerebralis* in the Pacific Northwest of the USA. During an epizootiological survey for the parasite, two methods for spore detection were compared, and an efficient procedure for determining *M. cerebralis* infection in adult fish was developed. The enzyme digest method was more efficient than the plankton centrifuge procedure for examination of numerous individual lots of fish processed during the survey. Sampling only the area around the otoliths was at least as effective as sampling entire heads for detection of spores in infected fish.

Whirling disease, caused by *Myxobolus* (*Myxosoma*) *cerebralis*, infects all species of salmonid fish except lake trout *Salvelinus namaycush* (O'Grodnick 1979). The disease occurs in Europe, the United Kingdom, New Zealand, South Africa, the USSR, and the USA (Halliday 1976; Hoffman 1976; Hnath 1983). Within the USA, the geographic range includes several eastern states and, until recently, only California and Nevada in the West (Halliday 1976; Hnath 1983). *Myxobolus cerebralis* is one of two fish pathogens covered in laws regulating importation of fish into the country. The disease is also included as one of concern in the fish disease control policies of other international, national, regional, and state governments (Rohovec 1983). Because of importation regulations, efforts have been made to improve the efficiency and accuracy with which the presence of *M. cerebralis* can be detected in fish tissues (Landolt 1973; Contos and Rothenbacher 1974; Kozel et al. 1980; Markiw and Wolf 1980). The enzyme digest method (Markiw and Wolf 1974a) and the plankton centrifuge method (O'Grodnick 1975) are techniques currently used to diagnose whirling disease. Microscopic examination of preparations that reveals spores showing morphology similar to that of *M. cerebralis* provides presumptive diagnosis of whirling disease. Confirmation of parasitism by *M. cerebralis* is made either by detection of spores in histological preparations of cartilaginous tissue (Plehn 1904) or by specific fluorescent antibody techniques (Markiw and Wolf 1978). Previously described methods are for diagnosis of whirling disease in juvenile fish, and none have been tested with infected adults.

In late 1986, *M. cerebralis* was detected in populations of juvenile rainbow trout *Oncorhynchus mykiss* (formerly *Salmo gairdneri*) and brook trout *Salvelinus fontinalis* at a privately owned site in northeastern Oregon. This first observation was followed by detection of the parasite in feral populations of these two species and of chinook salmon *Oncorhynchus tshawytscha* in nearby areas. The discovery provided both an opportunity to test methodologies for detection of the parasite in adults and an impetus to broaden an epizootiological survey already in progress.

In this report, we document the presence of *M. cerebralis* in Oregon and describe our detection method. During an epizootiological study of whirling disease in the state, we compared modifications of the currently used diagnostic procedures and examined methods for detecting *M. cerebralis* in adult salmonids.

Methods

Detection of M. cerebralis in Oregon.—Feral fish, primarily juvenile rainbow trout, steelhead (anadromous rainbow trout), brook trout, cutthroat trout *Oncorhynchus clarki*, kokanee (lacustrine sockeye salmon *Oncorhynchus nerka*), and coho salmon

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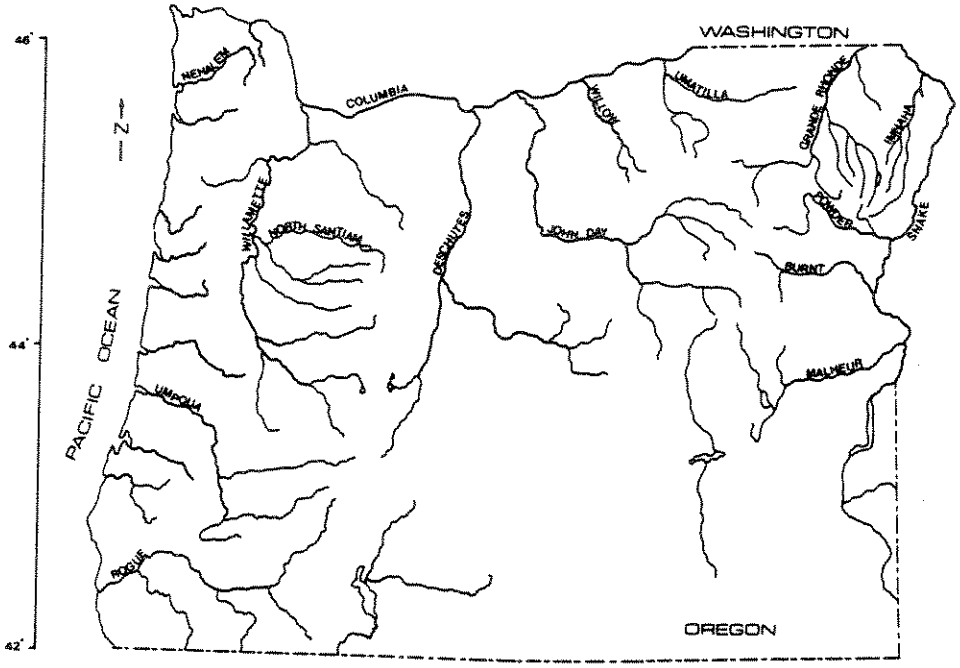

FIGURE 1.—Major watersheds in Oregon from which salmonid fish were collected for detection of *Myxobolus cerebralis*.

Oncorhynchus kisutch, were seined, angled, electrofished, and trapped from major watersheds throughout Oregon (Figure 1). Some samples were collected at state and private hatcheries. Adult steelhead and chinook salmon that had returned from the ocean to areas in northeastern Oregon were also examined for *M. cerebralis*.

In the epizootiological study, approximately 350 samples embracing more than 4,000 fish were collected. Entire fish or heads were frozen and delivered to the laboratory. At most sites, some heads were fixed in 10% buffered formalin, and these were included with many of the samples for histological examination. The frozen samples were processed and examined by methods similar to those in Amos (1985). Samples that contained spores of the size and shape typical of *M. cerebralis* were presumed positive; confirmation was made by histological examination. For histology, the portions of the preserved heads containing the semicircular canals and otoliths were decalcified for 3 d in CAL-EX II (Fisher Scientific, Pittsburgh, Pennsylvania) and then rinsed in flowing water for 3–4 h. Tissue samples were placed in 70% ethanol, processed in an ethanol–xylene series, and embedded in paraffin. Seven-micrometer-thick sections were cut, stained with May–Grünwald Giemsa, and observed microscopically.

Comparison of detection methods.—Fifty juvenile rainbow trout averaging 179 mm in fork length were collected from a hatchery where whirling disease was confirmed. The heads were severed just behind the opercula. The gills were removed and each head was cut in half longitudinally to provide material for detection of *M. cerebralis* by the enzyme digest and plankton centrifuge methods. The tissue was pooled (50 halves) and heated at 50–60°C for 15 min. The heads were defleshed and approximately 30 g of material was processed by one or the other method as described in Amos (1985). We varied the procedure for the enzyme digest by using formalin instead of serum to stop

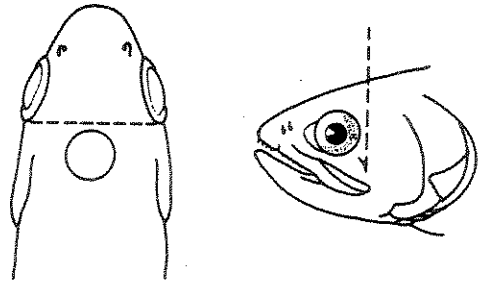

FIGURE 2.—Dorsal and lateral views of an adult salmon head, indicating the location for obtaining a core sample for examinations for *Myxobolus cerebralis*.

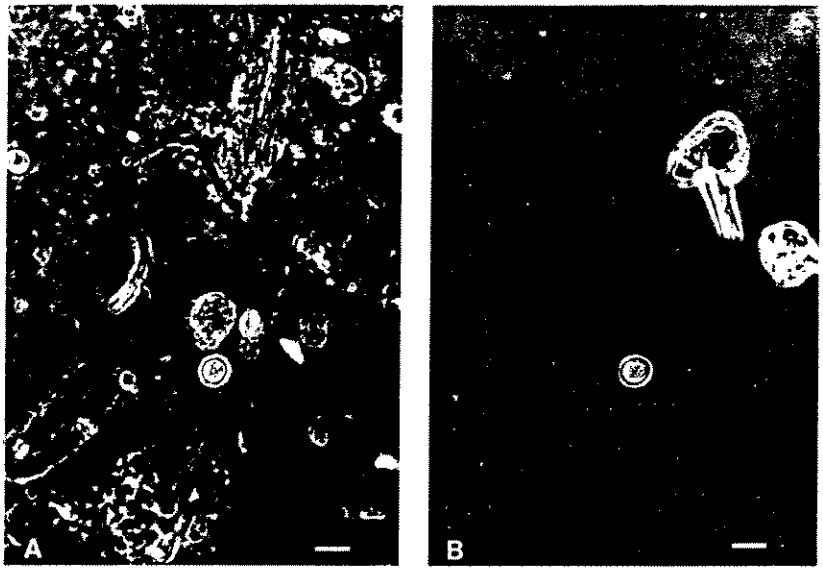

FIGURE 4.—Wet-mount preparations from juvenile rainbow trout containing spores of *Myxobolus cerebralis* obtained by (**A**) the plankton centrifuge method, and (**B**) the enzyme digest method. Bars = 10 μm.

slide was searched until *M. cerebralis* spores were detected or until an area of 22 mm^2 had been swept.

Results

Detection of M. cerebralis in Oregon

Spores of *M. cerebralis* were detected in feral fish collected in the Grande Ronde and Imnaha river systems. Furthermore, typical spores were found in captive fish that had been transferred from a single contaminated source into ponds in these two systems and in the John Day, Umatilla, Powder, and North Santiam systems. In some samples, two different sizes of spores with similar morphology were observed. When histological sections were examined, these spores could be differentiated by their tissue tropism (Figure 3). Spores parasitizing the nervous tissue of fish were from an unidentified species of *Myxobolus*.

Comparison of Detection Methods

Spores were easier to detect in the reduced level of background debris resulting from the enzyme digest method than they were after centrifugation (Figure 4). Averages of 12.5×10^2 and 3.75×10^2 spores/mL were detected in the enzyme digest and plankton centrifuge preparations, respectively.

Comparison of Sampling Methods

Two of 20 fish whose entire heads were processed were positive for *M. cerebralis*. The 20 fish from which cores were examined included 6 individuals positive for *M. cerebralis*.

Discussion

This report documents the occurrence of *M. cerebralis* in Oregon and the first observation of the parasite in the northwestern USA. An epizootiological survey indicated that *M. cerebralis* is confined to a relatively small area of Oregon, but has produced no indications of how introduction into Oregon occurred. Several possibilities exist. (1) The parasite may have been present for many years but was not detected until fish were reared in an environment ideal for development of clinical whirling disease (rainbow trout in earthen ponds with low water exchange). (2) The parasite may have been introduced with infected anadromous salmonids that strayed from regions where the disease is enzootic. (3) Contamination may have come from the Owyhee–Snake river system arising in Nevada, a state where *M. cerebralis* has been detected. (4) The disease may have been introduced with processed fish or (5) imported with fish that had been examined but in which the parasite went undetected. Epizootiological studies will continue to define the geographic range of the parasite more accurately.

During the epizootiological survey, we compared the efficiency of two different methods for detecting *M. cerebralis* in juvenile fish. Spores were easier to detect by the enzyme digest method be-

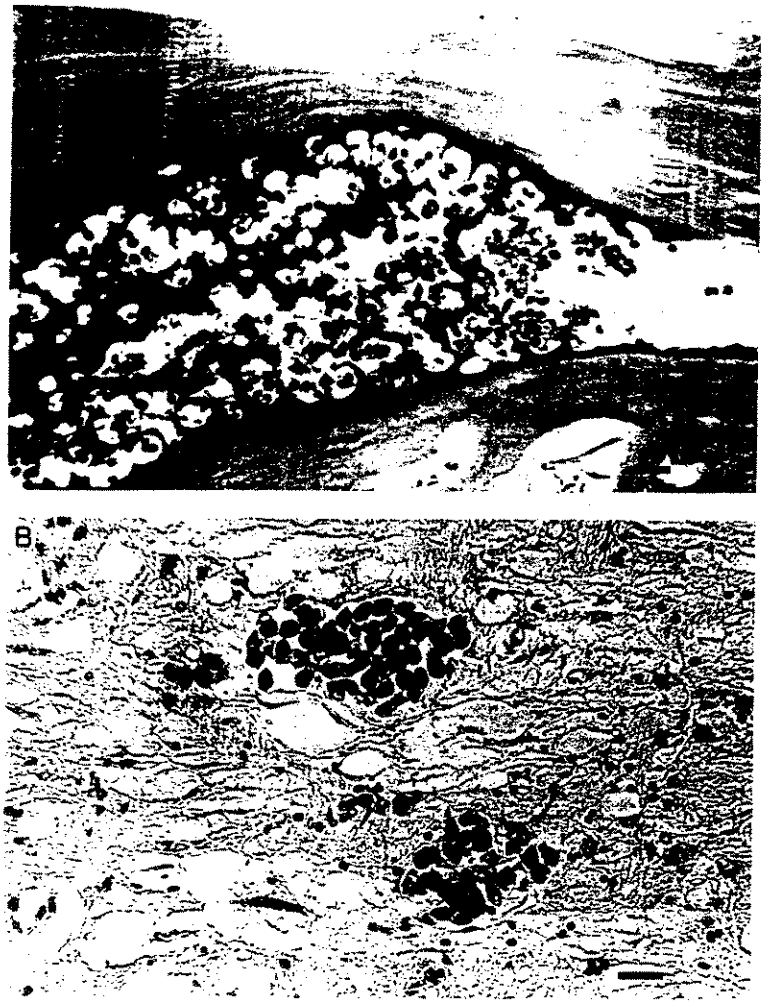

FIGURE 3.—Histological sections of salmonid heads. (A) Cartilage containing spores of *Myxobolus cerebralis*; bar = 36 μm. (B) Brain tissue containing spores of an unidentified species of *Myxobolus*; bar = 24 μm.

digestion and resuspend the pellets. The spores were counted with a hemocytometer.

Comparison of sampling methods.—To compare methods for sampling tissue to be screened for *M. cerebralis*, 40 adult chinook salmon were taken from a parasitized population returning to a northeastern Oregon hatchery. Twenty heads, some weighing as much as 1 kg, were used in each procedure and were processed individually.

The entire head was used in the first method. Heads were heated for 20 min at 121°C, cooled, and defleshed. The bone and cartilage (~18 g) were blended in 20 mL of pepsin and then processed by the enzyme digest method.

In the second method, a subsample was taken from each head with a cork borer 110 mm long and 19 mm in diameter. The borer was inserted into the head, dorsally and perpendicular to the long axis of the body, approximately 10 mm behind the eye and was pushed through the roof of the mouth (Figure 2). The sample contained the semicircular canals and, in the case of smaller fish, the otoliths. After the skin and some musculature were removed, each sample (~8 g) was blended in 10 mL of pepsin and processed by the enzyme digest method.

One milliliter of each sample was centrifuged through 5 mL of dextrose, resuspended in formalin, placed on a slide, and examined microscopically at 250× and 400× magnifications. The

cause it produced a cleaner preparation than centrifugation. Digestion also was the more efficient method when several samples were processed simultaneously. The plankton centrifuge technique is faster when single samples are examined (Markiw and Wolf 1974b), but a time-consuming decontamination of equipment is required between samples. In addition, only the number of samples for which there are plankton centrifuges available (usually one) can be processed at one time.

Although we cored only 20 chinook salmon heads, the resulting data indicate that this subsampling technique may be appropriate for detection of *M. cerebralis* in large fish. Not only could the technique be used in epizootiological studies, it might also be useful for examination of fish for compliance with international trade laws.

Acknowledgments

We acknowledge the assistance of personnel of the Oregon Department of Fish and Wildlife (ODFW) who collected samples. We also acknowledge the cooperation of owners of private trout hatcheries. We thank P. Allison of the College of Veterinary Medicine, Oregon State University, for her help with the histology and C. Pelroy for typing the manuscript. This research was supported by grants from the U.S. Department of Agriculture, Animal Health and Disease Research Funds, and from the ODFW through a contract with the Bonneville Power Administration (agreement DE-A179-87BP 33823) and by funds from the Anadromous Fish Act (Public Law 89-304) under project AFS-78 administered by the U.S. Fish and Wildlife Service. This is Oregon Agricultural Experiment Station technical paper 8524.

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PATHOLOGICAL CHANGES AND DIAGNOSTICS OF MYXOSOMOSIS OF THE RAINBOW TROUT (SALMO GAIRDNERI IRIDEUS)

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Abstract

Lucký Z.: *Pathological Changes and Diagnostics of Myxosomosis of the Rainbow Trout (Salmo gairdneri irideus).* Acta vet. Brno, suppl. 1, 1970: 19—29.

Pathological changes were studied of myxosomosis of the trout fry two months and half a year old. The trouts came from the fish farm in the Moravian Carst (drainage area of the river Punkva). In about two-month-old trout (July 6th) clinical symptoms of myxosomosis were observed and they were proved by histological examination as well. Amoebids with several nuclei were diagnosed in the cartilages of the skull. Towards the end of October (October 24th) further specimens of trouts from the breeding pond were examined. Some trout were found to have a deformed spine. By parasitological examination spores of the species Myxosoma cerebralis were diagnosed. By histological examination numerous foci of myxosporidia were found in the cartilage of the head bones. Vegetative stages were registered as well as sporoblasts and mature spores. Some foci were enclosed into an osseous pallium, others were surrounded by a granulative tissue of mesenchymal cells. The discussion deals with the determination of the age of pathological-anatomical processes and of the parasites. The examinations performed showed that even after 5 months the reproduction of the parasite was probably not finished; however, an expressive tissue proliferation occurs and also encapsulation of the parasites.

Myxosoma cerebralis, myxosomosis, Salmo gairdneri irideus, spores, sporoblasts, amoebids, granulative tissue, mesenchymal cells

A very severe parasitary disease of the fry of the rainbow trout (Salmo gairdneri irideus) and other salmonidae fishes bred in trout farms of the cold-water fish culture is myxosomosis, formerly called lentosporosis. This parasitosis is spread nearly always in those places where the rainbow trouts are bred; these fish are very sensitive to this parasitosis. Hoffman, Dunbar, Bradford (1962) evaluate its geographical distribution in their monograph. Besides the USA (Pennsylvania), where the authors found it in several trout cultures, they record that it has already been observed in Germany, France, Italy, Austria, Poland, the USSR, and also in our country.

In conditions of Czechoslovakia this parasitosis is also rather widely spread, even though its occurrence has not yet been registered in greater detail. The first case of myxosomosis of our salmonidae fish has been described by Volf (1957) in his work. Further data on its distribution can be found in the compendium Volf, Havelka (1958), Dyk (1961) considers the originator to be from the pathogenic point of view the most important member of its class because every year it causes losses of trout in the first year of their age.

The works of Plehn (1924), Schäperclaus (1931, 1954), Hoffman, Dunbar, Bradford (1962) and others deal with the pathology of myxosomosis.

Material and Methods

The trout fry (Salmo gairdneri irideus) came from the breeding farm in the drainage area of the Punkva river in the Moravian Carst. At the beginning of July, about two months after releasing the fish fry (with a loss of two thirds of the yolk sac), pathological symptoms appeared in the fish characterized by inappetence, uncoordinated swimming — sometimes in circles — and an increase of mortality.

8 fry specimens 18—25 mm long were examined by dissection. 4 specimens were elaborated histologically. Whole fishes were cut lengthwise. Specimens for the examinations were taken on July 6th, 1967. Next specimens of trouts were taken on October 24th, 1967 to enable the execution of a supplementary precise diagnosis. 15 specimens of trout fry 70—95 mm in size were examined. Two trouts were elaborated histologically — they were cut with a microtome across from the oral opening to the posterior periphery of the gill cover.

Parasitological examination and dissection were performed according to the instructions given in the compendium Lucký, 1966, special examination as to the presence of spores of Myxosoma cerebralis was performed according to a modified method given in the works of Plehn (1924) and Volf, Havelka (1958). In this examination a transversal incise or cut through the skull was performed behind the eyes, about 5 mm wide. On its lateral peripheries we inserted small eye scissors and cut about 10 mm of the skull in the oral line and we lifted and cut off the bony case. After removing the brain we performed a radical abrasion from the right and left side of the cranial cavity with a sharp eye scalpel. We transferred the osseous and cartilaginous abraded matter obtained onto a slide and crushed it as much as possible with a glass rod. The coarser parts were removed and after adding a drop of water and adjusting a micro-cover slip the material was examined enlargened 450×.

The Work Proper

As the anamnestic data were inaccurate and incomplete only the results of the examinations proper are described.

In the first specimen of trout fry, about 2 months old, no parasitary infestation was observed by parasitological examination. The intestine of the fish contained no food. In native preparations completed from the cartilage of the skull no parasitary formations could be diagnosed microscopically. It is necessary to realize that the whole cranial cavity measured only 1.5 mm in the line of the longitudinal axis and that the thickest wall of the cartilaginous skull was 0.230 mm. On histological cuts stained HE (H according to Ehrlich) we diagnosed parasitary formations on the periphery of the cartilages of the head bones only very sporadically. The osseous pallium covers the cartilages of individual head bones in a very thin layer as a thin folia in fish of this age. The parasitary formations were represented as amoebids of different shapes, 0.016—0.020 × 0.010—0.012 mm in size, with several nuclei (4—8) about 0.001 mm large. Walls of the amoebids were very indistinct and the cartilage in their surroundings was disintegrated only very slightly.

On the basis of histological examination we presumed that it is infestation of the trout fry by parasites whose vegetative stages localize in the cartilage. The final diagnosis of the disease could however be determined only with a certain exception because the vegetative stages of the parasites observed were found in the cartilages of the head bones only sporadically.

In cooperation with the fish breeder (eng. Němec) a further specimen of trout fry was obtained and examined towards the end of October. Some individuals were found to have a deformed spine and a deformed gill cover. Striking pigmentary changes have not been observed because the fry was nearly suffocated due to transport.

By examining in detail the bones and cartilages of the skull myxosporidia of the superfamily Platysporea with no iodinophilous vacuole were found in

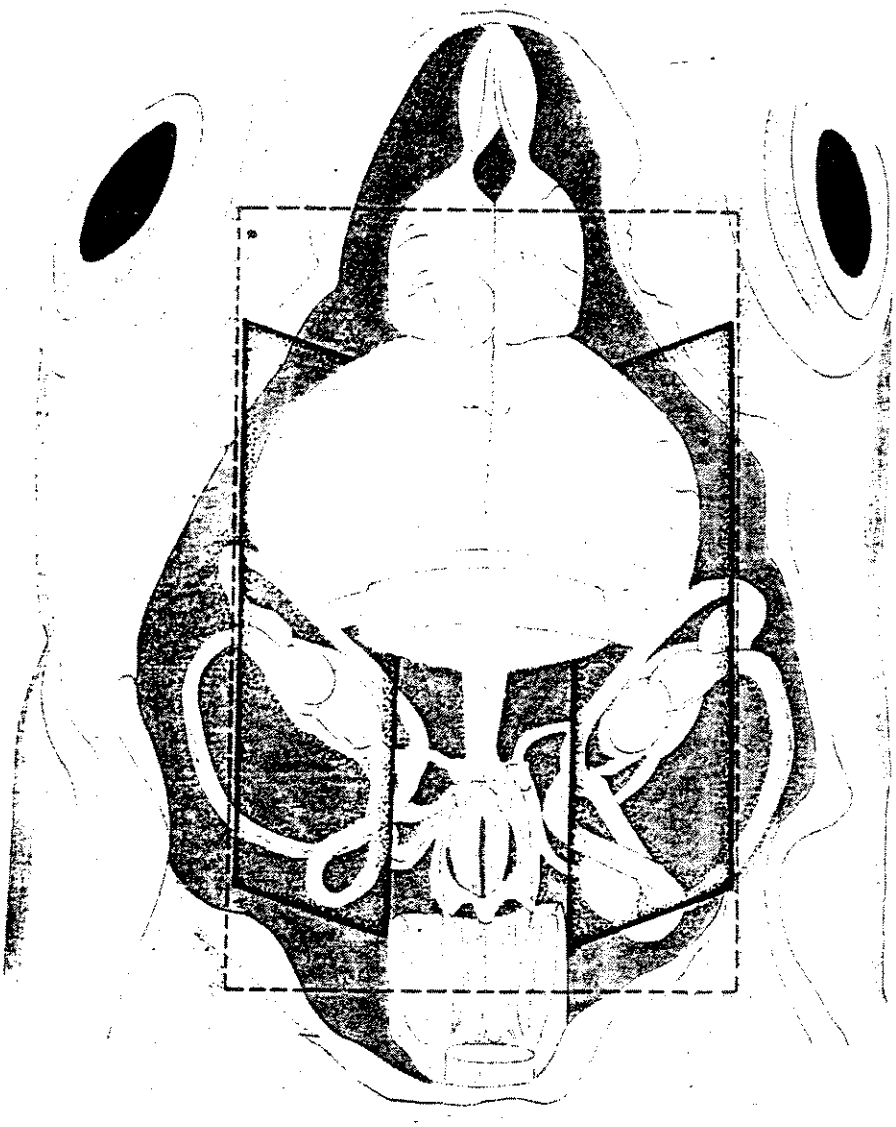

Fig. 1. Modified method of myxosomosis diagnostics: the skull is cut off behind the eyes (dotted line) and after preparation of the brain we perform an abrasion with a scalpel of the bones and cartilages from the walls of the cranial cavity in which the tubules of the labyrinth pass (thick black line) (model of the fish head has been drawn from Grodzinski, Z.: Anatomia i embriologia ryb. Warszawa, 1961.)

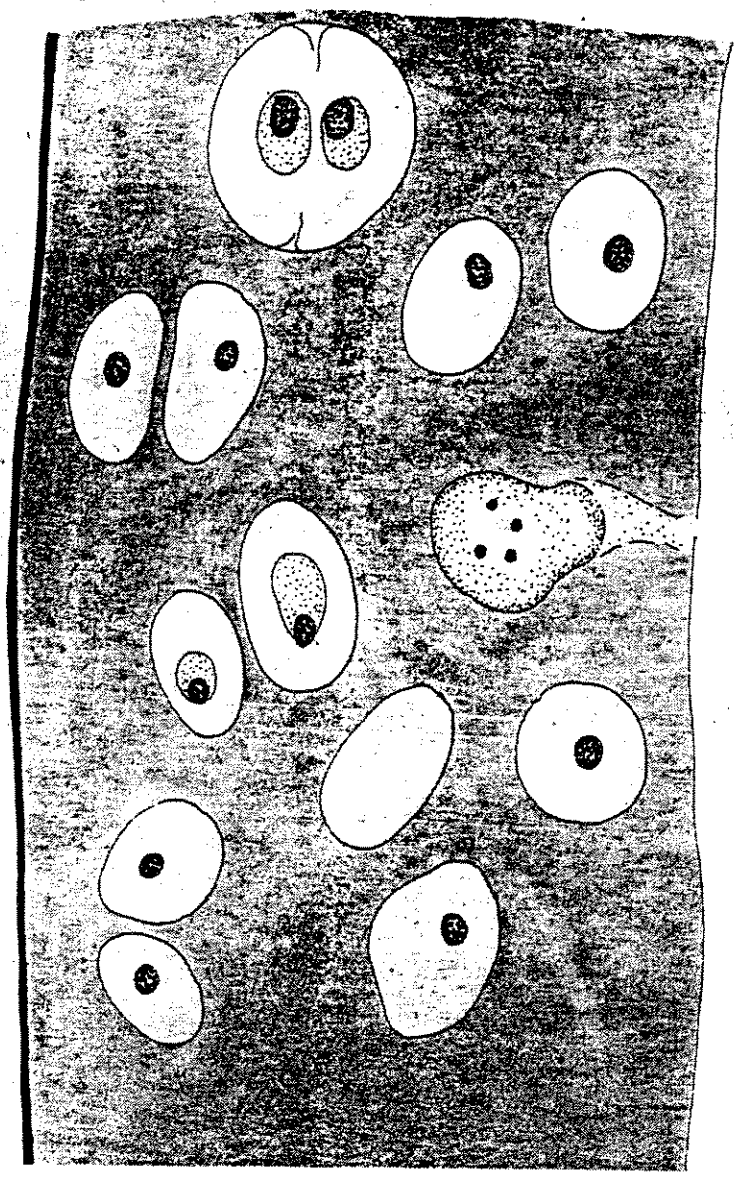

Fig. 2. Cartilaginous cranial bone of a two-month-old trout with an amoebid embryo of the myxosporidia Myxosoma cerebralis with 4 nuclei. On the left side of the cartilage the osseous pallium (in black).

4 trouts (out of the total number of 15), so that their appurtenance to the family Myxosomatidae, genus Myxosoma, was evident. Spores were found to occur only very sporadically in the native preparations, mostly 1—2 spores, when enlargened 450×.

Description of spores of Myxosoma cerebralis according to our own material

The spores are approximately of a circular shape with two expressive polar capsules. In a side position the spores are eliptic. The length of the spores is 0.008—0.010 mm, the width is either concurrent or somewhat smaller. The egg-like polar capsules are about 0.003 mm long and 0.002 mm wide. The difference of the dimensions of the polar capsules did not even reach 0.001 mm. The wall of the spore is thick and between the polar capsules it projects into a distinct beak-like intercapsular projection. After covering the native preparation of the spores with a solution of iodine (Lugol's solution) the bluish macula (iodinophilous vacuole) did not appear in the amoebid embryo.

Description of histological changes with myxosomosis

The knowledge proper on histopathology was obtained by studying some tens of histological cuts from two trouts with a slightly deformed spine, 73 and 84 mm long. They were specimens about 6 months old, naturally invaded about 5 months before. The age of the parasites and of the histopathological processes can therefore be determined only approximately.

In contradistinction to histological examinations of two-month-old trout fry it was possible to find extensive changes in the cartilaginous bones of the skull, of the mandibula, and of other — not accurately determined — head bones.

The parasites were represented by vegetative stages as well as by permanent spores with a thick wall. The cartilage is uneven in the place where it meets the parasite, and it is considerably eroded by vegetative stages of parasites. The ectoplasm of the parasite is very thin, the endoplasm in this period forms a strip about 0.025—0.035 mm wide in which we can find pansporoblasts with a various number of generative nuclei as well as differently developed sporoblasts. Vegetative stages of the parasite can be found only when in direct connection with the cartilage. In places where the parasite adheres to the bone only older sporoblasts can be found, or mature spores.

Foci of parasites in our material differ so that we might judge of a various age of the process or, if the age is the same, of a variously quick development according to the localization. Some foci are enclosed in the osseous pallium of the cartilage and parasites are mostly already changed into spores, only sporadically can vegetative stages be found. In the surrounding of the parasites no granulative tissue of the host was found in such cases.

The second group was formed by foci localized mostly on the periphery of the bones on the dividing line between the cartilaginous model of the bone and the perichondrial ossification of the formed osseous pallium. These foci of parasites, which contain vegetative stages and spores, are surrounded by an enormous mound of granulative tissue, which often surrounds and encloses small zones of cartilage and conglomerates of the osseous pallium. The granulative tissue is represented by mesenchymal cells. Around the foci no ligamentous

capsule was found and neither its forming. Around the foci we found no blood elements and neither blood capillaries. The foci are mostly of an elongated shape and they measure 0.6—0.75 × 0.35—0.39 mm.

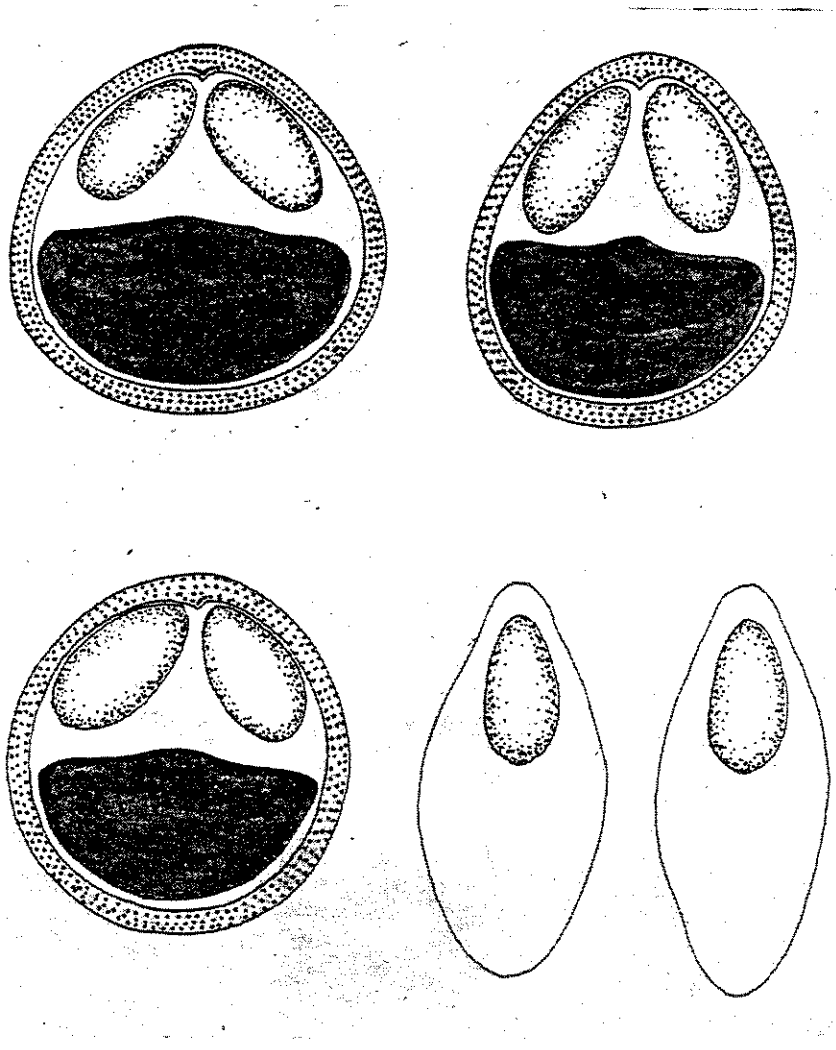

Fig. 3 Myxosoma cerebralis (spores) the originator of the myxosomosis of the trout fry. To the right below two spores from the side.

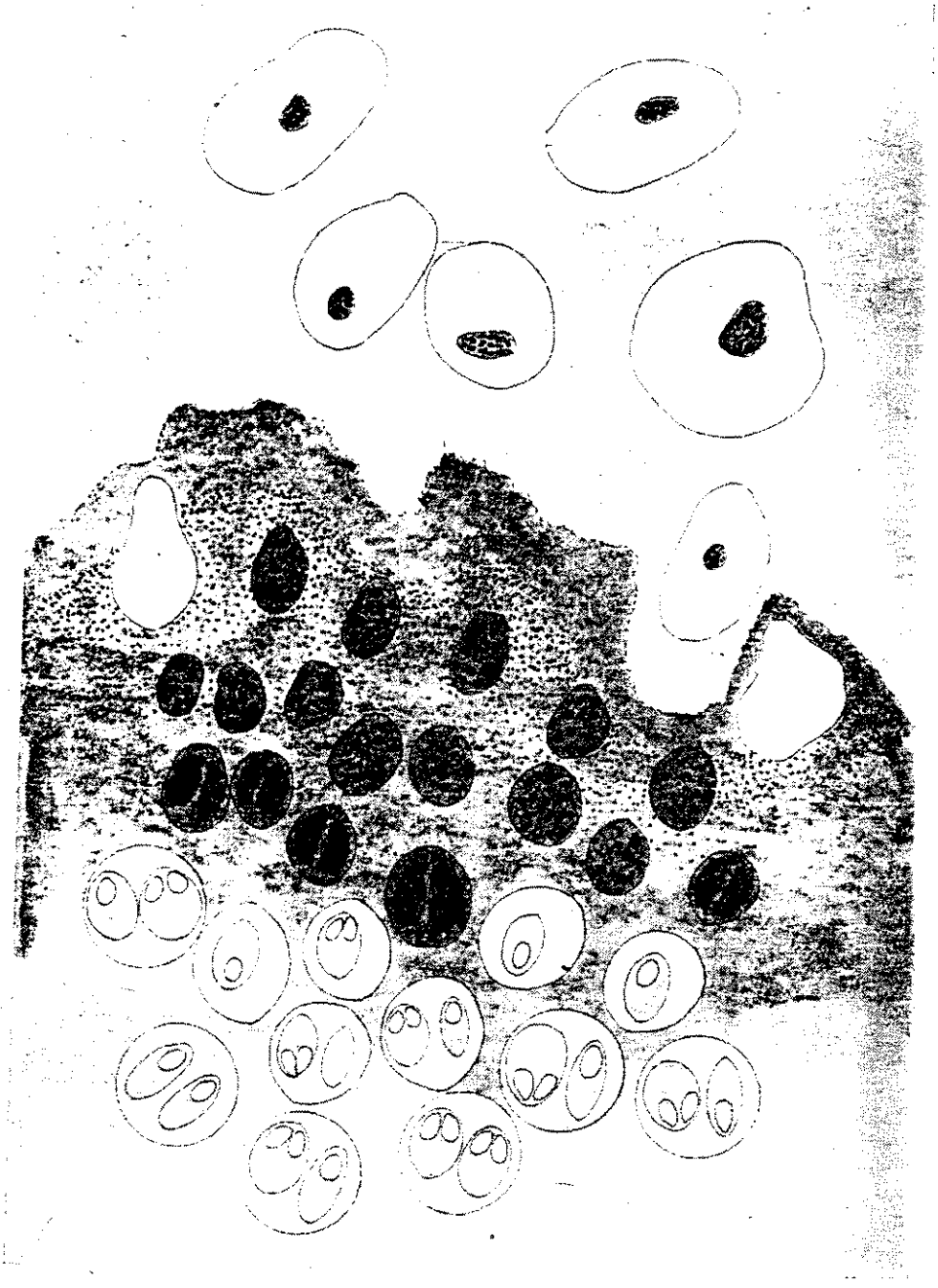

Fig. 4 By its erosive activity the parasite (Myxosoma cerebralis) gnaws variously deep projections in the cartilage. Above — normal cartilage, the darker zone underneath it represents the vegetative part of the parasite, below — sporoblasts with differently mature spores.

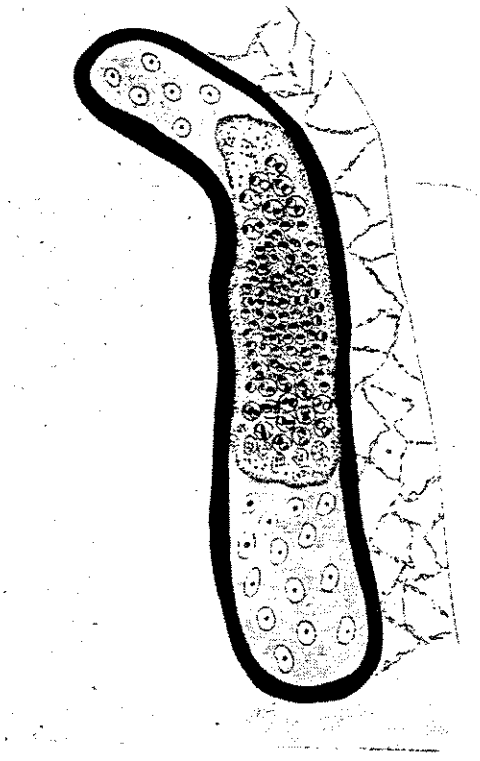

Fig. 5 Focus of the parasite Myxosoma cerebralis in the mandibula of the trout (age 6 months). In the focus mature spores predominate, vegetative stages can be found only in those places where the parasite comes into connection with the cartilage. The focus is enclosed into the osseous pallium of the cartilage.

Discussion

In our study we have elaborated in greater detail the diagnostics and pathology of myxosomosis of salmonidae fishes which had already been the object of study of other authors. Hoffman, Dunbar, Bradford, 1962 evaluated the present investigations and knowledge on judging the age of parasites and of the pathological processes. According to their observations no pathological changes have been found during the incubation period (up to 40 days). Schäperclaus (1954) found disintegration of the cartilage after a 40-day incubation. Schäperclaus (1931) stated the presence of singular pansporoblasts after 65 days of incubation, but no tissue proliferation, Hoffman, Dunbar, Bradford (1962) found neither tissue proliferation nor inflammation even after three months. After four months they found spores. After 11—12 months only spores are present in the foci, epithelioid proliferation takes place which afterwards surrounds the mass of spores.

Trouts examined by us were 2 months old in the first case and they could be naturally invaded at the age of about 14 days. When examining them we found amoebids of parasites very sporadically with no pathological changes but for a slight disintegration of the cartilage, similarly as Schäperclaus

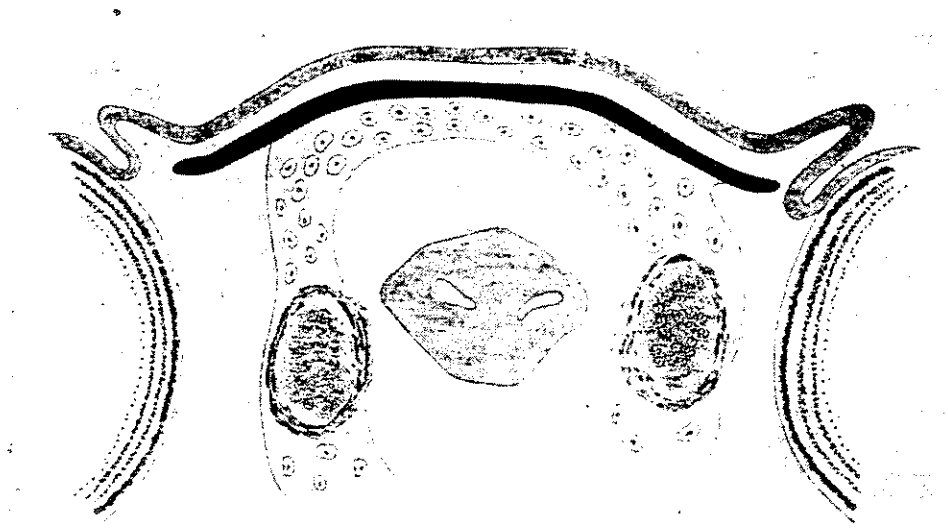

Fig. 6 The skull of the trout fry (age 6 months) with two encapsulated foci of Myxosoma cerebralis. The skull is mostly cartilaginous, only on the dorsal side it is covered by a bone (in black).

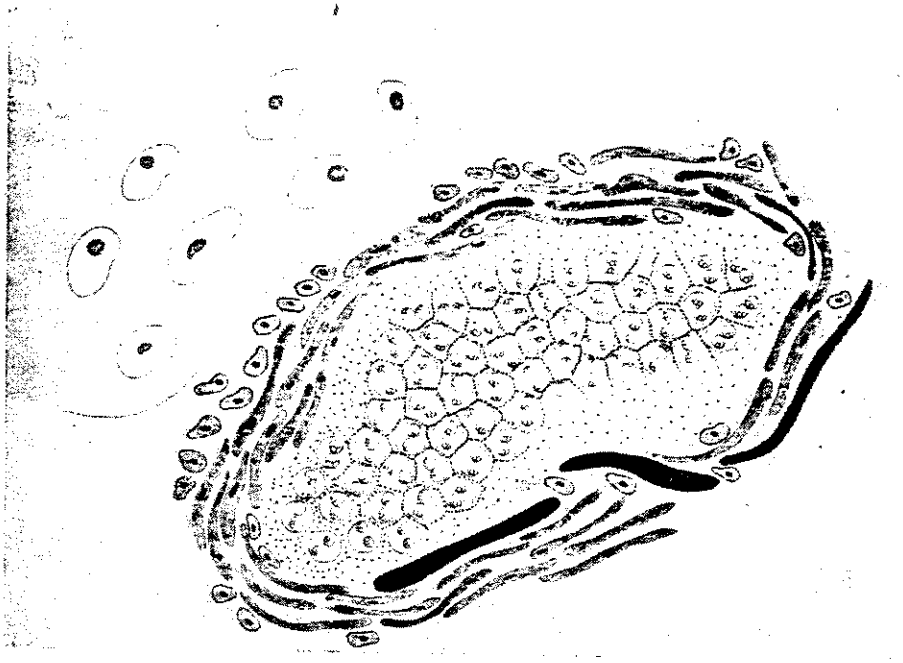

Fig. 7 An old focus of Myxosoma cerebralis encapsulated by a granulative tissue of mesenchymal cells among which we can find conglomerates of the osseous pallium.

(1954). In the second case in trouts 6 months old spores were found to a great extent after more than five months of incubation, similarly also extensive pathological changes were diagnosed.

On the basis of our results we can, therefore, complement the present survey of pathological changes with myxosomosis by stating that reproduction of the parasite is probably not finished even after 5 months. In the stage of the disease, however, an expressive tissue proliferation already takes place as well as the encapsulation of the parasites. It depends however on their localization.

Patologické změny a diagnostika myxosomózy (myxosomosis)

Byly studovány patologické změny myxosomózy u pstružího plůdku ve stáří dvou měsíců a půl roku. Pstruzi pocházeli z odchovny v Moravském krasu (povodí Punkvy). U asi dvouměsíčních pstruhů (6. července) byly zjištěny klinické příznaky myxosomózy, která byla potvrzena i histologickým vyšetřením. Byly diagnostikovány ameboidy s několika jádry v chrupavkách lebky. Koncem října (24. října) byl prošetřen další vzorek pstruhů z odchovného rybníka. Někteří pstruzi měli pokřivenou páteř. Parazitologickým vyšetřením byly diagnostikovány spory druhu Myxosoma cerebralis. Histologickým vyšetřením byla zjištěna početná ložiska rybomorek v chrupavkách hlavových kostí. Byla registrována vegetativní stadie, sporoblasty a zralé spory. Některá ložiska byla uzavřena v kostěném plášti, jiná byla obklopena granulační tkání z mezenchymálních buněk. Diskuse se zabývá určováním stáří patologicko-anatomických procesů a cizopasníků.

Патологические изменения и диагностика миксосомоза (myxosomosis) американских радужных форелей (Salmo gairdneri irideus).

Автор изучал вызванные миксосомозом патологические изменения у 2- и 6-месячных форелевых мальков. Форели были взяты из рыбоводного пруда в Моравском Красе (бассейн реки Пунквы). У примерно 2-месячных форелей (6 июля) были обнаружены клинические признаки миксосомоза, что подтвердилось также гистологическим исследованием. Были обнаружены амебоиды с несколькими ядрами в хрящах черепных костей. К концу октября месяца (24 октября) произвели обследование новых образцов форелей из рыбоводного пруда. У некоторых особей наблюдалось искривление позвоночника. Паразитологическим исследованием были обнаружены споры вида Myxosoma cerebralis. Гистологическим исследованием установлены многочисленные очаги слизистих споровиков в хрящах черепных костей. Регистрировались вегетативные стадии, споровласты и зрелые споры. Некоторые очаги были заключены в костяной капсуле, другие были окружены грануляционной тканью из мезенхимных клеток. Дискутируется проблема определения продолжительности патолого-анатомических процессов и возраста паразитов.

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THIN-SECTION ELECTRON MICROSCOPY OF MATURE *MYXOSOMA CEREBRALIS* (MYXOSPORIDEA) SPORES

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ABSTRACT: The morphology of purified, mature *Myxosoma cerebralis* spores was examined by thin-section electron microscopy. Surface architecture corresponded closely to that described in an earlier scanning electron microscopy study of this organism (Lom and Hoffman, 1971). A uniformly thick spore wall is composed of finely granular, electron-lucent material. Intrawall "valvoplasm" at the sutural border region contains small, light spheroidal profiles with dense centers situated in a background matrix; such structures are absent in the intermediate wall areas. Ovoid polar capsules contain complex filamentous profiles within the peripheral matrix region. Individual filaments appear twisted into S-shaped configurations and contain moderately electron-opaque material within their lumina.

Myxosoma cerebralis, a histozoic myxosporidian of salmonid fishes, is the etiologic agent of whirling disease. Unlike most myxosporidian infections, whirling disease is a serious problem in fish husbandry. Both the parasite and the disease have been comprehensively reviewed (Hoffman et al., 1962, revised 1969). Certain diagnosis of whirling disease is based upon identification of spores—in histologic section or more commonly in wet mounts of infected cartilage. The relative efficiencies of various physical and biophysical methods of spore detection have been determined (Markiw and Wolf, 1974).

There are relatively few studies of myxosporidians at the fine-structure level. Schubert (1968) described by thin-section electron microscopy the histozoic *Henneguya pinnae*, and included a brief review of the fine structure of myxosporidians to date. Relevant to our work, Grassé (1960) reported on a histozoic species, *Sphaeromyxa sabrazesi*. Lom (1964) described extruded polar filaments of *Myxobolus muelleri*. Later, Lom and Puytorac (1965a, b) studied *Henneguya psorospermica*, *Myxobolus* sp., *Zschokkela nova*, *Chloromyxum cristatum*, and *Myxidium lieberkühni*. Lom and Hoffman (1971) reported the scanning electron microscopy of two species of *Myxosoma*. That work compared *M. cartilaginis* of a centrarchid fish with *M. cerebralis* and found the latter to be smaller and more variable in size. More specifically, the salmonid myxosporidian possessed a distinctive circumsutural groove, a mucous envelope, and prominent polar filament pores.

The purpose of our report is to describe the fine structure of *M. cerebralis* as seen by transmission electron microscopy.

MATERIALS AND METHODS

Spores were obtained from fingerling rainbow trout (*Salmo gairdneri*) which when 5 months old had been experimentally exposed to whirling disease for 6 days with the method of Hoffman and Putz (1971), and kept for 5.5 months in 12.5 C spring water, the physical and chemical properties of which were reported by Warren (1963). Infected skeletal elements were manually stripped of soft tissues, gently crushed in a mortar with water, the resulting slurry filtered through 125-μm screen, sedimented at 1,200 *g* at 4 C for 10 min, resuspended and washed once with water, resuspended again, and the debris allowed to settle for 10 to 15 min. The supernatant fluid was centrifuged at 350 *g* for 10 min at 4 C, and the resulting pellet and fluid treated with 1,000 μg gentamicin per ml overnight to minimize bacterial contamination. Liquid and pelleted materials were purified through 65% dextrose solution at 1,250 *g*.

Pellets of *Myxosoma cerebralis* were fixed for 1 hr in 2% glutaraldehyde in 0.1 M cacodylate buffer, rinsed several times in the same buffer, and postfixed for 1 hr in 1% OsO_4. After several additional buffer rinses, an aqueous solution of 0.5% uranyl acetate was added. Pellets were then dehydrated in graded concentrations of alcohol and embedded in Epon "812" and sedimented in Beem capsules by centrifugation at 8,161 *g* for 30 min.

OBSERVATIONS

Figure 1 is a slightly oblique transverse section through the anterior region of a *M. cerebralis* spore illustrating its major structural

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FIGURE 1. Transverse section through the anterior polar capsule region of a *Myxosoma cerebralis* spore, showing the general morphology of shell valves, intravalve material (valvoplasm) sporoplasm, nuclei (N), and both polar capsules. × 16,700. Inset: Enlargement of sutural border area (outline of Fig. 1) showing spheroidal profiles in valvoplasm and intervalve material. × 36,250.

features, including the broadly lenticular morphology of both shell valves, two faintly resolvable, compressed nuclei (left) surrounded by sporoplasm, and two dense, ovoid polar capsules containing filamentous profiles.

Shell valves, constituting the spore wall, each display a pronounced protrusion at their lateral borders (Fig. 1). A narrow (17 nm) space containing a small amount of electron-lucent, cross-striated material separates the valves from each other in this region (Fig. 1, inset).

Progressing medially, each valve displays a short, flattened region soon followed by another well-defined, but less pronounced, protrusion. Hence, a lateral furrow exists between protrusions and is easily visualized as such in all transverse sectional images. While this protrusion pattern is similar for both valves, the size and shape of protrusions vary somewhat from section to section, and even from valve to valve, thus rendering a slightly asymmetrical profile to the spore.

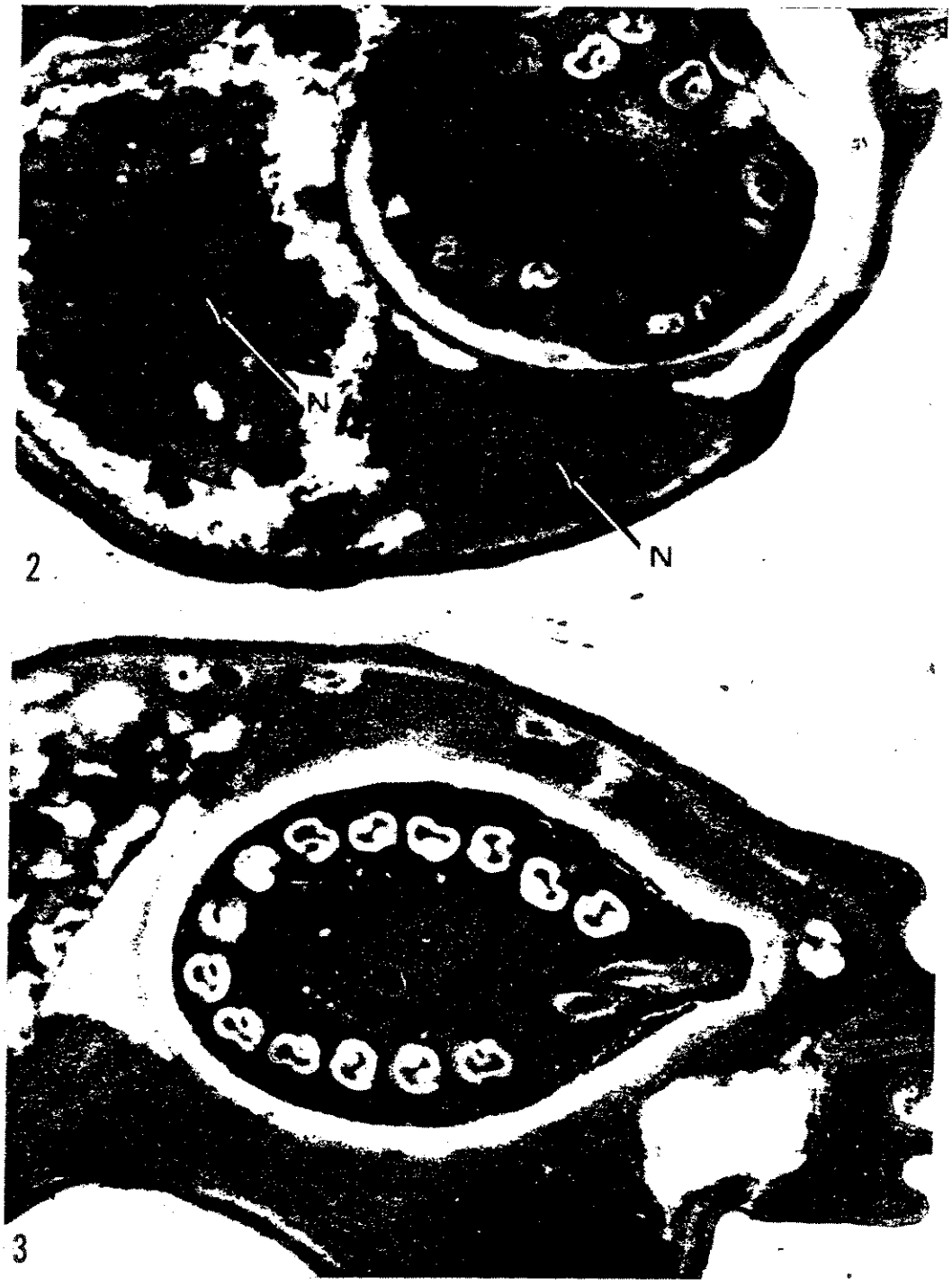
The remaining central region of the shell valves lacks protrusions, but shows a medial indented zone thought to represent a dorsoventral (upper–lower) groove running in an anteroposterior direction possibly corresponding to figure 6 of Lom and Hoffman (1971).

The outer surface of the valve (spore wall) is smooth (Fig. 1). Mucus strands were not observed even at the posterior regions of the spore (unpublished observations). Each surface consists of a finely granular, moderately electron-dense layer of uniform thickness (52 nm) throughout the entire structure. Beneath this surface within the sutural border region is a moderate amount of "valvoplasm" consisting of small (15 nm) spheroidal electron-lucent profiles with dense centers situated in a background matrix (Fig. 1, inset). Medially, the valvoplasm appears to consist of a narrow strip of granular matrix. Spheroidal profiles are absent in this intermediate region.

Nuclei of *M. cerebralis* are located lateral to or posterior to the polar capsules, and are either elongated if topographically close to a capsule, or ovoid if situated farther posterior (Fig. 2). Although the limiting membrane is resolvable, it is not well defined. Regardless of nuclear shape, the nucleoplasm exhibits a dense, irregular marginal region presumed to represent chromatin and a less-dense central zone of flocculent material. An accumulation of dense granules in this latter region (Fig. 2, left) may represent a nucleolar remnant.

Sporoplasm, located beneath the shell valves (spore wall), fills the spore centrum, and thus surrounds the two polar capsules and nuclei (Fig. 2). With optimal fixation it appears to consist of finely flocculent dense material (Fig. 3).

Spheroid profiles characteristically present in the valvoplasm were not observed in the sporoplasm. Other cytoplasmic organelles,



FIGURES 2, 3. *Myxosoma cerebralis*. **2.** Tangential, anteroposterior image of spore, illustrating a portion of one polar capsule and the morphology of the two nuclei (N). Granular material in the central portion of the left-hand nucleus may represent a remnant of the nucleolus. ×29,000. **3.** Longitudinal profile of polar capsule. Coiled filaments are seen, primarily in transverse section, at the peripheral region of the dense, capsular matrix. × 36,250.

such as mitochondria and Golgi bodies, typically found in developmental stages, were not observed in mature spores.

Ovoid polar capsules are anatomically complex organelles consisting of a peripheral sheath which surrounds an extremely electron-opaque, homogeneous matrix in which varying numbers of less-dense filamentous profiles are peripherally situated (Figs. 1–3). Capsules, including their surrounding sheaths, vary considerably in size and shape; the maximum total diameter of this organelle was 2.9 μm. The capsular sheath, clearly visualized in Figure 3 as a uniform electron-lucent region surrounding the matrix, is approximately 0.2 μm in width. Peripheral to the sheath proper is an irregular layer of moderately electron-dense material presumed to represent adhering sporoplasm (Fig. 2); in Figure 3, it can be seen that there is no structural distinction between material surrounding most of the capsule and the adjacent sporoplasm proper.

The morphology of capsular filaments is illustrated in longitudinal (Fig. 1) and semi-transverse (Figs. 1, 2) sections. Figure 3 shows 14 clearly transverse, and one or possibly two semilongitudinal, profiles of these complex structures in the outer region of the matrix. The interfilament space, consisting of matrix, ranges from 34 to 69 nm in width, confirming the regular coil or spiral of the structure. Individual filaments measure 276 nm in diameter in transverse section, and appear to be in a folded or twisted state often resulting in C- and S-shaped configurations. Filament convolution is further suggested by the presence of small, characteristically dense, capsular matrix holes within the filament profile. From these observations, as well as visualization of semilongitudinal images (Fig. 1), it can reasonably be concluded that the filament is a compound, flattened tube containing internal material within its lumen.

Structurally each filament is surrounded by a narrow (17 nm) layer of fine fibrillar material projecting into the surrounding matrix. Proximal to this layer is a thin (5 nm) electron-opaque region, followed by another less-dense area of variable width. The central or luminal portion of the filament consists of a moderately dense "core" zone.

DISCUSSION

From the foregoing observations it is evident that the basic architecture of mature *M. cerebralis* spores as determined by thin-section transmission electron microscopy corresponds closely to that of surface images obtained with the scanning electron microscope (Lom and Hoffman, 1971). These correlations are found to be the general morphology of the spore wall including various protrusions at the lateral surface, the circumsutural furrow, and the dorsoventral groove running in an anteroposterior plane.

The only major difference noted between the two studies was that in the TEM examination, the fine mucus strands were not observed on any region of the spore surface. Although the reason for this is not clear at the present time the absence of such strands may be related to differences in preparative techniques utilized. The spore surface of *M. cerebralis* is smooth in contrast to that of *H. pinnae*, which has been described as corrugated (Schubert, 1968).

In the current study we were unable to visualize the canal of the pore filament or the opening to the exterior. Such an image would require an extremely fortuitous section, particularly in a longitudinal axis.

Of particular anatomical interest was the presence of minute spheroidal profiles within the valvoplasm. While the chemical nature and functional significance of these structures is not currently understood, it is reasonable to hypothesize that they may be precursor material related to wall synthesis; their density is identical to that of the wall proper and they are restricted to the valvoplasm *per se*.

Polar capsules of *M. cerebralis* are structurally complex organelles composed of an outer sheath surrounding a homogeneously dense matrix. A single spirally twisted filament is situated in the peripheral matrix region. The sheath probably provides a protective and supportive function for the capsule; whether it also plays a role in filament extrusion is still problematical. Its density and texture closely resemble that of the chitinous perisarc surrounding certain marine hydroids (Lunger, 1963), thus rendering a contractile function improbable.

The polar capsule filament basically re-

sembles that described in intermediate developmental stages of *H. pinnae* spores (Schubert, 1968). Filaments of both organisms are situated in a coiled state at the matrix periphery, and consist of a surface layer of finely fibrillar material surrounding a more dense central zone. However, it should be noted that in this latter investigation difficulty was encountered in adequate fixation of polar capsules of mature spores; filaments in this stage were only faintly resolvable as dense outlines in a slightly less-dense matrix. Hence, a significant structural comparison of filaments between *M. cerebralis* and *H. pinnae* is not yet possible. In any event, the filament of *M. cerebralis* is visualized as a flattened tube or ribbon containing moderately dense luminal material.

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A Simplified Procedure for Detecting *Myxosoma Cerebralis* (Whirling Disease) Spores in Large Lots of Fish

With the advent of new rules and regulations for inspecting large numbers of fish to be transported to other States, it has become necessary to go through the tedious and time consuming process of examining numerous fish for *Myxosoma cerebralis.* The procedure described here is not really a new one but is a combination of several procedures. Picking out the easier, quicker steps and using them in sequence results in the following:

1. Pool the lots of fish into five fish per pool.
2. Excise the heads of the fish from one pool, and place them in a beaker of water at 40° to 45° C. for 2 to 3 minutes so that the tissue will separate easily from the bone and cartilage.
3. After the tissue is removed, cut out any cartilage around the brain case and the otolith region and place it in a mortar; add an equal volume of 10 percent formalin, and grind with a pestle.
4. Pour the ground material into a beaker and allow it to settle.
5. From the bottom of the beaker draw 5 to 10 drops of the material, spread it over most of a glass slide, and allow it to air dry.
6. Stain for 5 minutes with 1-percent aqueous malachite green.
7. Rinse with tap water.
8. Destain with 70-, 95-, and 100-percent alcohol for 30 seconds at each concentration.
9. Air dry; cover slide with 1 to 2 drops of low viscosity immersion oil, gently spreading the oil with the shaft of the dropper entirely over the stained film.
10. Scan the entire slide using a microscope equipped with 20× objective and 10× ocular.

In this procedure the spore is easily visible as a green oval with two darker green polar capsules against a nearly colorless background. At a magnification of 200×, there is not as great a danger of missing the spores, yet the magnification is low enough that a greater area is covered at each pass on the slide.

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Absence of the Protozoan *Myxosoma cerebralis* (Myxozoa: Myxosporea), the Cause of Whirling Disease, in a Survey of Salmonids from British Columbia

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MARGOLIS, L., T. E. MCDONALD, AND G. E. HOSKINS. 1981. Absence of the protozoan *Myxosoma cerebralis* (Myxozoa: Myxosporea), the cause of whirling disease, in a survey of salmonids from British Columbia. Can. J. Fish. Aquat. Sci. 38: 996–998.

Myxosoma cerebralis (Hofer, 1903) was not observed in 973 wild and 4496 cultured salmonids examined from collections made in widely separated localities in British Columbia from 1968 to 1980. Species investigated were *Salmo clarki* (180), *S. gairdneri* (3688), *S. trutta* (16), *Salvelinus fontinalis* (185), *S. malma* (300), *Oncorhynchus gorbuscha* (109), *O. keta* (17), *O. kisutch* (785), *O. nerka* (126), and *O. tshawytscha* (63). Previous reports notwithstanding, in the absence of corroborative evidence British Columbia (Canada) should not be accepted as a center of *M. cerebralis* infection.

Key words: *Myxosoma cerebralis*, salmonids, British Columbia, whirling disease

MARGOLIS, L., T. E. MCDONALD, AND G. E. HOSKINS. 1981. Absence of the protozoan *Myxosoma cerebralis* (Myxozoa: Myxosporea), the cause of whirling disease, in a survey of salmonids from British Columbia. Can. J. Fish. Aquat. Sci. 38: 996–998.

L'examen de 973 salmonidés sauvages et de 4496 salmonidés d'élevage dans des collections faites à des endroits très éloignés les uns des autres en Colombie-Britannique, de

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1968 à 1980, n'ont pas révélé la présence de *Myxosoma cerebralis* (Hofner, 1903). Les espèces étudiées étaient les suivantes : *Salmo clarki* (180), *S. gardneri* (3688), *S. trutta* (16), *Salvelinus fontinalis* (185), *S. malma* (300), *Oncorhynchus gorbuscha* (109), *O. keta* (17), *O. kisutch* (785), *O. nerka* (126) et *O. tshawytscha* (63). Nonobstant des rapports antérieurs, la Colombie-Britannique (Canada), ne devrait pas, en l'absence de preuves à l'appui, être acceptée comme centre d'infection par *M. cerebralis*.

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Myxosoma cerebralis (Hofer, 1903) Kudo, 1933 is widely distributed in Europe and the USSR and in recent years has been disseminated from central Europe to other parts of the world, including the USA (Hoffman 1970; Bogdanova 1973; Christensen 1973; Bauer 1975; Halliday 1976). The introduction and establishment of *M. cerebralis* in new geographic areas is a matter of considerable concern to fish culturists and fisheries resource managers because it is the cause of whirling disease, a serious ailment of cultured salmonids, particularly rainbow trout (*Salmo gairdneri*). In recent years, fish health protection regulations that include measures to prevent the introduction and spread of *M. cerebralis* have been established in a number of countries, including Canada.

Clinical whirling disease has not been observed in British Columbia (or elsewhere in Canada), but Bogdanova (1969, 1970a, b) claimed to have found low numbers of spores of *M. cerebralis* in wild Dolly Varden charr,[1] *Salvelinus malma*, from the Bella Coola River and its tributaries (central coast of British Columbia), and in pink salmon, *Oncorhynchus gorbuscha*, which she stated came from "southwestern Canada" (Bogdanova 1970b) or "western Canada" (Bogdanova 1977). Her pink salmon samples were actually collected in the southeastern Gulf of Alaska (Bogdanova 1970a, 1977), and the exact North American freshwater areas of origin of these fish are unknown. Based on the reports by Bogdanova, a number of review articles include Canada among the countries in which *M. cerebralis* occurs (Hoffman 1970; Lom and Hoffman 1970; Christensen 1973; Bogdanova 1973, 1974; Cordero del Campillo et al. 1975; Bauer 1975).

Following the appearance of Bogdanova's publications on the occurrence of *M. cerebralis* in British Columbia we undertook to confirm her results by examining wild Dolly Varden charr and other salmonids from the Bella Coola and other river systems in British Columbia. As noted by Halliday (1976), by 1972 we had examined over 500 wild salmonids, including 270 Dolly Varden charr from the Bella Coola River system, without detecting *M. cerebralis*. During the past 8 years we examined several thousand additional wild and cultured salmonids, belonging to 10 species of three genera, from widely separated localities in British Columbia (Table 1). Fingerlings to postspawned adults were included in the samples. Data on each sample examined and the method of its examination are provided in Margolis et al. (1981). From the Bella Coola River system a total of 646 fish of five species (95 *S. clarki*, 10 *S. gairdneri*, 296 *S. malma*, 145 *O. kisutch*, and 100 *O. gorbuscha*) has been examined.

TABLE. 1. Species and numbers of Salmonidae from British Columbia examined for *Myxosoma cerebralis*, 1968–1980.

Species	No. examined	
	Wild	Cultured
Salmo clarki	180	—
Salmo gairdneri	48	3640
Salmo trutta	16	—
Salvelinus fontinalis	30	155
Salvelinus malma	300	—
Oncorhynchus gorbuscha	109	—
Oncorhynchus keta	10	7
Oncorhynchus kisutch	175	610
Oncorhynchus nerka	105[a]	21
Oncorhynchus tshawytscha	—	63
Totals	973	4496

[a]Includes 50 smolts from the Klukshu River, Yukon Territory, north of the British Columbia boundary.

Samples were processed mainly by spore-concentrating techniques, although initially some were examined by more direct methods involving crushing skulls in a mortar (Hoffman et al. 1968) or between two large glass slides. In the earlier years the spore-concentrating technique described by Gillespie et al. (1974) was used, whereas in later years the digestion method of Markiw and Wolf (1974a, b) as detailed in Anon. (1977) was applied. Wild fish generally were examined individually and cultured fish were pooled in groups of 3 to 10. In all cases spores of *M. cerebralis* were not found.

A gross abstracting error in Protozoological Abstracts added to the confusion about the occurrence of *M. cerebralis* in Canada. The abstract (Anon. 1978) attributed to Christensen and Elleman (1976) a report of *M. cerebralis* in Pacific salmon from Canada. These authors refer to *M. cerebralis* as the cause of whirling disease in rainbow trout in Danish trout farms and make no comment about this organism in Pacific salmon. The error, clearly due to misunderstanding of the Danish text, was later corrected (Anon. 1979).

Earlier reports notwithstanding, the results of our extensive examinations of salmonids from British Columbia strongly indicate that *M. cerebralis* does not occur in this province. Since there are no records from other provinces, Canada should not be accepted as a focus of *M. cerebralis* infection without corroboration.

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[1]The spelling differs from that (char) used in *A List of Common and Scientific Names of Fishes from the United States and Canada*, by the American Fisheries Society, and is preferred by the authors.

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Salmonid Whirling Disease: Dynamics of Experimental Production of the Infective Stage — the Triactinomyxon Spore

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Markiw, M. E. 1986. Salmonid whirling disease: dynamics of experimental production of the infective stage — the triactinomyxon spore. Can. J. Fish. Aquat. Sci. 43: 521–526.

In two trials, spores of the myxosporean *Myxosoma cerebralis* were added to aquaria containing specific-parasite-free tubificid oligochaetes held at 12.5°C. After 104–113 d, the tubificids gave rise to and released actinosporean spores of triactinomyxon form, the alternate life stage of *M. cerebralis* and the initiator of salmonid whirling disease. Production of triactinomyxon spores peaked during days 154–190, and then declined almost as abruptly as it had risen. The triactinomyxon spores were released at trace levels for 9 mo after they first appeared. In a second trial, 26–50 million triactinomyxon spores were produced. The approximate yield from each tubificid was 730–3420 spores. The prevalence of experimentally infected tubificids was about 20%.

Au cours de deux essais, des spores de la myzosporidie *Myxosoma cerebralis* ont été ajoutées à des aquariums contenant des oligochètes du groupe des tubificidés exempts de parasites particuliers et gardés à une température de 12,5°C. Après une période de 104 à 113 jours, les tubificidés ont libéré des spores d'actinosporidies de la forme triactinomyxon, l'autre stade de développement de *M. cerebralis*, qui déclenche le tournis chez les salmonidés. La production de spores de type triactinomyxon a atteint un sommet entre les jours 154 et 190, puis a baissé presque aussi brusquement qu'elle avait augmenté. Les spores de type triactinomyxon ont été libérées en infimes quantités pendant neuf mois après leur première apparition. Lors d'un deuxième essai, de 26 à 50 millions de spores de type triactinomyxon ont été produites. La production approximative pour chaque tubificidé a varié de 730 à 3 420 spores. La proportion de tubificidés contaminés expérimentalement a été d'environ 20 %.

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Whirling disease is a protozoan parasite infection that localizes in cartilage of salmonid fishes, notably young rainbow trout (*Salmo gairdneri*) and brook trout (*Salvelinus fontinalis*). The disease is identified by finding the 8- to 10-μm lenticular spores of *Myxosoma cerebralis* in and about pathologic cartilage tissue.

Halliday (1976) reviewed the literature on the biology and other aspects of *M. cerebralis* and, like earlier authors, noted that the life cycle of the parasite was conjectural and only incompletely known. For most of its approximately 80-yr history, the cause or initiator of whirling disease was widely thought to be the 8- to 10-μm spores of *M. cerebralis*; however, such infectivity or causal role has not been critically demonstrated. Instead, Markiw and Wolf (1983) found that development of infectivity for fish was an exogenous process in which a tubificid oligochaete played an essential role. The long-sought infectivity of whirling disease and discovery of the complete life cycle were described and illustrated by Wolf and Markiw (1984).

In brief, the life cycle of the whirling disease parasite consists of two dissimilar stages, both of which are termed spores. One stage, the 8- to 10-μm lenticular *M. cerebralis* spores found in trout, is infectious only for the alternate host, a tubificid oligochate. Spores are freed and reach tubificids when infected fish die or are eaten by predators. Tubificids ingest the small spores, and the parasite increases in number and size and undergoes marked morphologic change. The result is numerous long three-parted spores, the triactinomyxon stage having grapple-like extended appendages 170–180 μm long. The large triactinomyxon spores are infective only for fish in which they initiate the condition known as whirling disease. Fish become infected when they feed on infected worms or encounter the waterborne triactinomyxon spore stage released by worms.

The objective of the present study was to determine at a constant temperature of 12.5°C the dynamics of development and production of the triactinomyxon spores produced by the tubificid. Additional information was critical for understanding the basic biology involved in the interrelation between *M. cerebralis* spores, tubificid oligochaetes, the triactinomyxon stage, and salmonid whirling disease.

Systematics

When one wishes to discuss the newly discovered biphasic life cycle of the whirling disease organism, certain conflicts in terminology become apparent. For one, the large three-parted spore produced in the tubificid is, by its shape and host, a member of the genus *Triactinomyxon*. That genus has a longer history than *M. cerebralis*, but triactinomyxons are known as obscure and little-studied parasites of certain annelids (Stolc 1899; Mackinnon and Adam 1924; Janiszewska 1955, 1957). The relationship of any *Triactinomyxon* to any kind of fish disease was not to be found in the literature; accordingly, Wolf

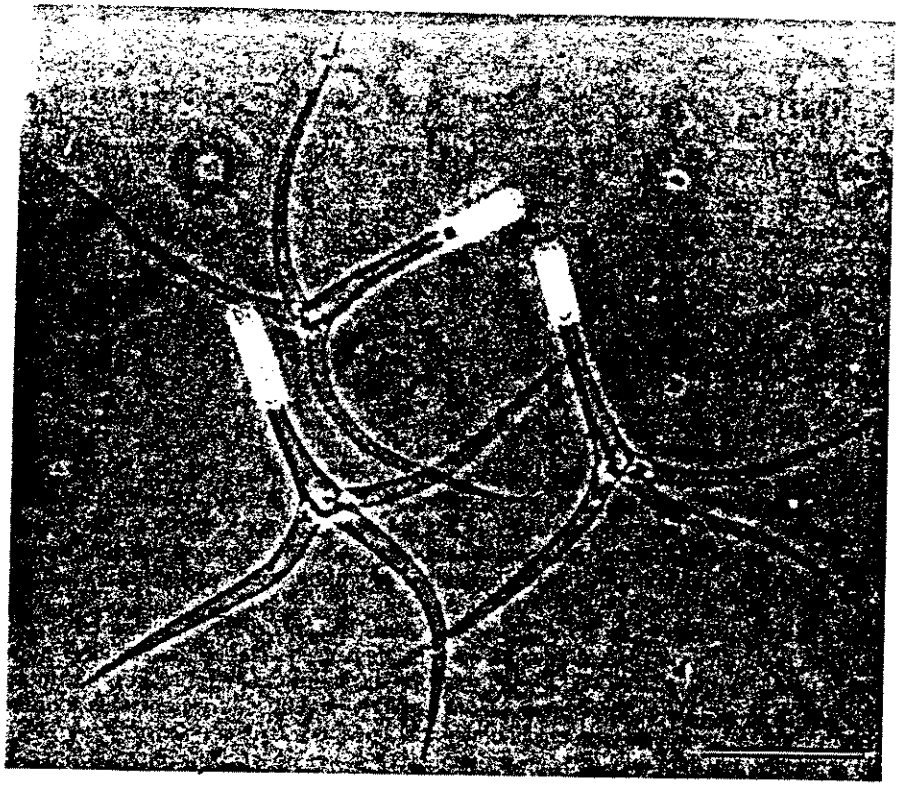

FIG. 1. Living unstained mature waterborne triactinomyxon stage produced in tubificid. Phase contrast. Bar = 50 μm.

TABLE 1. Experimental production of triactinomyxon spore stage.

Seasonal periods[a]			Triactinomyxons		
Number of *M. cerebralis* spores per worm	Date of exposure to *M. cerebralis*	First appearance of waterborne triactinomyxons (d)	Number produced ($\times 10^6$)	Mean per worm	Maximum release (no./min)
140	12 Nov. 1982	104	43.8	730	2430
380	29 June 1983	113	50.3	3420	2090
38	29 June 1983	110	26.3	1790	1190

[a]Worms were transferred from natural to experimental environment on the following dates (trial number in parentheses): 4 October 1982 (1); 3 June 1983 (2A, 2B).

and Markiw (1984) proposed the name *Triactinomyxon gyrosalmo*. However, it was later called to their attention that the name was "illegal" because, as the scientific name of an organism, *Myxosoma cerebralis* had priority and the triactinomyxon was simply a stage in the life cycle of *M. cerebralis*.

A second and larger problem becomes apparent when trying to reconcile the biology of whirling disease with the classification of Protozoa. According to Levine et al. (1980), *M. cerebralis* belongs in the phylum Myxozoa, Class I *Myxosporea*. Members of the genus *Triactinomyxon* are also myxozoans but are placed in Class II *Actinosporea*. Our thesis is that in the case of the whirling disease organism, only one parasite is involved; it alternates between two hosts and occurs in triactinomyxon form and as *M. cerebralis*.

For ease in understanding, the stage produced in the tubificid will be referred to as the triactinomyxon stage or phase.

Materials and Methods

Preparation of Soil and Tubificids

Samples of aquatic soil and tubificids were collected from a state trout hatchery (Ridge, West Virginia) that has always been free of whirling disease. As was verified by examination, fauna in a soil slurry was killed by heating at 70°C for 1 h. The slurry was then cooled overnight, and then washed with several changes of pathogen-free spring water to remove possible heat-induced toxicity. Particulate matter was allowed to settle after each washing. So prepared, the axenic soil was distributed to a depth of about 8–10 cm in 9-L glass jars, 19 cm in diameter, with an outlet at the 6-L level. Four experimental jars were prepared and kept at 12.5°C.

The collected tubificids, a mixed population consisting of

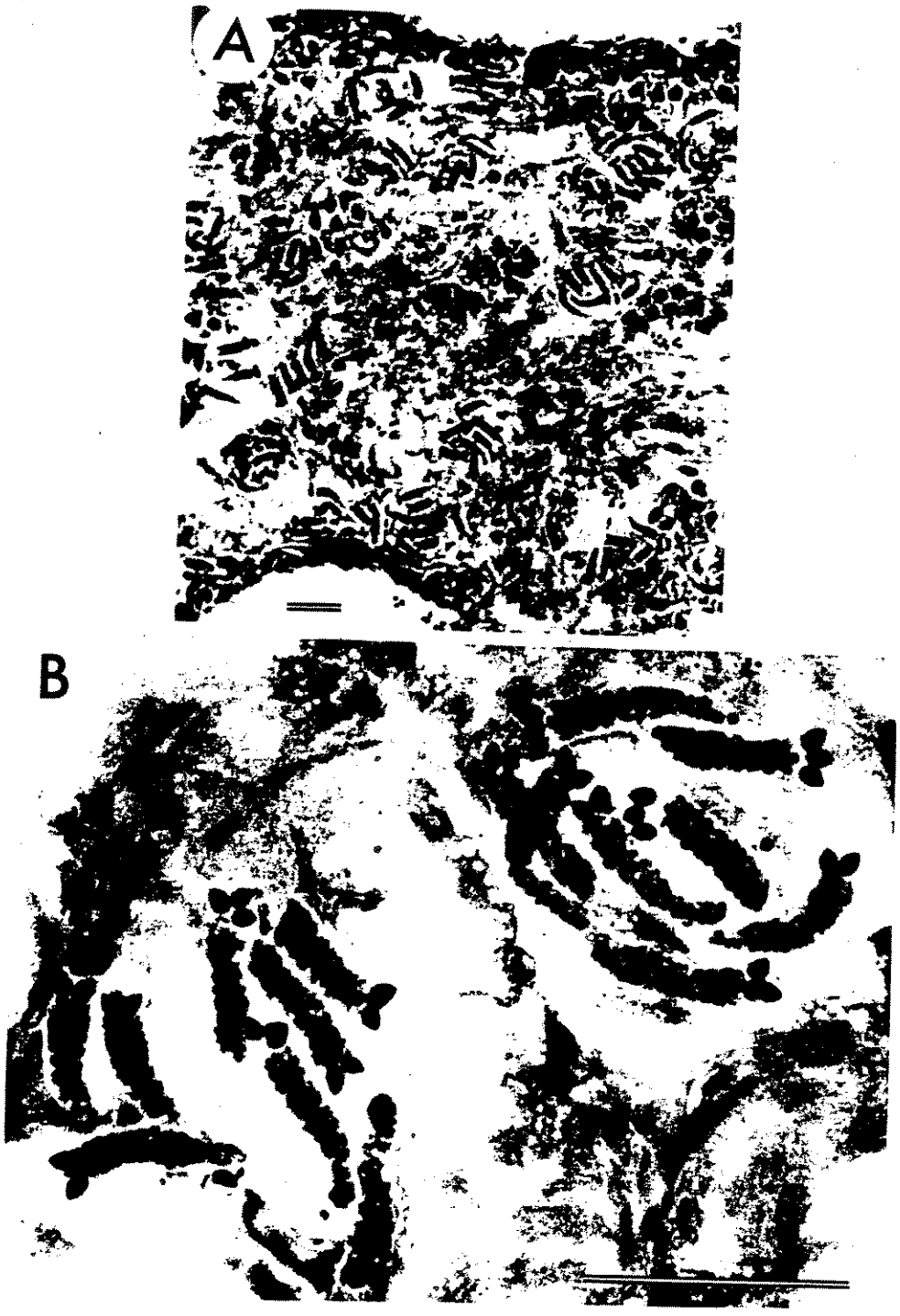

FIG. 2. (A) Portion of crushed, formalin-fixed tubificid oligochaete infected with the triactinomyxon stage 130 d after exposure to *M. cerebralis* spores. May–Grunwald Giemsa stain. Bar = 50 μm. (B) Two mature ruptured cysts of the triactinomyxon stage in tubificid. Each cyst contains eight spores with numerous internal sporozoites. The distinctive three polar capsules of the upper body or epispore are intensely stained in May–Grunwald Giemsa. Bar = 50 μm.

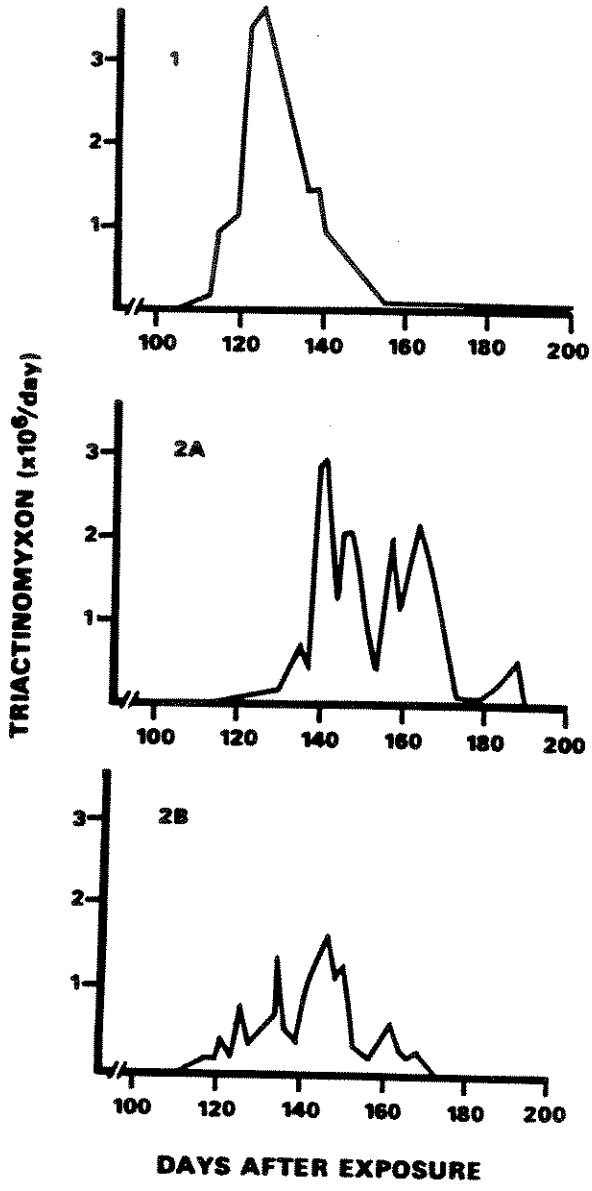

FIG. 3. Comparative production of waterborne triactinomyxons at 12.5°C. (1) First trial with 140 spores of *M. cerebralis* per tubificid oligochaete; (2A) second trial with a multiplicity of exposure of 380 spores of *M. cerebralis* per tubificid; (2B) multiplicity of exposure of 38 spores per tubificid.

several genera, were washed several times in spring water, weighed, and number per gram determined. Measured weights of the tubificids were distributed to the jars of the heat-treated and washed soil and allowed to establish themselves for 2–3 d.

Collection of Spores

Spores of *M. cerebralis* were physically harvested from defleshed heads of experimentally infected rainbow trout. The material was kept at 12°C or lower to maintain spore viability. Cranial elements and gill arches of fingerlings were gently macerated, but bones and cartilage of larger fish were passed through a precooled household food grinder. The ground tissues were pooled, suspended in spring water, and sequentially passed through screens with mesh sizes of 1000, 500, and 250 μm. The final filtrate was held at 12.5°C overnight to allow spores to settle from the suspension. Sedimented spores were harvested by aspiration and quantified by hemocytometer count.

The harvested spore suspension was gently mixed with the top several centimetres of axenic soil in the 9-L jars, thus providing an even distribution and contact with the anterior or feeding end of the worms.

A negative control consisted of one jar that contained tubificids to which cranial elements from specific-pathogen-free trout were added. All jars were filled with water to the 6-L level and held without flow until the next day when a gentle flow of water was provided at a rate of 120–175 mL/min.

Collection of Triactinomyxons

The waterborne triactinomyxon stage, as described by Wolf and Markiw (1984), is anchor-shaped and topped by three polar capsules (Fig. 1). The anterior body or enispore measures about 32–36 × 10 μm and contains up to 50 spherical sporozoites, each about 2.2 μm in diameter. The three tail-like appendages of the valves extend 170–180 μm.

Waterborne triactinomyxon spores were harvested by selective screening of effluent from the jars. The effluent was first passed through a 200-μm-mesh screen to remove gross debris and then through a 50-μm-mesh screen to retain the organisms. The two screens were tightly fitted together and immersed in circulating spring water held at 12.5°C. Triactinomyxon spores were collected every 1–3 d. Trapped spores were removed from the 50-μm screen by washing and aspirating; harvests were quantified by hemocytometer count.

Examination of Tubificids for Triactinomyxons

Experimentally infected tubificids were randomly selected from the first trial 130 d after initial exposure to spores of *M. cerebralis*. The naturally infected tubificids were selected from two different trout hatcheries where whirling disease has been confirmed. Collected worms were fixed in 5% neutral buffered formalin. Each worm was crushed on a microscope slide and examined under 10× magnification for the presence of triactinomyxon spore stages (Fig. 2A, 2B).

Test for Whirling Disease Infectivity

Twelve months after triactinomyxon spores were first released, I exposed 15 specific-pathogen-free rainbow trout, 5–6 cm long, to soil/worm contact for 1 mo. After 4 mo of incubation, 10 survivors were examined by the pepsin–trypsin–dextrose (PTD) method (Markiw and Wolf 1974) for the presence of *M. cerebralis* spores.

Results

The dynamics of the triactinomyxon stage in tubificid oligochaetes were determined in two trials at 12.5°C. In both trials, the organism appeared 3.5 mo after tubificids had been exposed to *M. cerebralis* spores.

Production of the waterborne triactinomyxon stage showed a rather consistent pattern of yield that began at 104–113 d and was greatest during the following 15–60 d (Fig. 3, trial 1). In the first trial, each tubificid was exposed to a mean of 140 *M. cerebralis* spores, and the triactinomyxon stage was found 104 d after initial exposure. As determined by microscopic examination, production peaked during the next 50 d

and continued at trace levels for 8.9 mo. The waterborne triactinomyxon stage was not evident microscopically during the next 3 mo, but rainbow trout exposed to the soil for 1 mo became infected. Each of the 10 surviving fish was found to have had an average of 340 *M. cerebralis* spores. The triactinomyxon stage could not be found in negative controls, and exposed fish remained free of *M. cerebralis* spores.

In the second trial, I tested two concentrations of *M. cerebralis* spores (380 or 38) for tubificid infectivity. Release of waterborne triactinomyxons from the 380-spore exposure (Fig. 3, trial 2A) was first detected at 113 d; production rose slowly, with several irregular peaks, during the next 87 d. The release of spores then dropped to a trace and was microscopically undetectable when the trial was discontinued after 208 d. The unit in which tubificids were exposed to 38 spores (Fig. 3, trial 2B) yielded the first triactinomyxons at 110 d. The production peaked, with some irregularities, for 62 d; the trial was discontinued at 182 d at which time the stage was no longer detectable microscopically.

Production of triactinomyxons in the first trial peaked at 125 d (Fig. 3) at 2430/min, and produced its major yield in a period of 35–40 d (Table 1). In the second trial, with 380 spores per worm, production peaked at 141 d (Fig. 3), when the rate of spore production was 2090/min. The period of major yield spanned about 45 d (Fig. 3). When 38 spores per worm were used, production peaked at 145 d (1190 spores/min), and the major yield was produced over a period of about 50 d.

The calculated yield of the triactinomyxon spore stage was 730 from each worm in the first trial and 3420 and 1790, respectively, in the second trial. In two of three experiments the total number of triactinomyxons produced was about 50 million.

Among 50 worms from the first trial randomly selected after 130 d, the prevalence of experimental infection was 20%. In comparison, among 200 worms from two different natural sources, the prevalences of infection were only 1.5 and 2.0%.

Discussion

Consistency in the pattern of experimental production of the triactinomyxon stage by tubificid worms is evident. The first appearance of spores, the development of a major period of spore production, and the rapid cessation of spore production were similar in all trials. In all three trials the triactinomyxon stage first appeared at 3.5 mo (104–113 d) after exposure of tubificid oligochaetes to spores of *M. cerebralis*. The time of first appearance could not be determined by Mackinnon and Adam (1924) or Janiszewska (1955, 1957) because they were limited to examining naturally infected populations. However, the 3.5 mo is in total agreement with the well-established fact that, at 12.5°C, a 3.5-mo period is needed for spores of *M. cerebralis* to develop into the infectious stage of whirling disease (Hoffman and Putz 1969). I have provided details of a method for experimentally producing triactinomyxons, and the work strongly supports the relation between the myxosporean *M. cerebralis* and its alternating life phase, the actinosporean for which the name *Triactinomyxon gyrosalmo* was suggested by Wolf and Markiw (1984).

The experimentally produced triactinomyxon that infects fish with whirling disease morphologically resembles forms of *Triactinomyxon* described by earlier investigators; however, the relationships among *Triactinomyxon* species in unknown.

The dominant period of spore production of this study (Fig. 3) is attributed to the simultaneous exposure of the confined tubificids to *M. cerebralis* spores in high concentration. Such massive exposure is unlikely in natural environments where continual exposure at a low level is assumed. However, results from the first trial demonstrated that residual infectivity for trout peristed for at least 1 yr.

Different stages of maturity of tubificids, and consequently different sizes, were used in the two trials (Table 1) to produce triactinomyxons. Although the production of spores per worm differed significantly, there was little difference in total yield. Predominantely small worms (2–3 cm long) that were exposed in mid-November to 140 spores produced an average of 730 triactinomyxons. Larger worms (6–8 cm long) exposed in late June to 380 or 38 spores produced averages of 3420 and 1790 triactinomyxons per worm, respectively. The differences in yield per worm might be attributed to differences in the maturity of worms at exposure. About 680 of the small worms or 147 of the larger worms weighed 1 g. The yields for similar weights of worms (88 and 100 g) exposed to two higher concentrations of spores (140 and 380 per worm) were somewhat correlated — 44 and 50 million triactinomyxons.

A 10-fold difference in concentration of *M. cerebralis* (380 and 38 spores per worm) produced only a twofold increase in yield of triactinomyxon spores. Exposure to the higher concentration of *M. cerebralis* spores might have killed some worms. Accordingly, higher numbers of *M. cerebralis* spores might not necessarily result in a larger yield of triactinomyxons.

For the two lower concentrations of *M. cerebralis* spores (140 and 38 spores per worm), the higher concentration produced only about a twofold larger yield (44 versus 26 million) of triactinomyxons. But the total yield produced from such a small number (38) of *M. cerebralis* spores was large.

Overall, the data indicate that there is no correlation between the concentrations of *M. cerebralis* spores available to tubificids and an optimal production of triactinomyxons. The production of the triactinomyxon stage seems to depend on at least four factors: (1) the presence and density of the tubificid host, (2) the degree of maturity or size of the tubificids, (3) an environment that ensures maintenance of the tubificids throughout several months and longer, and (4) viable *M. cerebralis* spores.

The prevalence of tubificid oligochaete infection with triactinomyxons derived from the present study differed from those reported by Stolc (1899) and Mackinnon and Adam (1924). The present experimental infection produced an incidence of 20%, 10-fold greater than was found in two trout hatcheries where whirling disease was established. In truly natural situations, Stolc (1899) reported a prevalence of 0.3% and Mackinnon and Adam (1924) reported 0.4%.

Although earlier investigators described actinosporeans of the genus *Triactinomyxon*, it should be emphasized that the authors described the organism as infective only for tubificids. Accordingly, any relationship of *Triactinomyxon* to salmonid whirling disease was not considered. I have described a method of experimentally producing large quantities of relatively pure triactinomyxons, and this work supports strongly our contention that there is a biological relationship between *M. cerebralis* and its alternating triactinomyxon phase (Wolf and Markiw 1984).

The ability to produce the triactinomyxon stage should open the way for other investigations into the immunoserology and biochemical characterization of specific antigenic components that could be tested for the development of a vaccine and the genetic evaluation studies to control whirling disease.

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Salmonid whirling disease: myxosporean and actinosporean stages cross-react in direct fluorescent antibody test

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Abstract. Serologic relatedness of the two life stages of the salmonid whirling disease parasite *Myxosoma cerebralis* Hofer, 1903 — myxosporean spores from fish cartilage and actinosporean triactinomyxon spores from aquatic tubificids — were investigated. When the direct fluorescent antibody technique was used, anti-triactinomyxon and anti-*M. cerebralis* rabbit sera conjugated with fluorescein isothiocyanate cross-reacted with the respective heterologous life stage. Both stages showed similar locations of specific fluorescence with conjugates of either homologous or heterologous serum. Thus, serology supports the relatedness of the myxosporean *M. cerebralis* and the actinosporean triactinomyxon stages.

Introduction

Salmonid whirling disease has been known for more than 80 years, but only recently have studies by Markiw & Wolf (1983), Wolf and Markiw (1984), Markiw (1986) and Wolf, Markiw & Hiltunen (1986) demonstrated that the infection is caused by a parasite with a two-stage life cycle. The first stage is a disk-like myxosporean *Myxosoma cerebralis* Hofer, 1903 spore 8–9 μm in diameter that occurs in and damages fish cartilage. The second stage is an actinosporean, provisionally named *Triactinomyxon gyrosalmo* by Wolf & Markiw (1984), that develops in the gut of the small aquatic tubificid, *Tubifex tubifex*. The triactinomyxon stage is an anchor-shaped spore having three 160–180 μm grapple-like appendages.

When the spores of *M. cerebralis* are released from decaying fish or shed by fish-eating predators, they settle and are ingested by tubificids. In the gut of the worm, the spores morphologically change into the triactinomyxon, the stage that initiates whirling disease. Susceptible salmonids become infected by ingesting infected worms or having contact with waterborne triactinomyxons.

The purpose of this study is to report the serological relatedness between the *M. cerebralis* from fishes and the *Triactinomyxon gyrosalmo* from tubificids.

Materials and methods

Propagation, harvest and antigen preparation

Propagation of the triactinomyxon spore stage was initiated as described elsewhere (Wolf & Markiw 1984; Markiw 1986). About 52000 (75 g) of a mixed population of actinosporean-free tubificids, consisting of about 67% *Tubifex tubifex* were exposed to $7{\cdot}8 \times 10^6$ viable *M. cerebralis* spores. The tubificids were maintained in flowing spring water at 12°C. The first

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waterborne triactinomyxons appeared at about 3 months and, thereafter, were collected or 50- μm screen during the period of peak release, 140–160 days after the initiation. The m concentrated one-day harvests, easily seen as a dense fluffy suspension, were collected 10–20-ml conical tubes and set in ice for 20–30 min. After gross debris had settled, the spo suspension was pipetted into clean tubes. Water at 4°C was used for washing spores, a sedimentation of debris was repeated until a clean preparation was obtained. Spores we quantified by haemocytometer count and concentrated at 800 × *g* for 10–15 min at 4° Concentrated spores were aspirated from the sediment and stored at −20°C.

Spores were disrupted with three passes through a chilled French press pressure cell at 14 kg/cm^2. Ruptured spores were concentrated by pressure dialysis at 4°C, using a PM 10 Amic Diaflow Ultrafilter (Amicon Corp., Lexington, Mass 02173, USA) under nitrogen at about kg/cm^2.

Production of antiserum

Antiserum against triactinomyxon spores was produced in a New Zealand white rabbit th had been immunized with antigen from 1·7 × 10^7 spores containing 1·6 mg of soluble prote (Bradford 1976). Initial inoculation was with 1·3 × 10^7 disrupted spores in 2 ml of Hank balanced salt solution emulsified with an equal volume of Freund's incomplete adjuvant. A initial dose of 1 ml was given subcutaneously in each foreleg and intramuscularly in each hin leg. After 12 days, the rabbit was desensitized with an inoculum of 0·2 ml and boosted 4 h lat by an intraperitoneal injection of 2 ml of pooled antigen (1 × 10^6 disrupted and 3 × 10^6 inta spores) without adjuvant. After 6 days, the rabbit was bled by cardiac puncture, and 2-n aliquots of the serum were lyophilized.

Antiserum against the *M. cerebralis* spore stage was prepared as described by Markiw & Wolf (1978) with one change: spores and prespore stages were ruptured by four passes throug a French press pressure cell instead of by freeze-thawing.

Direct fluorescent antibody test

For the direct fluorescent antibody test (FAT), normal sera collected from the same rabbi before immunization and specific antisera were conjugated with fluorescein isothiocyanat (FITC) according to the method of Rinderknecht (1962). One millilitre subsamples of conjugate sera were lyophilized or stored at −20°C.

Serial two-fold dilutions of anti-triactinomyxon and anti-*M. cerebralis* were made in 0·02 sodium phosphate buffer at pH 6·5.

Both antigens — *M. cerebralis* spores isolated from rainbow trout, *Salmo gairdne* Richardson cartilage by the pepsin-trypsin-dextrose (PTD) method of Markiw & Wolf (1974 and triactinomyxon spores obtained as described (50 μl of a suspension of 1 × 10^6 spores pe ml) — were placed in parallel spot wells on microslides coated with 50% egg albumin Duplicate tests were carried out. Antigens were dried at 50°C for 30 min. Slides were fixed i absolute methanol for 2–5 min and air dried: the spots of antigen were then flooded with 50 μ of FITC conjugated antisera. Conjugated normal rabbit serum was used as a control. Afte 60-min incubation in a covered humid chamber at 22–24°C, the slides were dipped two o three times in diluted (1:4) 0·05 M sodium carbonate-bicarbonate buffer at pH 9–9·5, placed in full-strength buffer, and gently shaken for 5 min. The slides were blotted and examined at 1250 × under low fluorescence immersion oil. Intensity of the fluorescence was scored as + to ++++.

Spores of *Myxosoma cartilaginis*, isolated from bluegills, *Lepomis macrochirus* Rafineague, by the PTD method and stored at −20°C, were also tested for reactivity.

Results

When the direct FAT was used, the conjugated anti-triactinomyxon serum reacted strongly with *M. cerebralis* spores. Brightness was highest (++++) at dilutions of 1:10 to 1:80 (Table 1). Reactivity with the homologous triactinomyxons was observed at higher dilutions, ++++ at 1:320 and +++ at 1:640.

Comparative cross-reactivity with anti-*M. cerebralis* serum also showed a strong reaction with triactinomyxon spores, although maximum brightness was observed at lower concentrations ++++ at 1:10 and +++ at 1:20.

The spores of *M. cartilaginis* showed no reactivity with anti-triactinomyxon serum, although they reacted weakly with the anti-*M. cerebralis* conjugate (+++ at 1:10 dilution).

Conjugated normal rabbit sera did not react with any of the spores.

In addition to cross-reactivity with the heterologous antiserum, both stages showed reactivity at similar locations. The two polar capsules of *M. cerebralis* spores (Fig. 1a) were typically unstained voids in a fluorescent background, whereas fluorescence was densely concentrated in the sporoplasm. In triactinomyxon spores, the three polar capsules were unstained voids, but the sporoplasm and the sporozoites were strongly reactive (Fig. 1b & c). After discharge, the polar filaments of both spore types were also fluorescent. Triactinomyxon spores that contained sporozoites showed no fluorescence in the three appendages (Fig. 1d); however, the appendages showed slight (++) fluorescence in occasionally seen triactinomyxons in which the sporoplasm containing sporozoites had been liberated.

Discussion

Serological techniques have been widely used to detect myxosporean antigens (Halliday 1974; Pauley 1974; Markiw & Wolf 1978; Amandi, Holt & Fryer 1985). Cross-reactions between antisera prepared for specific myxosporean parasites and heterologous spore antigens occur infrequently, particularly when direct FAT is used (Markiw & Wolf 1978; Amandi *et al.* 1985). Therefore, the strong serological responses of anti-triactinomyxon and anti-*M. cerebralis* FITC

Table 1. Fluorescent antibody response of *Myxosoma cerebralis* spores, triactinomyxon spores and *Myxosoma cartilaginis* spores to FITC conjugated antisera

	Dilutions of antisera						
Spore antigen	1:10	1:20	1:40	1:80	1:160	1:320	1:640
	Anti-triactinomyxon serum conjugate						
M. cerebralis	++++	++++	++++	++++	−	−	−
Triactinomyxon	++++	++++	++++	++++	++++	++++	+++
M. cartilaginis	−	−	ND*	ND	ND	ND	ND
	Anti-*M. cerebralis* serum conjugate						
Triactinomyxon	++++	+++	−	−	−	−	−
M. cerebralis	++++	+++	−	−	−	−	−
M. cartilaginis	+++	−	ND	ND	ND	ND	ND

* ND = not done.

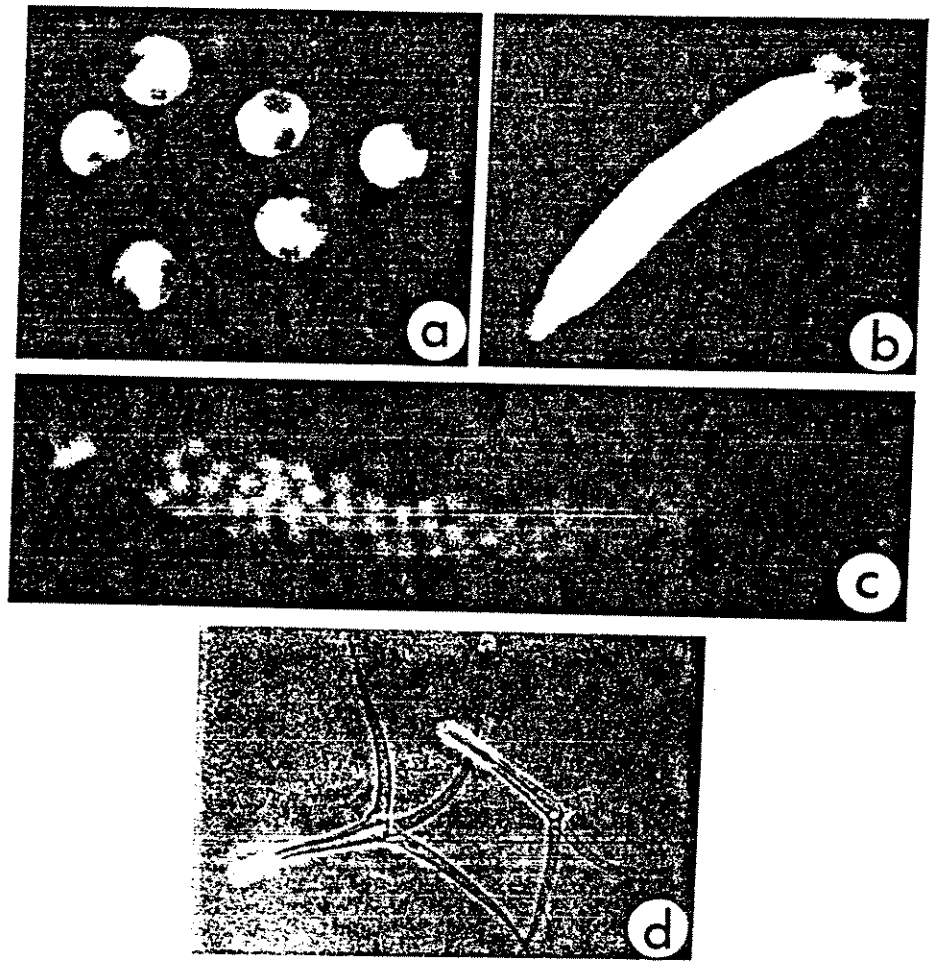

Figure 1. Direct FAT micrographs of the two life cycle stages of salmonid whirling disease organism comparabl in size, responding to the fluorescein isothiocyanate conjugated heterologous antisera and unstained triactinomyxon stage at lower magnification for comparison. (a) Positive reaction of mature *M. cerebralis* spore from fish, to a 40 × dilution of anti-triactinomyxon conjugate. Note strongly reactive sporoplasm and two nonreactive polar capsules (×1200). (b) Positive reaction of epispore of mature waterborne triactinomyxon, from tubificid, to a 10 × dilution of anti-*M. cerebralis* conjugate. Note sporoplasm containing sporozoites of an epispore react strongly and homogenously (×1200). (c) Mature epispore of the triactinomyxon showing 64 sporozoites and three nonreactive polar capsules (×1200). (d) Non-reactive three polar capsules and three appendages of triactinomyxon shown under light microscopy (phase contrast ×200).

conjugated sera to their respective heterologous spores indicate that the two stages are related

Notwithstanding the fact that cross-reactivity was evident, the anti-triactinomyxon serur was potent at a greater dilution than was the antiserum prepared against the spore stage fron fish. The difference could be due to variation in the immune response of the rabbits used, or t differences in preparation of antigen. Triactinomyxon antigen was prepared solely by physica means at 12·5°C or less. In contrast, spores from fish were obtained by pepsin digestion a 37°C, followed by trypsin digestion at 22°C and additional purification through dextrose, afte

which they were physically disrupted. The proteolytic action of the enzymes might have degraded antigenicity. Such degradation, probably of nuclear proteins, is suggested by the demonstration that the procedure of sequential pepsin-trypsin digestion and purification through dextrose inactivates infectivity of the spores (Markiw 1985). It is unlikely that small amounts of nuclear protein constitute the principal spore antigenicity.

The differences in quantity of antigen used for immunization of rabbits (soluble protein content) had no apparent effect on potency of the antiserum. The protein content of the triactinomyxon inoculum was 1·6 mg per rabbit, and that of *M. cerebralis* was 1 mg or 20 mg per animal. The 20-fold greater concentration for *M. cerebralis* did not result in proportionally greater antiserum potency. Spores are simply poor antigens.

Spores of *M. cartilaginis* were moderately reactive with antiserum prepared against spores and prespore stages of *M. cerebralis* from fish, but only at a low dilution of serum. No reactivity occurred between *M. cartilaginis* and antiserum against the triactinomyxon spore stage from tubificids. The reactivity of *M. cartilaginis* spores to anti-*M. cerebralis* serum supports the classification that places both organisms in the same genus, but it also suggests that a relationship exists between some antigens that occur only in the myxosporean stage.

Although the two stages differed in fluorescent reactivity, both produced cross-reactive antisera.

Antiserum against the triactinomyxon offers a tool for discovering modes of entry of fish by the triactinomyxon.

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Markiw, M.E. 1991. Whirling disease: Earliest susceptible age of rainbow trout to the triactinomyxid of *Myxobolus cerebralis*. Aquaculture. 92: 1-6.

Various developmental stages of eyed eggs and newly hatched sac fry of rainbow trout (*Oncorhynchus mykiss*) were exposed to several concentrations of laboratory produced spores of the triactinomyxon stage of *Myxobolus cerebralis*, the infective stage of salmonid whirling disease. Exposed eggs and sac fry and appropriate unexposed controls were examined microscopically immediately after challenge for the presence of initial forms of the disease and 4 months later for the presence of spores of the myxosporean stage of *Myxobolus cerebralis* in resulting fingerlings. Although initial forms of whirling disease shown as intracellular aggregates of small sporozoites (sporoplasms) 1.5-2.0 μm in diameter were found in the epithelium of eyed eggs a few hours before hatching and in 1-day-old sac fry, the resulting fingerlings examined 4 months later were free of *M. cerebralis* spores. The youngest trout that became infected with whirling disease and yielded spores of *M. cerebralis* was the 2-day-old sac fry. This result indicates that the distribution of eyed eggs from water contaminated with whirling disease should not pose a threat of spreading the disease into non-endemic areas.

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Experimentally Induced Whirling Disease I. Dose Response of Fry and Adults of Rainbow Trout Exposed to the Triactinomyxon Stage of *Myxobolus cerebralis*

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Abstract.—The intensity and prevalence of whirling disease was tested by exposure of 2-month-old fry and 1-, 2-, and 3.5-year-old adults of rainbow trout *Oncorhynchus mykiss* to a known number of laboratory-produced *Myxobolus cerebralis* at the actinosporean triactinomyxon stage. Fry exposed to graded concentrations of infectivity (triactinomyxons) for 3 h were individually examined for spores of *Myxobolus cerebralis* 5 and 6 months later. Exposure of fish to the lowest doses, 1 and 10 triactinomyxons per fish, did not result in detectable myxosporean spores. Fish that became lightly infected by a dose of 100 triactinomyxons per fish experienced a decrease in the incidence of infection between 5 and 6 months after exposure. A linear relationship was found between the numbers of recovered myxosporean spores and doses of 100–10,000 triactinomyxons per fish, and the spore burden appeared to plateau at doses of 10,000–100,000 triactinomyxons per fish. Adult fish continuously exposed to the highest dose of triactinomyxons for 3.5 months were infected and asymptomatic; however, the severity of myxosporean infection decreased with increased age of fish. This information may help in controlling whirling disease in salmonids.

Whirling disease is internationally recognized as one of several major communicable diseases of the family Salmonidae. Import or transfer of salmonids from unknown locations are restricted to prevent the spread of the disease to nonenzootic areas. Whirling disease is caused by the myxosporean *Myxobolus cerebralis* (also known as *Myxosoma cerebralis*). This disease is chronic and may cause heavy mortality, particularly among young fish. The parasite has a selective tropism for cartilage and thus damages the skeletal structure of the host; therefore, moderately infected survivors are often disfigured. Asymptomatic, lightly infected carriers pose a threat of spreading the disease. As reported by O'Grodnick (1979), rainbow trout *Oncorhynchus mykiss* are most susceptible to the disease, and brook trout *Salvelinus fontinalis* less so. Newly hatched 1-d-old rainbow trout sac fry are refractory to the infection (Markiw 1991).

The biological characteristics of the infective triactinomyxon stage have to be known to design control measures. Although the disease has been known for nearly a century (Hofer 1903), the two-host life cycle of the parasite, which involves a tubificid oligochaete as intermediate host for the development of the stage infecting fish, was discovered not long ago (Markiw and Wolf 1983; Wolf and Markiw 1984). The life cycle of the whirling disease organism described by the above authors has been confirmed by El-Matbouli and Hoffmann (1989). The primary sites of infection of fish by the entry of triactinomyxon through epithelial cells were also demonstrated (Markiw 1989).

The purpose of this study was to determine the virulence of quantified doses of laboratory-produced *M. cerebralis* triactinomyxon spores used to infect several age-groups of rainbow trout.

Methods

Fish and water quality.—All rainbow trout used in this study were reared at this laboratory from eyed eggs obtained from the White Sulphur Springs (West Virginia) National Fish Hatchery. This hatchery was free of whirling disease, and egg populations were certified as being free of other disease agents. Pathogen-free spring water at 12.5°C and a pH of 6.8 was used. Alkalinity was 260 mg/L. Other water quality characteristics included sulfate, 21.3 mg/L; calcium hardness, 243 mg/L as $CaCO_3$; magnesium hardness, 51 mg/L as $CaCO_3$; and total hardness 294 mg/L, as $CaCO_3$. Experimental fish were fed a commercial dry starter or pellets, and observed daily for clinical signs of whirling disease. The appearance and mortality of fish were recorded daily.

Triactinomyxon spore production.—Triactinomyxons were propagated in tubificid worms as described by Markiw (1986). In brief, actinospo-

TABLE 1.—Numbers of *Myxobolus cerebralis* myxosporean spores recovered from 2-month-old rainbow trout fry 5 and 6 months after initial exposure to graded concentrations of triactinomyxon spores.

Dose (number of triactinomyxons per fish)[a]	Time after exposure (months)	Number of fish examined	Number of fish infected	Number of spores recovered per fish	
				Range	Mean
0	5	12	0		
	6	9	0		
1	5	12	0		
	6	8	0		
10	5	12	0		
	6	8	0		
100	5	12	6	0–120,000	16,460
	6	11	2	0–6,440	900
1,000	5	12	12	8,120–341,870	78,850
	6	9	8	0–295,000	98,080
10,000	5	12	12	262,500–1,737,500	680,340
	6	8	8	25,000–1,337,500	570,140
100,000	5	12	12	68,750–1,262,500	691,670
	6	9	9	93,750–1,693,750	750,690

[a] Fish were exposed to parasites for 3 h.

rean-free tubificids collected at the trout hatchery were exposed to viable *M. cerebralis* spores and maintained in flowing spring water at 12.5°C. The triactinomyxons released by the experimentally infected tubificids into the water columns (effluents) of aquaria were quantified with a hemocytometer before use.

Exposure of fry to quantified doses of triactinomyxon spores.—Waterborne triactinomyxons were siphoned from an experimental aquarium deprived of incoming spring water and left undisturbed for 16–18 h. Seven containers were prepared, each holding 1,800 mL of spring water with suspended triactinomyxons at concentrations of 0, 1, 10, 100, 1,000, 10,000, and 100,000 spores per fish. Twenty five healthy 2-month-old fry were exposed to spores under static conditions (no water flow) without aeration at 12.5°C for 3 h. These fish and unexposed control fish were transferred to aquaria containing spring water, where they were held for 5 or 6 months. After 5 months, 12 fish from each group were individually processed by the enzymatic method of Markiw and Wolf (1974a) to determine the presence of *M. cerebralis* spores. The remaining fish were processed similarly 6 months after initial exposure.

Exposure of subadult and adult trout to triactinomyxon spores.—Thirty 1-year-old, twelve 1.5–2-year old, and six 3–3.5-year-old healthy rainbow trout were placed in one of three 134-L tanks, according to their ages. In each tank, 100 2-month-old rainbow trout fry were held in screened buckets as positive controls for infectivity. Triactinomyxons were propagated in three aquaria. Mature waterborne triactinomyxon spores were collected from the effluent of one aquarium on 50-μm-mesh screen and counted. Effluents containing triactinomyxons from the other two aquaria were distributed among the three tanks of fish. About 67 million waterborne triactinomyxons (2–3 million/d at the peak of production; average, 638,000 daily) were continually provided to each test tank for 3.5 months. Seven months after initial exposure, heads of all exposed fish (pooled by age-group) were processed according to the enzymatic digestion method (Markiw and Wolf 1974b). Fry were examined earlier, 3 months after initial exposure, because they were severely infected and sustaining heavy mortality.

Results

Response of Fry to Triactinomyxons

The 2-month-old rainbow trout fry exposed to concentrations of fewer than 100 triactinomyxons per fish produced no recoverable *M. cerebralis* spores (Table 1). A concentration of 100 triactinomyxons per fish resulted in recoverable myxosporean spores in 6 of 12 fish examined at 5 months and in 2 of 11 fish at 6 months postexposure; this decrease in the incidence of infection in lightly infected fish between 5 and 6 months after exposure was statistically significant at $P = 0.10950$. For this group, the average number of *M. cerebralis* myxosporean spores recovered per fish decreased from 16,460 at 5 months to 900 at 6

months postexposure. After a dose of 1,000 triactinomyxons per fish, only one of nine fish was negative for myxosporean spores 6 months after initial exposure. The severity of infection (number of spores per fish) increased linearly with doses from 100 to 10,000 triactinomyxons per fish ($r = 0.999$), and plateaued at 10,000 to 100,000 triactinomyxons per fish ($P \leq 0.05$). Mortality among infected trout was not significantly different ($P > 0.05$) from that of unexposed control fish. The majority of infected fish were asymptomatic, although a few showed the clinical sign of whirling, and one exhibited a black tail.

Response of Subadults and Adults to Triactinomyxons

All three age groups of adult trout were susceptible to infection with triactinomyxon spores, but *M. cerebralis* spore production was lower in older trout (Table 2). All infected adult trout were asymptomatic and none died. An average of 48% of the fry exposed together with each age-group of adult fish survived 3 months, and similar numbers of *M. cerebralis* spores were isolated per fry (134,000, 126,000, and 131,000), indicating equal infectivity in the three fish tanks.

TABLE 2.—Numbers of *Myxobolus cerebralis* myxosporean spores recovered from adult rainbow trout 7 months after initial exposure to triactinomyxon spores.

Age of fish at initial exposure (years)	Number of fish exposed	Approximate daily dose (number of triactinomyxons per fish)	Average number of spores recovered per fish
1	30	21,270	569,670
1.5–2	12	53,170	227,080
3–3.5	6	106,350	6,150

Discussion

This study demonstrated that very low concentrations of *M. cerebralis* triactinomyxon spores (1 or 10 triactinomyxons per fish) did not produce whirling disease in 2-month-old fry. In an earlier study (Markiw 1991), newly hatched 2-d-old sac fry exposed under similar conditions were severely infected with a dose of 10 triactinomyxons per fry. This indicates that older fish are more resistant than younger fish.

Among fish dosed with 100 triactinomyxons each, the number of myxozoan spores recovered and the incidence of infection clearly decreased (from 50 to 18% infected) between 5 and 6 months after exposure (Table 1). Spores of *M. cerebralis* were found in significant numbers when the fish were examined 5 months after exposure, but representatives from the same group examined 1 month later either were not infected or were infected with only a few spores. Although a reduction of the level of infection over time has been observed repeatedly in similar studies of whirling disease, mass production of triactinomyxons in the laboratory allowed this phenomenon to be demonstrated experimentally. Elimination or reduction of a light myxosporean infection may have been effected by a fish host tissue reaction. Cell-mediated immunity involving recruitment and stimulation of effector cells, which function either alone or in combination with a certain antibody to eliminate or destroy the original pathogen and thereby control the infection, was observed in the study of human parasitism by James and Scott (1988).

In other words, the severity and not simply the presence or absence of infection determines the onset of disease. This implies that control measures do not have to be 100% effective to reduce the disease. Any measure such as vaccination, chemotherapy, or selecting for natural resistance to myxosporean infection may have a great effect on the number of trout infected and level of infectivity.

The level of parasitism, defined as the number of myxosporean spores per host, plateaued in 2-month-old fry. The highest doses (10,000 and 100,000 triactinomyxons per fish) produced about the same average number of *M. cerebralis* spores per fish head examined 5 or 6 months after exposure. There were also extreme differences in susceptibility to the disease among individual fish. Clinical signs of the disease, such as whirling, black tails, or mortality, are not always an indication of the severity of the infection.

It is not surprising that adult trout can be infected by the whirling disease agent because myxosporean spores have selective tropism for the cartilage tissue that fish possess throughout their lives. However, the apparent resistance to infection observed among older trout might be explained by physiological changes of the skin. For instance, heavier mucus and a more complete scale cover could reduce infectivity of waterborne triactinomyxons because target epithelial cells of the skin and fins are prime sites of entry (Markiw 1989). Also, the infection might be partially prevented by a strong tissue immune response in adult fish, as seen in human responses to parasites (James and Scott 1988).

Finally, detection of whirling disease in the aquatic environment when infective agents (triactinomyxons) are low in number is difficult until the parasite is numerous enough for *M. cerebralis* spores to be detected in fish. Examination of the water supply (filtration through 50-μm-mesh screen) or tubificids for the presence of the specific actinosporean triactinomyxon could be a useful means of detecting whirling disease in suspected areas.

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Experimentally Induced Whirling Disease II. Determination of Longevity of the Infective Triactinomyxon Stage of *Myxobolus cerebralis* by Vital Staining

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Abstract.—Effects of temperature and aging on viability and infectivity of laboratory-produced actinosporean triactinomyxon spores (infective stage of the organism causing whirling disease) were studied. In vitro staining of triactinomyxon spores with vital fluorescein diacetate correlated with the ability of the spores to infect fry of rainbow trout *Oncorhynchus mykiss*. The experimentally produced actinosporean stage of *Myxobolus cerebralis* was short-lived, persisting for only 3–4 d at 12.5°C and for less time at warmer temperatures. The vital staining method has potential for screening therapeutants intended to control myxosporean infection of fish.

In developing methods to control salmonid whirling disease, the length of the viability of both stages of the parasite *Myxobolus cerebralis* (also known as *Myxosoma cerebralis*) must be known. Particularly important is the retention of viability of the tubificid-produced triactinomyxon spores, the stage that infects fish. Studies showed that a population of tubificids infected by a single exposure to *M. cerebralis* myxosporean spores can release infective triactinomyxon spores for as long as a year at a level detectable only with sentinel fishes (Markiw 1986). Until now, a vital staining technique for determining spore viability has not been available.

Viability is easily checked by staining with fluorescein diacetate (FDA) alone, which has been widely applied with animal cells, or in combination with ethidium bromide, propidium iodide (PI), or other fluorochromes, which detect dead cells. The method was reported reliable for determining the viability of protoplast and cultured plant cells (Rotman and Papermaster 1966; Widholm 1972). Simultaneous staining with FDA and PI has been used for many years to characterize viability and fertility of spermatozoa. Matyus et al. (1983) used FDA–PI double fluorescence labeling of bull seminal fluid to determine the viability of sperm cells measured by flow cytometry. With this technique, these authors studied semen samples from several bulls with known fertility indexes, and results showed good agreement with conception rates obtained in field tests. Jones and Senft (1985) used FDA–PI double labeling to determine the viability of mouse spleen cells in suspension and compared the results with those from the widely used trypan blue dye exclusion method. The staining of cells with FDA–PI is more consistent with longer exposure to the dyes.

The objectives of this study were to determine the viability of waterborne triactinomyxon spores held at 7–24°C by vital staining and to document the persistence of infectivity of the actinosporean triactinomyxon spore of *M. cerebralis*.

Methods

FDA–PI vital staining.—Working solutions were prepared. First, 0.05 mL of FDA (number F-7378; Sigma Chemical Co., St. Louis, Missouri) stock solution (5 mg/mL dissolved in acetone and stored at −20°C) was added to 8.3 mL of phosphate-buffered saline at a pH of 7.2. An aqueous solution of PI (number P-5264; Sigma) at a concentration of 2 mg/100 mL was kept at −20°C in 2-mL aliquots. Then, 0.5 mL of triactinomyxon suspension was combined with 0.25 mL each of freshly diluted FDA at a concentration of 30 μg/mL and PI at a concentration of 20 μg/mL. The mixture was gently rotated for 5 min and then diluted with 2 mL of a 20% (weight/volume) aqueous solution of bovine albumin (fraction V; Sigma). Stained materials were held in glass tubes on ice until they were examined with fluorescence microscopy (430× or under oil at 970× magnification). Microscope slides with stained spores can be stored at 4°C for 2–3 weeks in a humid chamber, or simply refrigerated, allowed to dry on the slide, and examined under oil. Viable and dead triactinomyxon spores were enumerated on the basis of a total of 100 spores counted. Fluorescent epispores of triactinomyxons containing sporozoites (spo-

roplasms) were considered viable if they stained bright green and dead if red. Those epispores containing few (two, three, or four) fluorescent sporozoites were not counted. Formalin-killed triactinomyxons served as dead controls.

Effect of temperature on viability of triactinomyxon spores in vitro.—The triactinomyxon spores were produced in tubificids as described by Markiw (1986). Experimentally infected worms were held in an aquarium (12.5°C) that was deprived of incoming spring water and left undisturbed; released spores were collected from the water column after 4 h and again after 16–18 h. Ten-milliliter aliquots of waterborne triactinomyxons in a suspension containing 50,000 spores/mL, determined with a hemocytometer, were distributed into four sets of eight glass tubes and held at 7, 12.5, 19–20, and 23–24°C. Spore viability was determined daily at room temperature (23–24°C) over 8 d. Before FDA–PI staining, tubes were centrifuged at 650 × gravity for 10–15 min at 4°C, and the supernatant fluid was withdrawn to leave 1 mL of concentrated spores in each tube. Staining was done in duplicates.

Exposure of fry to aged triactinomyxons.—Triactinomyxon spores collected from an aquarium that was left undisturbed for 16–18 h were distributed to eight containers. Each container held 1,800 mL of suspension containing triactinomyxons at a concentration of 1,000 or 10,000 spores per fish (14 or 140 triactinomyxons/mL; four containers each) to test the effects of aging spores for 1–4 d on infectivity. Spores were not added to two additional containers that served as negative controls. Containers were set in flowing water at 12.5°C. During 4 d, 25 healthy 2-month-old fry of rainbow trout *Oncorhynchus mykiss* reared at this laboratory from eyed eggs were exposed daily to one of the two concentrations of triactinomyxons for 2 h without aeration. The trout were then transferred to covered holding aquaria supplied with pathogen-free spring water at 12.5°C. Control fish not exposed to triactinomyxons were maintained in the same manner. Half of the trout were held for 5 months and half for 6 months. Then, pools of 10 fish heads were processed according to the enzymatic digestion and concentration method of Markiw and Wolf (1974), and isolated *M. cerebralis* myxosporean spores were enumerated.

Pretrials to test the effects of aging spores for 1–8 d on infectivity were conducted under the same conditions. Two concentrations of triactinomyxon spores were tested (10 and 1,000 triactinomyxons per fry) and exposed fish (pools of five heads) were examined 3 and 4 months later.

Results

A double FDA–PI staining that allows a form of reciprocal fluorochroming visible under fluorescence microscopy (living spores fluoresce in bright green; dead spores show up red) proved to be a rapid, reliable technique for determining the viability of spores. The results of this staining technique also correlated to some extent with those obtained by in vivo exposure of fish. Epispores, or upper portions of viable triactinomyxon spores, were stained bright green, and no intermediate faded fluorescence was observed. Few epispores showed only several reactive internal sporozoites. The three appendages of the spores did not react and remained unstained. The epispores of formalin-killed triactinomyxon spores stained red. Stained spores remained intact and retained fluorescence for at least 2–3 weeks.

The effects of temperature on the viability of laboratory-produced triactinomyxon spores, as determined by FDA–PI staining, is shown in Figure 1. The viability of triactinomyxon spores was retained the longest at the lowest temperature (7°C): 80% of the spores fluoresced at day 4, 10% were still viable at day 7, and all were nonviable by day 8. At 12.5°C, viability decreased more rapidly: only 50% survived by day 4, and only 10% survived by day 5. Spores were reactive for 2 d at 19–20°C and for only 1 d at 23–24°C.

The results of exposure of rainbow trout fry to two concentrations of triactinomyxon spores aged for 1–4 d are shown in Table 1. Typical clinical signs of whirling disease, such as whirling and black tails, developed in fish that were each exposed to 10,000 triactinomyxon spores aged for 1 or 2 d or to 1,000 triactinomyxon spores aged for 1 d. Fish developed light infection, but without clinical signs, after exposure to either concentration of triactinomyxons aged for 3 d; however, *M. cerebralis* spores were not found in fish exposed to triactinomyxons aged for 4 d. All unexposed control fish tested negative.

Pretrial tests of fish exposed to a concentration of 10 triactinomyxon spores per fish with spores aged for 1 d revealed about 125 *M. cerebralis* spores in five fish (25 spores per fish) examined 3 months after initial exposure, and no spores in 10 fish examined 1 month later. Tests after exposure of fish to a higher concentration of 1,000 triactinomyxon spores per fish revealed 31,000 myxo-

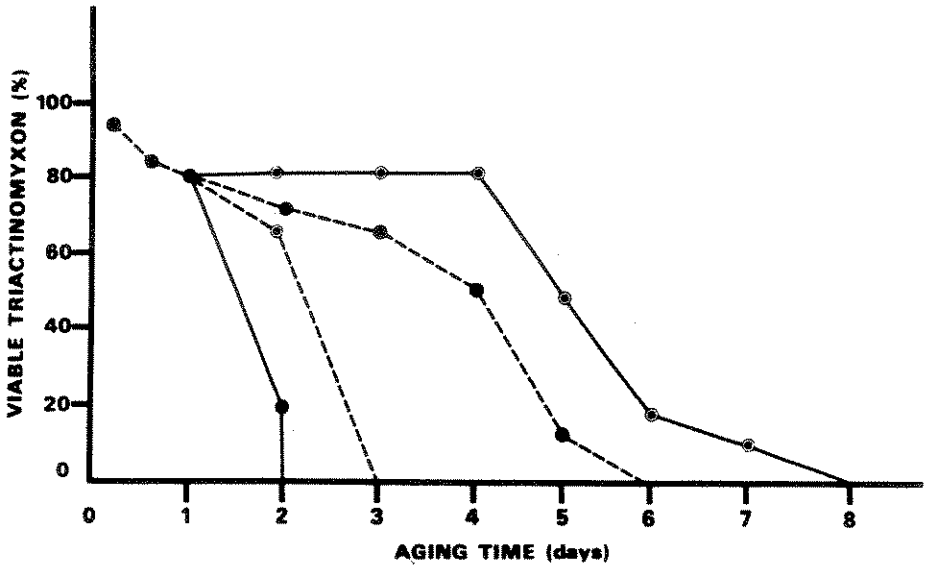

FIGURE 1.—Effect of temperature on viability of triactinomyxon spores of *Myxobolus cerebralis*, as determined by vital staining with fluorescein diacetate and propidium iodide. ⊙ —— ⊙ = 7°C; ● ----- ● = 12.5°C; ⊙ ----- ⊙ = 19–20°C; ● —— ● = 23–24°C.

sporean spores per fish at 3 months and 68,000 spores per fish at 4 months postexposure.

Discussion

These results demonstrate that the causative agent of salmonid whirling disease, the actinosporean triactinomyxon spore, persists only for a few days in water and has a decreasing ability to infect trout with increasing water temperature.

The triactinomyxon spores of *M. cerebralis* retained their infectivity to 2-month-old rainbow trout for 3 d, which correlated to some extent with spore viability determined by FDA–PI staining. Triactinomyxon spores aged for 1 and 2 d showed 80 and 70% viability by staining and produced the expected increase (approximately 10-fold) in the number of myxosporean spores in fish when the exposure dose increased from 1,000 triactinomyxon spores per fish to 10,000 spores per fish, as shown in Table 1. This correlation disappeared when spores were aged for 3 and 4 d, because these spores rapidly lost their infectivity. The triactinomyxon spores aged for 3 d, although showing 65% viability by staining, infected fish very lightly, particulary at the lower exposure dose (1,000 spores per fish). Spores aged for 4 d did not infect fish even at the higher dose (10,000 spores per fish), even though staining revealed 50% viability. This can be explained if, during the critical time when triactinomyxon spores are rapidly losing their ability to infect fish, the vital fluorescein diacetate stain can still penetrate the spore membrane and show fluorochromasia. Using a medium controlled at physiological pH during staining could contribute to such a reaction.

Low concentrations of infective units appeared to result in uninfected individuals (as seen in the preliminary study) or infections with a low number of myxosporean spores per fish (Table 1; 1,000

TABLE 1.—Infectivity of triactinomyxon spores of *Myxobolus cerebralis* aged for 1–4 d, as determined by exposure of 2-month-old rainbow trout fry to aged spores for 2 h at 12.5°C. Infectivity was measured as the number of *M. cerebralis* myxosporean spores found in fish 5 and 6 months after exposure.

Exposure (number of triactinomyxon spores per fish)	Age of spores at time of exposure (d)	Approximate number of viable spores based on results of vital staining	Number of myxosporean spores per fish[a]	
			5 months after exposure	6 months after exposure
1,000	1	800	33,250[b]	123,080[b]
	2	700	17,330	15,770
	3	650	190	230
	4	500	0	0
10,000	1	8,000	302,450[b]	1,713,750[b]
	2	7,000	236,380[b]	828,640[b]
	3	6,500	12,350	14,170
	4	5,000	0	0

[a] Value shown is the calculated average per 10 fish.
[b] Clinical signs of whirling disease were present.

triactinomyxon spores per fish; spores aged 3 d). Lom (1987) reviewed the basic features of several myxosporean life cycles and their mode of entry into the host. He stated that myxosporeans usually have primary cells that eventually disintegrate and release secondary cells that may repeat the cycle, go into a sporogonic phase, or be destroyed by the host tissue reaction.

In conclusion, the use of fluorescein diacetate vital staining in combination with propidium iodide proved to be a rapid technique for distinguishing viable triactinomyxon spores. The vital staining method applied to actinosporean, myxosporean, or microsporean spores should be useful for screening the candidate therapeutants for their effectiveness in controlling diseases of fish, instead of performing time-consuming in vivo exposures.

Acknowledgments

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Myxosoma cerebralis: Isolation and Concentration from Fish Skeletal Elements — Sequential Enzymatic Digestions and Purification by Differential Centrifugation

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MARKIW, M. E., AND K. WOLF. 1974. *Myxosoma cerebralis*: isolation and concentration from fish skeletal elements — sequential enzymatic digestions and purification by differential centrifugation. J. Fish. Res. Board Can. 31: 15–20.

An effective sequential procedure for recovery of *Myxosoma cerebralis* spores from infected trout was developed, and quantification of spores was carried out at each step of release and concentration. Methods are described for fresh and frozen material. Effective concentration of from 1100- to 9000-fold and an estimated efficiency of recovery of about 80% has been achieved. Tabular and graphic data are presented with recommendations for diagnostic applications. The immediate applications of these procedures are in implementing more effective detection and in preparing antigen for the immunologic studies that should provide the most sensitive detection.

MARKIW, M. E., AND K. WOLF. 1974. *Myxosoma cerebralis*: isolation and concentration from fish skeletal elements — sequential enzymatic digestions and purification by differential centrifugation. J. Fish. Res. Board Can. 31: 15–20.

Nous avons mis au point une technique séquentielle pour la récupération des spores de *Myxosoma cerebralis* chez des truites infectées, et en avons effectué la quantification à chaque palier de libération et de concentration. Nous décrivons des méthodes applicables à du matériel frais et congelé. Nous avons réussi une concentration effective de 1100–9000 fois plus grande et une efficacité de récupération d'environ 80%. Nous présentons des données sous forme de tableaux et de graphiques, avec recommandations en vue d'une application diagnostique. Les applications immédiates de ces techniques sont, d'une part, une détection plus efficace et, d'autre part, la préparation d'un antigène pour des études immunologiques, qui donnerait la détection la plus sensible.

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Myxosoma cerebralis is a myxosporidian parasite of European origin that has been introduced into North America, and is now established in the effluent waters in several hatcheries and presumably in waters stocked with hatchery-infected fishes. Depending upon the severity of infection and upon salmonid host species — notably in rainbow trout (*Salmo gairdneri*) and brook trout (*Salvelinus fontinalis*), *M. cerebralis* can produce mortality, cripple, or disfigure. The clinical disease is known as "black tail" or more commonly as "whirling disease," and is readily diagnosed on the basis of signs and behavior changes. Identification is based upon biophysical characteristics of the spores.

At present, histologic examination or microscopy for spores are the only techniques available for identification of *M. cerebralis* and hence for verification of whirling disease. Both approaches are time-consuming and have serious limitations in sensitivity of detection; the carrier state, pre-spore incubation stages, and light infections are all apt to be missed. As has been shown with many vertebrate pathogens, serologic procedures often provide a simple, rapid, and sensitive approach to detection and the most accurate identification. Methods that release and concentrate *M. cerebralis* spores with minimal residues of fish tissue or debris will greatly increase sensitivity of detection and also provide a means of obtaining antigen needed for developing serologic procedures. With that goal in mind, Landolt (1973) has developed a procedure whereby trypsin is used to release spores from decalcified skeletal elements and further biophysical steps are employed to minimize fish-tissue contamination and to concentrate spores. The methodology requires 18–24 h and effects upon spore antigenicity are as yet undertemined.

The purpose of our report is to describe an alternative and more rapid procedure for releasing and concentrating spores of *M. cerebralis*. The

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newer method is suitable either for young fish with predominantly cartilaginous skeletons or for older fish in which ossification has occurred.

Materials and Methods

Fish

Fresh and frozen infected fingerling rainbow trout were used for all work. Fresh, 4- and 5-month-old specimens were experimentally infected at 12.5 C by the method of Hoffman and Putz (1971). Frozen 4-month-old fingerlings with natural infection were also used. They were provided by the Pennsylvania Fish Commission, Benner Spring Fish Research Station, Bellefonte, Pa.

Removal of Soft Tissue and Mincing Cartilage

Fish were immersed in water of 45–50 C for 5–10 min (MacLean 1971; Taylor personal communication). Following heating, eyes, skeletal muscle, and adherent soft tissues were teased away. Gill arches were excised intact and pooled with the cleaned skeletal parts. Skeletal parts were cut into 3–5 mm pieces with scissors.

Peptic Digestion

Minced skeletal fragments were initially digested in freshly prepared pepsin (Pepsin, N.F.) 0.5% (w/v) at *p*H 1.8–1.85 in 0.5% HCl solution. One volume of 25–30 ml pepsin solution was used for each gram of skeletal fragments; digestion was carried out in 250-ml Erlenmeyer flasks containing three marbles, in a 37.5 C shaker bath for 1–3 h. When young fish were used, 1 h was adequate. Skeletal fragments from older fish required two to three times greater digestion time. If cartilage remained after 2 h, fluid was harvested and an equal volume of fresh pepsin added. Digestions tried at 23 C required 4–4.5 h. At the end of pepsin digestion, all material was centrifuged in a horizontal head at 22 C at 1200 *g* for 10 min. Supernatant fluid was aspirated and each volume of pelleted sediment was resuspended in 12.5–15 ml water for secondary digestion.

Tryptic Digestion

Secondary digestion was effected with purified trypsin 0.5% (w/v) dissolved in calcium- and magnesium-free Tyrode's solution as modified by Rinaldini (1959).[1] Trypsin solution was filtered through Whatman #114 filter paper. Phenol red at a concentration of 0.002% was added as a *p*H indicator.

Resuspended peptic sediments were mixed with equal volumes of trypsin solution to give a final concentration of 0.25%. The starting *p*H was adjusted to 8.0–8.5 with 1N NaOH. Digestion was at 22 C for 30 min with occasional gentle agitation.

[1]Composition: NaCl 8.0 g, KCl 0.2 g, $C_6H_5Na_3O_7$-$2H_2O$ (sodium citrate) 1.0 g, $NaH_2PO_4H_2O$ 0.05 $NaHCO_3$ 1.0 g, glucose 1.0 g, water to make 1000 ml.

Secondary digestion with 0.25% trypsin of material from fish that had been frozen resulted in excessive spore damage; during counting procedures spores were seen to disintegrate. A 0.05% concentration was tested and found to be satisfactory for use on material from frozen fish.

Tryptic digestion was arrested by addition of fetal bovine serum at a final concentration of 20% (v/v) or 1% (w/v) bovine serum albumin powder. Tryptic digests were filtered through a gauze-thin layer of glasswool, and centrifuged at 1200 *g* for 10 min at 22 C. Pelleted material was resuspended in 20–25 vol of half-strength Hanks' balanced salt solution containing 20% fetal bovine serum.

Purification, Isolation, and Concentration by Differential Centrifugation

Five ml of resuspended tryptic digest pellet were layered onto 3 ml of 55% (w/v) aqueous dextrose solution contained in a 12 ml conical centrifuge tube or for quantification, onto 1 ml dextrose in a 6.5-ml Shevky-Stafford albumin tube. Both were centrifuged at 1200 *g* for 30 min at 22 C. Five-gram aliquots of cartilage from healthy fish were finely minced, transferred to Erlenmeyer flasks, and seeded with 1000, 10,000, or 100,000 spores. Control cartilage was not seeded with spores. A parallel series of flasks without cartilage received the same numbers of spores. The contents of both sets of flasks were treated first with pepsin and then with trypsin. Residues were centrifuged through dextrose solution and aliquots were quantified for estimates of remaining spores.

Quantification

Spore number was determined from duplicate or quadruplicate counts of 0.4 mm^3 in a Neubauer hemacytometer. Only typical spores were counted: those that had uniform size and two clearly recognizable polar capsules of normal size. Spores were occasionally encountered with three or four polar capsules or with abnormally small capsules.

Efficiency of Recovery

There is no known procedure for establishing uniform infection with *M. cerebralis* or for determining beforehand the actual number of spores occurring in an infected fish. Accordingly, the only practical method of assessing the efficiency of recovery of our procedures was to seed uninfected cartilage fragments with known numbers of spores. To assure minimal degradation, spores for such seeding were obtained by mechanical means; they were gently rubbed from infected gill arches with a porcelain pestle. Debris was allowed to settle, and the suspended spores were decanted and counted.

Effects of Freezing and Storage on Spore Recovery from Cartilage

Cartilage from 7- to 8-month-old trout was pooled, finely minced, distributed in 2-g aliquots in screw-cap vials, and frozen at –2 C. A control aliquot was not

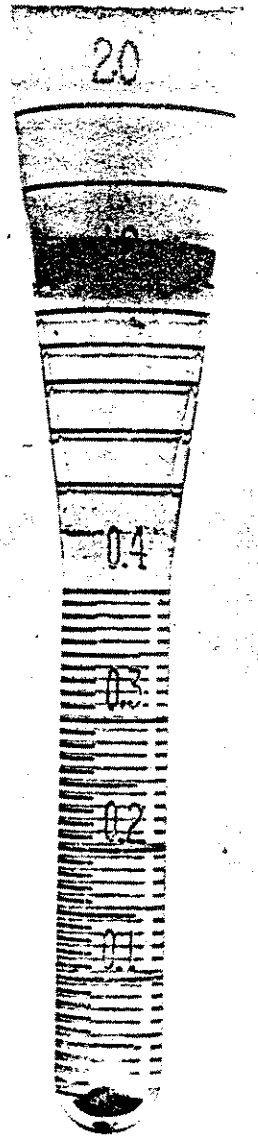

FIG. 1. Typical results of purification of *Myxosoma cerebralis* spores by centrifugation of tryptic digest residues layered onto 55% dextrose column. Spores pelleted at bottom of tube have passed through optically clear sugar solution, but dense band of debris and opaque material has remained at the top.

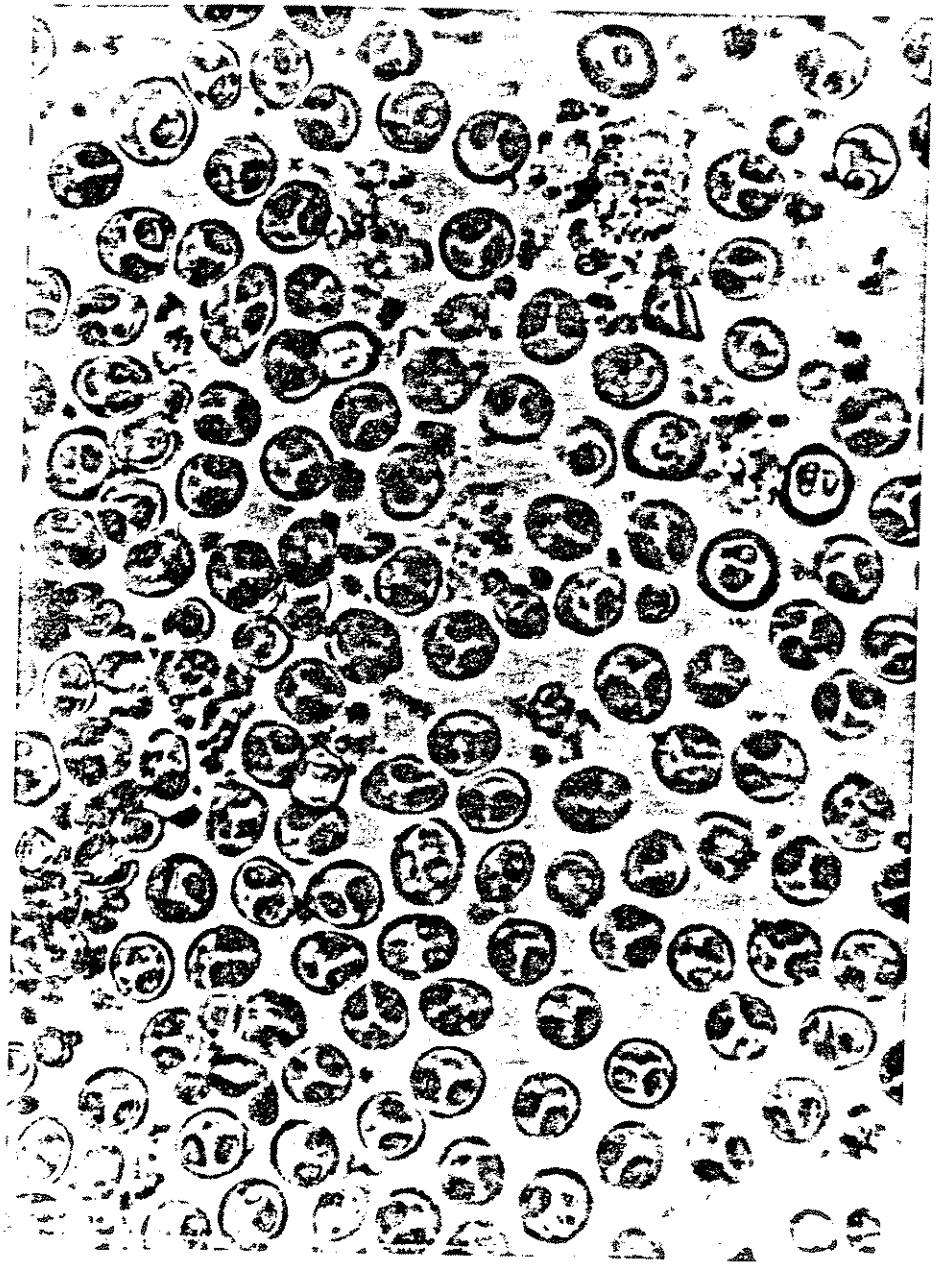

FIG. 2. Concentrated suspension of *M. cerebralis* spores obtained by sequential digestions and purification by centrifugation through 55% dextrose.

frozen but was processed immediately. Periodically, frozen samples were thawed and processed.

Results

Sequential enzymatic digestion of minced skeletal elements released spores of *M. cerebralis*, which were concentrated in a relatively pure condition by differential centrifugation through dextrose solution (Fig. 1 and 2). The total time required was about 8 h and the recovery was at least 80% efficient.

Removal of soft tissue from skeletal elements gave an 8.3–8.9-fold reduction of bulk and effected an equivalent spore concentration; the resulting cartilaginous-bony elements constituted about 12% of the total fish weight. Removal of soft tissue did, however, result in loss of some spores.

The incidence of spores in tissues in 10 4–5-month-old fingerlings was determined to provide guidelines for recovery and concentration. As determined by peptic digestion alone, excised gills contained from a trace to 96% of total spores (average 37%), the head itself, minus gills, contained 0–100% (average 27%), the axial skeleton minus the head contained 0–48% (average 16%), and soft tissues plus fins contained from 0–57% (average 20%). Most of the spores (an average of 79%) occurred in the axial skeletal elements (Table 1). That fact and the fact that digest residues from soft tissues made microscopic examination difficult lead to a decision to discard soft tissues from the balance of the study. However, soft-tissue removal was thereafter done gently to minimize spore loss from the surface of the head and axial skeleton.

Peptic digestion of cartilage uniformly resulted in a 9–11-fold additional spore concentration (Table 2). Cartilage from young fish (2–3-month-old) was digested in 1 h, and that from 3- to 4-

TABLE 1. Occurrence of *Myxosoma cerebralis* spores in various rainbow trout (*Salmo gairdneri*) tissues.

Fish Number	1	2	3	4	5	6	7	8	9	10	Average
Tissue											
Gills	19%	18%	42%	42%	26%	20%	59%	45%	Trace	96%	37%
Head	10%	39%	31%	10%	17%	47%	8%	11%	100%	None	27%
Spinal column	48%	34%	13%	None	None	33%	15%	17%	Trace	4%	16%
Soft tissues	23%	9%	17%	48%	57%	None	18%	27%	None	None	20%
Percentage in skeletal elements	77%	81%	83%	52%	43%	100%	82%	73%	100%	100%	$\bar{m}$ 79%
Number of spores per fish $\times 10^5$	20.3	12.4	13.5	2.3	2.9	2.8	4.5	1.5	0.2	0.9	

[a]Spores detected after centrifugation through dextrose. Pellet resuspended in 0.1 ml and an 0.8-μl aliquot counted. Number $\geq$ two spores per aliquot.

TABLE 2. Comparative yield of *M. cerebralis* spores during release and concentration sequence.

Material	Series	Weight or volume I[a]	Weight or volume II[b]	Spores per ml I	Spores per ml II	Concentration factor I	Concentration factor II
Intact fish		57.60 g	339.00 g	Unknown	Unknown	None	None
Minced skeletal parts		6.70 g	40.40 g	4.4×10^4	6.5×10^4	8.6×	8.3×
Peptic digest residue		0.60 ml	4.5 ml	6.1×10^6	5.8×10^6	11.2×	9.0×
Tryptic digest residue		0.22 ml	2.3 ml	3.2×10^7	2.0×10^7	2.7×	2.0×
Pellet after differential centrifugation through dextrose		0.5 ml	0.04 ml	1.1×10^8	1.2×10^8	4.4×	61.3×
					Cumulative concentration	1144×	9041×

[a]Series I data are mean values from three trials each with five fish.
[b]Series II data are mean values from one trial with 25 fish.

month-old fish required about 2 h. Seven- to 8-month-old fish required the longest time; pepsin solution was changed after 2 h and an additional charge of fresh pepsin was allowed to act for 30–60 min to effect complete digestion — a total time of 2.5–3 h.

Tryptic digestion resulted in release of additional spores; this was particularly true when older fish were used. This step gave about a two- to three-fold greater concentration than was present in peptic digest pellets. As shown in Fig. 3, trypsin concentration and time both affected spore recovery.

Centrifugation through dextrose, the final step in the recovery sequence, varied widely in degree of concentration. At a minimum, there was a four-fold concentration, at a maximum, 60-fold.

Quantification of the entire process showed an effective spore concentration per unit volume of from 1100 to 9000 times that present in the intact fish. This does not take into account the spores known to be present in soft tissues that were discarded during recovery of cartilage.

The efficiency of recovery from normal cartilage seeded with 2000 or 20,000 spores per ml and processed with pepsin, trypsin, and dextrose centrifugation was 81.5–86.75% (Table 3). A series of 32 counts gave a mean recovery of 83% with a standard deviation of ±1.12%. When the initial spore level was only 200 per ml, a few spores were always recovered, but the percentage was too low to permit valid calculation of the efficiency of recovery.

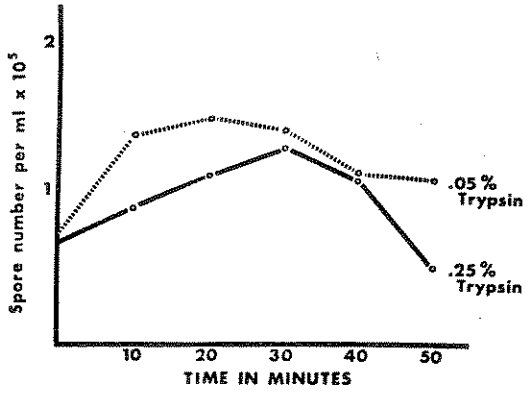

FIG. 3. Effects of trypsin concentration and time on release of spores from resuspended peptic digest pellets.

TABLE 3. Results of recovery efficiency tests using normal cartilage seeded with *M. cerebralis* spores.[a]

Number of spores seeded	Mean number recovered	Mean percentage recovery
Replicate 5-g aliquots of minced normal cartilage in 125 ml pepsin solution		
1,000	<2 per .4 mm³[b]	<0.2%
10,000	8,175	81.75%
100,000	82,250	82.25%
Replicate 125 ml aliquots of pepsin solution without cartilage		
1,000	<2 per .4 mm³	<0.2%
10,000	8,150	81.5%
100,000	86,750	86.75%

[a]Mean values for two trials. Dextrose pellet was resuspended in 0.5 ml for counting.

[b]At most, two spores were found in any 4-mm² field. Accordingly, dilution factor calculations would result in spore estimates that exceeded the number actually seeded.

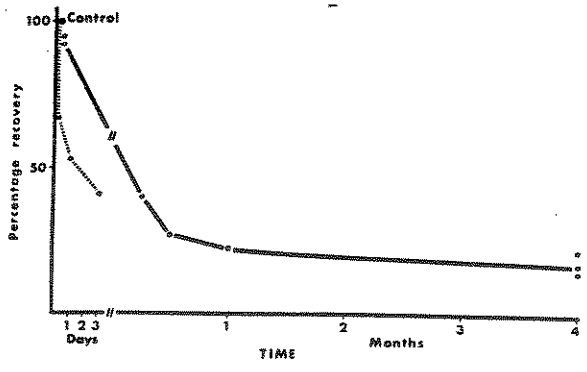

FIG. 4. Effect of freezing and storage on spore recovery by centrifugation through dextrose.

When frozen specimens were used as a source of spores, centrifugation in 55% dextrose solution was found to distort the normal shape of the sporoplasm and many damaged spores remained near the top of the liquid. Accordingly, the effect of freezing and thawing as it related to damage in dextrose solution was investigated.

Freezing at –2 C and thawing within 1 h reduced the recovery by one-third. Additional loss occurred during longer storage (Fig. 4). By 72 h, 60% of the spores were damaged beyond recovery. Still longer storage resulted in even greater damage, and after 4 mo the mean of three samples showed that only 18% of the spores could be recovered intact, with apparently normal morphology.

Discussion

The procedures reported here provide a highly efficient means of releasing and concentrating *M. cerebralis* spores in a relatively pure condition.

The significance of the work is at least twofold. It provides a means of preparing spore antigen that can be tested for applicability in immunological–serological studies. Secondly, the methodology greatly increases the probability of detection when spores are present in small numbers. This quantified sensitivity has no parallel in published reports on *M. cerebralis*.

In its entirety, the sequence of spore release and concentration requires up to 8 h, but that is still only 40–80% of the time reported by Rydlo (1971). Moreover, for diagnostic and detection work the entire sequence need not be carried out; instead, spores can be sought at each step. The methods have further value in being applicable, with suitable precautions, to frozen specimens.

The finding of microsporidian parasites in certain of the specimens is evidence that the procedures will be applicable to other small parasites.

Recommendation for Detection, and Diagnostic Applications

Based upon the present need for reliable means of detecting *M. cerebralis* spores, the following procedures are recommended:

1. Gill arches should be removed, grossly trimmed of their filaments, and the cartilage itself gently crushed in a mortar and pestle. The crushed tissue should be mixed with 5 vol of water, the gross fragments allowed to settle for 1 or 2 min, a sample taken from the top of the supernatant fluid and examined for spores. If spores are not quickly found, specimens should be heated and the skull and axial skeleton processed. About 1 min is required to strip soft tissue from a heat-treated fingerling.

2. Gill arches and skeletal elements should be minced and digested with pepsin, and the digest fluid examined for spores. From 1 to 3 h are required for pepsin digestion, depending upon fish age. If no spore is found, the total volume should be centrifuged, the pellet resuspended in about 10 vol of water and also examined. Spores will usually be found in this step, even in very lightly infected fish, but if not, digestion with trypsin should be carried out.

3. The peptic digest residue should be trypsinized for about 30 min, then serum added to a final concentration of 20% or powdered bovine serum albumin to a final concentration of 1% to stop tryptic action. At this time, the maximum number of spores will have been released, and the fluid should be examined microscopically.

4. In cases where very small numbers of spores are present, they will be difficult to find in the relatively diluted trypsin solution, and debris will interfere with microscopic search. The neutralized tryptic digest should be centrifuged, the pellet resuspended in about 20 vol of half-strength physiological salt solution containing either 20% serum or 1% bovine serum albumin. The resuspended pellet should be carefully layered onto 55% dextrose solution in 12 ml conical centrifuge tubes at the rate of 5 ml per 3 ml of solution. After centrifugation, the spores will be pelleted and easily found in microscopic examination.

Where spore detection in frozen specimens is critical, trypsin should be employed only at a 0.05% concentration, and centrifugation through dextrose should not be used because it will result in excessive spore damage and loss.

For diagnostic purposes, the material that has been pelleted through dextrose solution should be examined promptly. If spores are to be kept for other purposes,they should be resuspended in half-strength physiological salt solution with 20% serum or 1% bovine serum albumin.

Acknowledgments

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Myxosoma cerebralis: Comparative Sensitivity of Spore Detection Methods

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MARKIW, M. E., AND K. WOLF. 1974. *Myxosoma cerebralis:* comparative sensitivity of spore detection methods. J. Fish. Res. Board Can. 31: 1597–1600.

Physical and biophysical methods of detecting spores of *Myxosoma cerebralis* were used in sequence on 87 individual fingerling rainbow trout (*Salmo gairdneri*) from two populations with a low incidence of infection. Physical methods of releasing spores from the organs of equilibrium, gill arches, or the axial skeleton gave an estimated rate of infection of 2.3–4.6%. Each succeeding step — pepsin digestion, trypsin digestion, and differential centrifugation through 55% dextrose solution — revealed additional infected fish. The final step of the sequence detected 10.5 times more infected fish than were found by examination of the organs of equilibrium or the axial skeleton with physical means; the true incidence was at least 24.1%. Procedures are described for using biophysical methods on 60-fish pools for hatchery inspection work and also on the more bony skeletons of mature fish.

MARKIW, M. E., AND K. WOLF. 1974. *Myxosoma cerebralis:* comparative sensitivity of spore detection methods. J. Fish. Res. Board Can. 31: 1597–1600.

Nous avons utilisé en succession des méthodes physiques et biophysiques pour détecter les spores de *Myxosoma cerebralis* sur 87 alevins individuels de la grosseur du doigt de truite arc-en-ciel (*Salmo gairdneri*) provenant de deux populations à basse fréquence d'infection. Les méthodes physiques de libération des spores des organes d'équilibre, des arcs branchiaux ou du squelette axial donnent un taux d'infection estimé de 2.3–4.6%. Chaque étape successive — digestion à la pepsine, digestion à la trypsine et centrifugation différentielle à travers une solution de dextrose à 55% — découvre encore plus de poissons infectés. L'étape finale de la série révèle 10.5 fois plus de poissons infectés que l'examen des organes d'équilibre ou du squelette axial par des moyens physiques; la fréquence réelle est d'au moins 24.1.% Nous décrivons la marche à suivre pour utiliser les méthodes biophysiques sur des bassins de 60 poissons lors de l'inspection d'établissements de pisciculture, de même que sur les squelettes plus osseux des poissons mûrs.

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To control *Myxosoma cerebralis*, the cause of whirling disease, one must be able to determine whether a population harbors infected individuals. Clinical whirling disease is readily diagnosed and the spore stage, critical to accurate identification, easily recognized. In contrast, subclinical infection, early incubation stages, the carrier state, and low incidences in a population are difficult to detect. Although serological or immunological methods may eventually provide definitive and sensitive detection, only physical, histological, and biophysical methods of detection are now available (MacLean 1971; Prasher et al. 1971; Rydlo 1971; Landolt 1973; Halliday 1973; Markiw and Wolf 1974). Of these, the biophysical methods offer decided advantages because they incorporate digestion to release spores gently for concentration and offer a practical approach to use of pooled samples without risk of loss of sensitivity. The methods vary in the time required for completion of the procedures; they have not been compared for sensitivity, and both factors are important in control programs.

The purpose of our work was to compare several physical methods with biophysical methods for sensitivity of spore detection.

Materials and Methods

FISH

We used rainbow trout (*Salmo gairdneri*) with low population incidences of *M. cerebralis* infection, but none of the fish showed any clinical signs of whirling

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disease. There were four lots of experimentally infected trout — survivors of other research — and one lot of naturally infected trout from a hatchery with a history of whirling disease (Table 1).

Sampling procedure for comparison of physical and biophysical methods

Although they were in four tanks, the 256 fish of Lot I were considered to be a single population. According to calculations given by Ossiander and Wedemeyer (1973), in a population of 250 animals having a disease incidence of 5%, one must sample 49 fish to detect at least one infected individual at the 95% confidence level. We sampled 65 of the 256 trout. All 22 fish of Lot II were individually assayed.

To promote uniformity in testing, one person performed all detection procedures. Four specimens were processed each day, and separate sets of instruments and laboratory ware were used for each fish. After each day's work, instruments and equipment were washed and held in 1:1 (v/v) dilution of household bleach (5.25% sodium hypochlorite) to destroy spores and to prevent their carry-over to the next day's work. A "single blind" approach was used to minimize bias; all specimens were coded and their health or disease status was unknown to the processor.

Stepwise sequence in comparison of physical and biophysical methods

Each fish, in its own container, was killed with anesthesia, measured, weighed, held at 45 C for 3–5 min, and defleshed. The organs of equilibrium and gill arches were removed, the total axial skeleton was weighed, and the material processed in six consecutive steps. A uniform protocol for the spore search was followed throughout the study. Samples of the fluid materials resulting from each of the steps (corresponding with a detection method) were placed in a hemacytometer and both of the ruled areas (1.8 mm^3) were methodically searched at 430× until 5 min had elapsed or spores were found, whichever occurred first. Only typical intact spores having two polar capsules were counted. Occasionally, when only one or two spores were detected in a preparation, identification was confirmed by a second observer.

Table 1. Length, weight, and age of rainbow trout (*Salmo gairdneri*) used.

Lot no.	Length (*cm*)	Wt (g)	Age (*mo*)	Kind of infection
I	6 to 14	4.5 to 36	5	Experimental
II	8 to 14	7 to 32	5	Experimental
III	11 to 14	20 (avg)	13	Natural
IV	10 to 12	25 (avg)	8	Experimental
V	16 to 28	191 (avg)	18	Experimental

Step 1. (Organs of equilibrium method)
Organs of equilibrium were crushed in a mortar, and held in 1 ml of 1% HCl for 20 min.

Step 2. (Gill arches method)
Gill arches were trimmed of filaments, crushed in 2–3 ml H_2O, and allowed to settle 1–2 min.

Step 3. (Axial skeleton method)
Axial skeleton was combined with residues of Step 1 and 2, crushed in 10 ml 0.5% pepsin (w/v) at pH 1.8–1.85 in 0.5% HCl, and allowed to settle 3–5 min.

Step 4. (Pepsin–trypsin–dextrose centrifugation method [Markiw and Wolf 1974])
Residue from Step 3 was brought to 20 ml with pepsin solution and shaken at 37 C for 1–1.5 h. Collected fluid was centrifuged at 1200 × *g* for 10 min at 22 C. The pellet was resuspended in 2 ml water.

Step. 5. Residue from Step 4 was brought to 4 ml and trypsinized. The pellet was resuspended in 2 ml half-strength Hanks' balanced salt solution (BSS), containing 1% bovine albumin fraction V (BSA).

Step 6. Suspended residue from Step 5 was layered onto 1 ml of 55% dextrose in a Shevky-Stafford tube and centrifuged at 1200 × *g* for 30 min at 22 C. The pellet was resuspended in 0.1–0.2 ml half-strength Hanks' BSS with BSA.

Comparison of two biophysical methods

Defleshed axial skeletons from 20 trout from Lot I were pooled, minced, and divided into four 17.5-g samples. Two of the samples were processed with the trypsin method of Landolt (1973) and two with the pepsin–trypsin–dextrose method of Markiw and Wolf (1974). Final residues of each sample were collected in Shevky-Stafford tubes, resuspended in 0.1 ml half-strength BSS, and searched for spores.

Modifications of pepsin-trypsin-dextrose method for large-scale sampling and for use on mature trout

As reported by Markiw and Wolf (1974) about 60% of *M. cerebralis* spores are localized in the head and gills of infected fish. Consequently, only defleshed heads were used in adapting the pepsin–trypsin–dextrose methodology to the kind of population sampling now being done in hatchery inspection work.

Two trials were run in which 60 rainbow trout heads were pooled and processed as a single batch. Processing was carried out according to procedures of Markiw and Wolf (1974) except that, after digestion with trypsin, the material was filtered through a thin layer of glass wool to remove gross particulate matter before concentration and differential centrifugation. Total weight of defleshed and cranial elements was 86 g for 60 Lot IV fish, and 111 g for 60 Lot III fish.

The pepsin–trypsin–dextrose method was also applied to material from two pools of 20 mature fish (Lot V). The only departure from the original de-

scribed procedure was the use of a meat grinder to mince the axial skeletal elements.

Results

Comparison of physical and biophysical methods

Biophysical methods of spore release and concentration revealed more infected fish than did physical methods (Table 2). The pepsin–trypsin–dextrose centrifugation method revealed 21 infected fish among the 87 examined. The biophysical method was 10.5 times more sensitive than the examinations of organs of equilibrium and axial skeleton (each of which revealed two infected fish) and 5.2 times more sensitive than the examination of gill arches (which revealed four infected fish).

The least sensitive physical methods yielded incidences of infection for Lot I and II of 3.1 and 0%, respectively; the most sensitive method (biophysical) gave incidences of 26.2 and 18.1%, respectively.

As stated by Ossiander and Wedemeyer (1973), sample sizes derived in their table were determined for infectious pancreatic necrosis (IPN), and the program could be varied for such factors as population size and disease incidence, and used for other diseases. Accordingly, using the basic program for IPN, the carrier incidences detected by the various methods and also the number of trout in the two populations (278) were inserted and the program was rerun. The analysis showed that at the 95% confidence limits for a population of 278 the numbers of trout that would have to be examined to detect spores by the different methods were as follows: organs of equilibrium or axial skeleton, 94; pepsin digestion, 36; pepsin and trypsin digestion, 18; and enzymatic digestion plus centrifugation through dextrose, 9. The number of trout used in the present study was sufficient to detect spores in the organs of equilibrium or axial skeleton at 90% (but not 95%) confidence limits.

Comparison of trypsinization with pepsin–trypsin–dextrose method

The pepsin–trypsin–dextrose method proved to be more sensitive than the use of trypsin alone. Although a few spores were present in residues from the latter, only a single spore from one sample was found within the ruled grids of a total of eight hemacytometer chambers. In contrast, both samples from the pepsin–trypsin–dextrose method showed spores in the ruled areas of every one of four counting chambers; the averages were 8 and 10.5.

Large-scale sampling and assay of mature specimens

The pepsin–trypsin–dextrose procedures worked effectively in large-scale sampling. A total of 8800 spores was recovered from the 60 experimentally infected trout examined, or an average of 147 spores/head. Although physical methods failed to show spores in the naturally infected fish, the sequential processing released a total of 93,000 spores or an average of 1550/head.

For pooled samples of 60 fish, 6–7 h were required for processing, divided among four steps: 1) decapitation, heating, and defleshing, 1.5 h; 2) pepsin digestion, 3–4 h; 3) trypsin digestion,

Table 2. Comparison of the incidence of *Myxosoma cerebralis* spores detected by physical and biophysical methods.

		Physical methods			Biophysical methods		
Lot no.	Fish no.	Organs of equilibrium	Gill arches	Axial skeleton	Pepsin digestion	Pepsin and trypsin digestions	Pepsin and trypsin digestions + dextrose centrifugation
I	1–15	0	0	0	0	0	0
	16–33	0	1	0	1	1	3
	34–51	2	3	2	4	8	12
	52–65	0	0	0	1	2	2
	Subtotals	2 (3.1%)	4 (6.2%)	2 (3.1%)	6 (9.2%)	11 (16.9%)	17 (26.2%)
II	1–22	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (4.5%)	4 (18.1%)
Totals							
No. 87		2	4	2	6	12	21
Percentage		2.3%	4.6%	2.3%	6.9%	13.8%	24.1%

0.5 h; and 4) concentration and centrifugation through dextrose, 1.0 h.

Skeletal elements from mature (Lot V) trout were readily digested. Spore counts were high, averaging 6700/fish in one pool and 10,000/fish in the second pool.

Discussion

Our results indicate that the pepsin–trypsin–dextrose processing sequence is the most sensitive procedure yet developed for detection of spores of whirling disease. Although developed for use on young (fingerling) fish, the method also worked well with mature trout whose skeletons had more bone. Trials in which pools of 60 fish heads from populations with low incidences of *M. cerebralis*, through either natural or experimental infection, showed clearly the suitability of the procedures in practical hatchery inspection work.

Although the total elapsed time required to complete processing 60 fish heads is 6–7 h, the actual working time is about half of that. In hatchery inspection work, the intervening time can be used for bacteriological, parasitological, and virological examinations. As now conducted by federal personnel, the inspection of hatcheries for *M. cerebralis* requires 4–8 h, and much of the time is spent at a microscope (L. L. Pettijohn personal communication). The procedures we describe require less working time and provide a far greater measure of confidence in detection.

Provided that the infection process has progressed to the stage of spore development, the results suggest that the pepsin–trypsin–dextrose method is broadly applicable, as in surveillance, where light infection is sought among mature fish or among fingerlings or fry.

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Myxosoma cerebralis: Fluorescent Antibody Techniques for Antigen Recognition

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MARKIW, M. E., AND K. WOLF. 1978. *Myxosoma cerebralis:* fluorescent antibody techniques for antigen recognition. J. Fish. Res. Board Can. 35: 828–832.

Rabbits were immunized with antigens extracted from mature spores or prespore stages of *Myxosoma cerebralis*, and the resulting antisera and their globulins were used in direct and indirect fluorescent antibody techniques. Both kinds of antisera reacted with homologous spores and with stages of the organism that precede spores. When tested for specificity against spores of 12 other myxosporidans the direct fluorescent antibody technique showed cross-reactivity with only one other and that was a species of *Myxosoma*. The indirect fluorescent antibody technique showed some reactions across generic lines. The antisera have application in studies of the parasite's life cycle and in diagnostics.

Key words: spores, parasites, direct fluorescent antibody techniques, indirect fluorescent antibody techniques, diagnosis, myxosporidans, whirling disease

MARKIW, M. E., AND K. WOLF. 1978. *Myxosoma cerebralis:* fluorescent antibody techniques for antigen recognition. J. Fish. Res. Board Can. 35: 828–832.

Des lapins ont été immunisés par antigènes extraits de spores mûres ou de stades de préspores de *Myxosoma cerebralis*. Les antisérums qui en résultèrent et leurs globulines ont été ensuite utilisés dans des techniques d'anticorps fluorescents directes et indirectes. Les deux genres d'antisérums réagissent avec des spores homologues et avec des stades de l'organisme précédant les spores. Lorsque essayée en vue de déterminer la spécificité contre les spores de 12 autres myxosporidies, la technique d'anticorps fluorescents directe montre une réactivité réciproque avec seulement un autre, une espèce de *Myxosoma*. La technique d'anticorps fluorescents indirecte réagit avec certains organismes au-delà des frontières génériques. Les antisérums trouvent une applications dans des études du cycle biologique et dans le diagnostic du parasite.

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THE life cycle of *Myxosoma cerebralis*, cause of salmonid whirling disease, is a challenging puzzle to parasitologists and because it is so poorly understood, one of the more difficult problems of those who are concerned with matters of fish health. *Myxosoma cerebralis* has been known for more than 70 yr, but as is true for all myxosporidans, the greater part of its life cycle is an enigma (Fig. 1). Fundamental properties of myxosporidans are in some dispute; most parasitologists consider them to be protozoans, but others note bilateral symmetry and multinucleation — features found in metazoans. Also, with the exception of the term *spores* for the mature stage in the fish, there is a notable lack of unanimity in terminology used for the morphologically different life stages that are presently recognized.

The fish health specialist can diagnose frank whirling disease from its clinical signs and by finding spores that have the size and shape of *M. cerebralis*. In such cases, spores are abundant and easily found. When the incidence of infection is low or if the intensity is light, spores are not abundant. Therefore, the diagnostician would have a difficult task were it not for several procedures that were developed to release and concentrate spores (Landolt 1973; Markiw and Wolf 1974; O'Grodnick 1975). However, whirling disease has a long incubation and at least 3 mo elapse before spores are formed (Fig. 1). During that time, few persons are qualified to make a critical diagnosis, i.e. a positive identification; and such identifications are presumptive.

Serological–immunological methods are widely used to substantiate diagnoses of bacteriological and virological diseases of fishes and the methods have great potential for being keys to the secrets of the *M. cerebralis* life cycle. In addition, the methods can provide the general fish diagnostician with up-to-date means of identification — methods that augment and in time will replace morphometry. Because serological methods can reveal biological relationships, systematists too can profitably use them.

Pauley (1974) was the first to apply immunological methods to *M. cerebralis*. He sought a way of detecting asymptomatic carriers, and found that spore extracts

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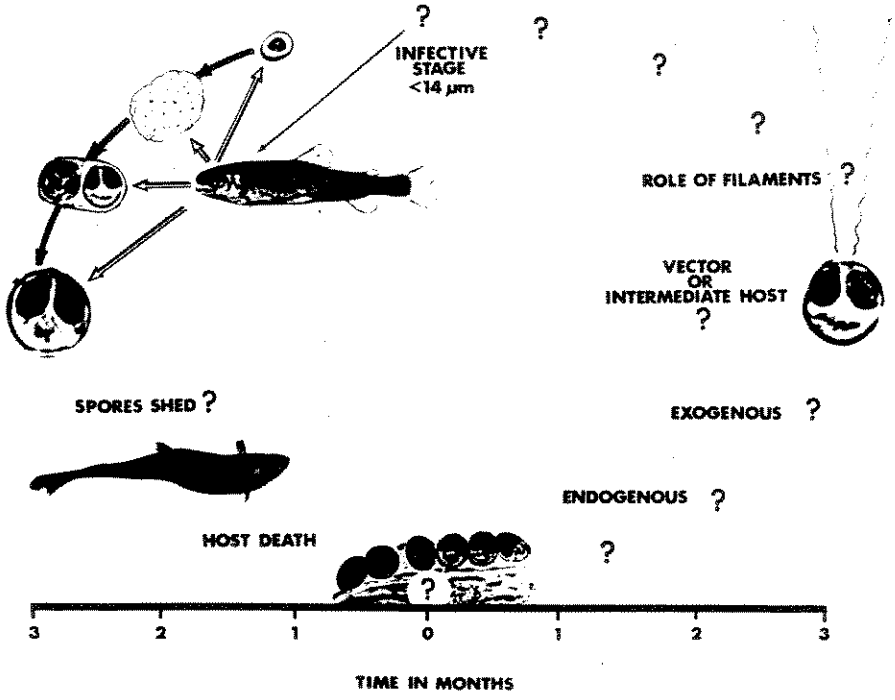

FIG. 1. Schematic life cycle of *Myxosoma cerebralis*. Bicapsular multinucleate spores form in host fish skeletal elements and are assumed to be the mature stage of the organism. Starting at *bottom center*, mature spores enter the environment by host death and decomposition, or defecation by fish-eating predator, or possibly shedding by the host fish itself. Through completely unknown processes requiring at least 3 mo, each spore ultimately gives rise to one or more infective stages. The process may be endogenous or it may involve an intermediate host. The enigmatic coiled filaments within the polar capsules undoubtedly play a crucial role in the life cycle. Virtually nothing is known of the infective stage or of how and where it enters its fish host (*top left*). Several weeks or more after infection, foreign bodies may be found in cartilage and in time give rise to multinucleate stages — whether or not a sexual stage exists is unknown. At a minimum of 3 mo from first infection, sporulation occurs and spores persist for the life of the host. About two thirds of the minimum life cycle is unknown.

were antigenic in rabbits but not in rainbow trout (*Salmo gairdneri*). He presented evidence to show that the parasite's antigens mimicked those of rainbow trout even though the proteins were immunoelectrophoretically different. Halliday (1974) used indirect fluorescent antibody techniques (FAT) and confirmed the reactivity of rabbits to immunization with *M. cerebralis*. However, neither naturally nor experimentally infected rainbow trout showed an immune response, and he concluded that FAT could not be used for diagnosis of whirling disease. Halliday (1974) did find that rabbit antiserum did not react with spores of an unidentified species of *Myxobolus*, and he suggested that serology might be of value in clarifying myxosporidan taxonomy.

The antigenicity of *M. cerebralis* spore extracts in rabbits has been amply demonstrated. We think it likely that some of the parasite's antigens persist throughout all stages of the life cycle, therefore our objective was to ascertain whether fluoresecent antibody techniques would provide specific serological recognition of antigens in spores and in other life stages of *M. cerebralis*.

Materials and Methods

SOURCE AND PREPARATION OF ANTIGENS AND ANTISERA

The method of Markiw and Wolf (1974) was used to release and concentrate spores, as well as prespore stages from experimentally infected rainbow trout. Prespore stages were predominantly ovoid forms, and in unstained wet mounts they showed no obvious internal structures. The method of harvesting spores was modified slightly in that 0.05% (wt/vol) soybean trypsin inhibitor was substituted for bovine serum albumin.

TABLE 1. Summary results of direct and indirect fluorescent antibody tests on spores of 13 myxosporidans reacted with rabbit antiserum against *Myxosoma cerebralis*.

Organism	Test	
	Direct	Indirect
Myxosoma cerebralis	+	+
M. cartilaginis	+	−
M. cephalus	−	+
M. cyprini	−	+
Myxobolus insidiosus	−	+
M. argenteus	−	−
M. sp. (cyprinid)	−	+
Henneguya exilis	−	−
H. zschokkei	−	−
Unicauda sp.	−	+
Ceratomyxa shasta	−	−
Unidentified (salmonid)	−	+
Unidentified (catostomid)	−	+

We used the method of Pauley (1974) to extract soluble antigens from two different preparations — the first being 3.1×10^7 mature spores from adult fish, and the second 4.6×10^7 prespore stages from fingerling fish.

Antigens from each preparation were brought to 3 mL and protein determined by the Folin phenol method of Lowry et al. (1951). The protein content of antigens from mature spores was 1.8 mg/mL and that of prespore stages was 4.2 mg/mL. For immunization, a mixture of 1 mL antigen, 1 mL Freund's incomplete adjuvant, and 2 mL Hanks' balanced salt solution (BSS) was homogenized.

Normal serum was taken from New Zealand white rabbits before they were immunized with intramuscular injections of 0.8 mL antigen homogenate in each hind leg. The inoculum was equivalent to 4.12×10^6 spores each for two rabbits immunized with the mature spore extract and 6.13×10^6 prespore stages for two immunized with the prespore antigen extract. After 12 d the rabbits were bled and their sera separated, distributed in 5-mL subsamples, and lyophilized. For use, the sera were reconstituted with water and diluted with equal volumes of pH 7.0 buffered NaCl (NaCl 8 g, $NaH_2PO_4 \cdot H_2O$ 1.38 g, and NaOH 0.333 g dissolved in 1 L). Sera were then adsorbed with acetone-dried powders of cartilage–bone and liver tissues from healthy adult rainbow trout. Two adsorptions were made at 10°C; the first was static for 1 h and the second continually mixed for 16 h. Tissue powders were used at the rate of 50 mg of each per millilitre of diluted serum, and they were removed by centrifugation for 30 min at 48 200 × *g* at 5°C.

Sera or their precipitated globulins or both from 10 normal rabbits — preinoculation serum from the 4 immunized rabbits and individual sera from 6 others — were tested for suitability as negative controls. We also tested two lots of pooled normal rabbit serum.

Globulins were precipitated at 0°C with ammonium sulfate at 50% saturation washed twice in ammonium sulfate at 60% saturation, reconstituted with 0.05 M potassium–sodium phosphate buffer at pH 7.3 to the original serum volume and extensively dialyzed against 0.002 M potassium–sodium phosphate buffer at 4°C.

For direct FAT we conjugated whole normal rabbit serum, serum globulins, or antisera against *M. cerebralis* with FITC on diatomaceous earth (Celite®, Calbiochem, San Diego, Calif.) according to the method of Rinderknecht (1962). For the indirect FAT we used FITC conjugated with IgG fraction goat antirabbit serum at a dilution of 1:25 in conjunction with a 1:10 dilution of rhodamine conjugated bovine albumin (Cappel Laboratories, Downington, Pa.).

TESTING MYXOSPORIDANS FOR FLUORESCENT ANTIBODY REACTION

In addition to the homologous organism, formalin-fixed spores (the only life stage realistically available) from 12 other myxosporidans were tested for reactivity with both the direct and indirect FAT (Table 1).

For both the direct and the indirect FAT, spores were attached to microscope slides lightly coated with either 50% egg albumin (vol/vol) or Tissue Bond® (Harleco, Gibbstown, N.J.). Slides were dried 15–20 min at 50–60°C, and fixed 5–10 min in methanol. Serum, serum conjugate, or globulin conjugate was tested at dilutions of 1:10, 1:20, 1:40, 1:80, and 1:160. Each dilution was used at 0.1 mL, which was spread over slides for 30–60 min in a darkened humidified container at 20–25°C.

Slides for direct FAT were then washed for 10–15 min in freshly prepared 0.5 M sodium carbonate–bicarbonate buffer (pH 9.0–9.5), blotted dry, covered with low fluorescence immersion oil, and examined microscopically.

For indirect FAT, slides were washed for 10–15 min in pH 7.0 buffered NaCl and blotted dry. Diluted fluorescein conjugated IgG goat antirabbit serum, mixed with rhodamine conjugated bovine albumin, was applied for 30 min in the light-proof humidified chamber at 20–25°C. Slides were then washed with freshly prepared 0.5 M sodium carbonate–bicarbonate buffer as in the *direct method*, blotted dry, and examined as in the direct method.

Slides were coded and a single blind method of scoring was used throughout the work.

Results

Used in either the direct or the indirect FAT, both kinds of rabbit antisera implemented serological recognition of antigens of mature spores and of earlier life stages of *M. cerebralis* (Fig. 2). The direct method, however, was the more specific for with it the only other reactive myxosporidan spores were those of *M. cartilaginis* (Table 1). Conjugates prepared from spore or prespore antiserum or their globulins produced equally fluorescent spores of *M. cerebralis* and *M. cartilaginis*, and the reaction was blocked by unconjugated globulin.

With the direct method maximum brightness of spores was found at dilutions of 1:20 and 1:40, and polar capsules were typically apparent as unstained voids against a fluorescent background that included the sporoplasm and intercapsular substance (Fig. 2). Some spores of *M. cerebralis* were immature — in others the valves or polar capsules were evidently open and in such the developing polar capsules or the polar filaments were also fluorescent. We found no such reactivity in *M. cartilaginis*, but the only spores we had were fully mature.

The two kinds of antisera differed in the intensity of their reaction on *M. cerebralis*. Although measurements were not practical, the oval prespore stages seemed to

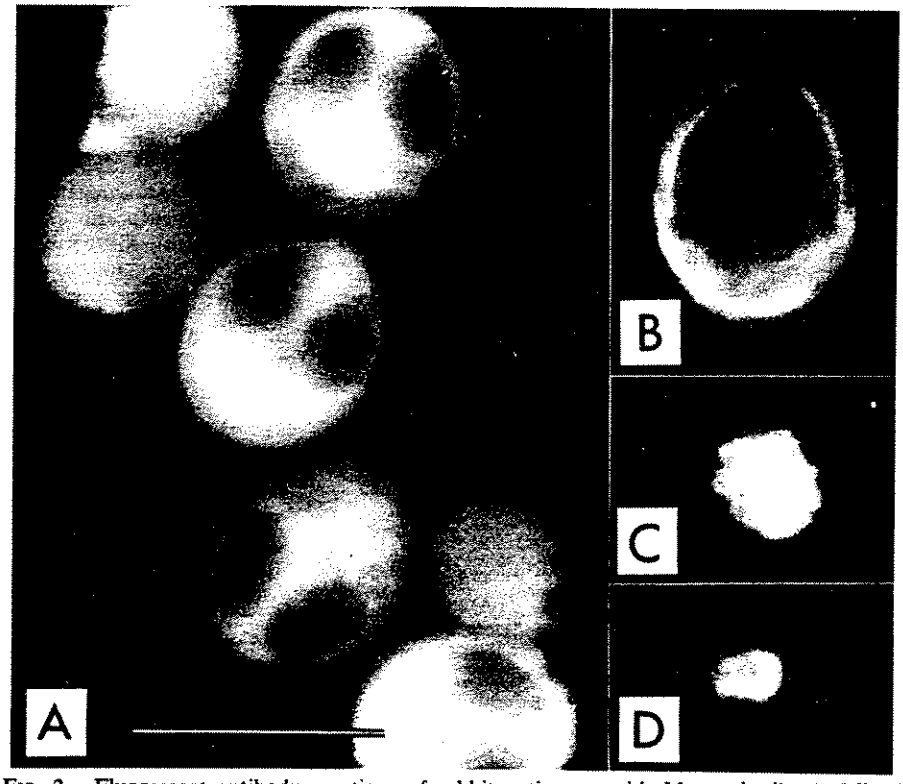

FIG. 2. Fluorescent antibody reactions of rabbit antiserum with *M. cerebralis*. A, Mixed spores and prespore stages (smaller ovals) stained with the direct method. Note the unreactive polar capsules; B, a mature spore stained with the indirect method shows halo-like surface fluorescence; C, 40-d-old form presumptively identified by its reactivity with antiserum in the direct method; D, 2-d-old form in rainbow trout head presumptively identified by the direct method. Bar scale is 10 μm.

stain more brightly with prespore antiserum and fully mature spores with spore antiserum. When two lots of pooled normal rabbit serum or their precipitated globulins were conjugated with FITC, both reacted with *M. cerebralis* to give a false positive fluorescence. We found it necessary to conjugate and test precipitated globulins from individual rabbits to find negative normal serum for the direct FAT. Only two of six rabbits so tested proved suitable and we have no explanation for the unexpected reaction.

With the indirect method, reactivity of *M. cerebralis* occurred at all dilutions of antiserum, but optimum brilliance was obtained at 1:40. The fluorescence of indirectly stained life stages was at the surface, giving a halo-like effect in which polar capsules were not readily apparent (Fig. 2).

With the indirect FAT, 7 of 12 other myxosporidans showed surface fluorescence (Table 1). The fluorescence of heterologous spores was variable from species to species; it was seldom entire and tended toward concentration at the distal end. In *Unicauda* sp. the condition was extreme; fluorescence was confined to the most distal portion of the caudal appendage. Neither *Ceratomyxa shasta* nor the two species of *Henneguya* fluoresced.

With the indirect method, neither *M. cerebralis* nor any other myxosporidan showed fluorescence with normal rabbit serum.

The two methods of fluorescent staining worked with either fresh or preserved spores.

Discussion

The common denominator in our tests of 13 different myxosporidans for FAT reactivity with rabbit anti-*M. cerebralis* serum was the spore stage. Our results leave no doubt that of the two techniques, the direct method was by far the more specific, and this agrees with serological tenets. The FITC conjugated *M. cerebralis* antisera, or their globulins, reacted with homologous spores and with spores of *M. cartilaginis* but not with spores of the other two species of *Myxosoma*. These results suggest a close relationship between the two reactive species, and they also suggest that *M. cephalus* and *M. cyprini* have quite different antigens and may not belong to the genus *Myxosoma*. These findings lend

support to Halliday's contention that serology could clarify myxosporidan taxonomy. We believe serology can greatly improve classification based solely on morphometry.

In the homologous system, the reactivity or prespore stages with antiserum prepared with spore antigens and vice versa supports our hypothesis that some of the parasite's antigens are present throughout its life cycle. Accordingly, while we are not yet prepared to describe them by light microscopy, we believe that the kinds of fluorescent forms illustrated in Fig. 2C and D may be stages of the life cycle.

We recognize that selective purification of *M. cerebralis* antigens will probably be required to provide antisera that will give absolutely specific serological identification. In the meantime, the direct FAT should be used in diagnostic work for it lends added credence to morphometric methods of spore identification. Perhaps most importantly, the direct FAT provides the best available means of identifying life forms of *M. cerebralis* prior to sporulation, and thus extends to many persons the expertise currently possessed by a very few skilled parasitologists. In monitoring a suspect facility we have used the direct FAT to identify an incipient outbreak of whirling disease 2 mo before its actual occurrence.

Members of the genera *Myxosoma* and *Myxobolus* have spores of similar size and shape, and both occur in salmonids. The two are presently distinguished by demonstration of an iodinophilus vacuole in *Myxobolus*, but that test is not reliable with preserved spores. Halliday (1974) found that one unspeciated member of the genus *Myxobolus* did not react to an indirect FAT with *M. cerebralis* antiserum. We found that with the indirect FAT two of three *Myxobolus* species and other genera reacted with anti-*M. cerebralis* serum. The location of the fluorescence suggests that the outer mucoid material is reactive. Such reactivity is indicative of a common external antigen or antigens. According to Lom and Hoffman (1971) *M. cerebralis* has a mucous envelope and *M. cartilaginis* does not. That fact may explain why in the indirect FAT, *M. cartilaginis* was not reactive to antiserum against *M. cerebralis*. In this light, the direct FAT may depend upon antigens from within the valves, and that is consistent with the described appearance of fluorescence throughout the spores of the two species of *Myxosoma*. That being the case serologic comparisons of myxosporidans should utilize internal antigens and direct FAT.

Some normal rabbit sera and their precipitated globulins that were conjugated with FITC gave fluorescence with *M. cerebralis* spores. It seems most unlikely that the reactive sera represented prior antigenic experience of the rabbits to the myxosporidan. Rather, we attribute the nonspecificity to vagaries of the rapid conjugation method we employed, or to possible affinity of the spores to unidentified protein of the conjugate. The false positive reaction was not universal among the rabbits tested, but it is a strong reason for testing rabbits and demonstrating them to be negative before immunizing them with antigens of *M. cerebralis*.

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Myxosoma cerebralis: Trypsinization of Plankton Centrifuge Harvests Increases Optical Clarity and Spore Concentration

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MARKIW, M. E., AND K. WOLF. 1980. *Myxosoma cerebralis*: trypsinization of plankton centrifuge harvests increases optical clarity and spore concentration. Can. J. Fish. Aquat. Sci. 37: 2225–2227.

Pepsin, pronase, and trypsin were compared for their ability to digest the tissue residues that constitute the overwhelming mass of plankton centrifuge harvests. Trypsin was preferred; a treatment with 0.25% for 30 min at pH 7.2–7.5 released an average of 22% additional spores and resulted in an average 11-fold increase in spore concentration. The procedure significantly enhances the sensitivity of spore detection by the plankton centrifuge method.

Key words: Myxosoma cerebralis, myxosporidans, spore detection

MARKIW, M. E., AND K. WOLF. 1980. *Myxosoma cerebralis*: trypsinization of plankton centrifuge harvests increases optical clarity and spore concentration. Can. J. Fish. Aquat. Sci. 37: 2225–2227.

Nous avons comparé l'apptitude de la pepsine, de la pronase et de la trypsine à digérer les résidus de tissu qui constituent la presque totalité de la masse des récoltes de plancton au centrifuge. Nous préférons la trypsine; un traitement à 0,25% pendant 30 min au pH de 7,2–7,5 met en liberté, en moyenne, 22% de spores supplémentaires et produit une augmentation de concentration des spores de 11 fois en moyenne. Cette méthode rehausse de façon significative la sensibilité de la détection des spores par la méthode de centrifugation du plancton.

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THE literature on whirling disease contains an array of procedures that have been used to release and isolate spores of the causal organism, *Myxosoma cerebralis*; however, the methods are not equally applicable for diagnosis and detection. The *Fish Health Blue Book* (McDaniel 1979) indicates that where *M. cerebralis* is thought to be causing disease, a rather simple diagnostic procedure can be used: heads of suspect fish are defleshed and ground and the resulting materials examined microscopically. Spores are typically abundant in fish with clinical whirling disease, and the speed and simplicity of the method are advantageous. In contrast, spores are typically far less numerous in asymptomatic carriers and simple diagnostic procedures are usually not sufficient for sensitive and reliable detection. For that reason, the American Fisheries Society sanctions only two procedures for spore detection — the digest method developed by Markiw and Wolf (1974) and the plankton centrifuge method described by O'Grodnick (1975).

Each procedure has its particular advantages and disadvantages. Harvest volume of the digest method is but a millilitre or less and although it consists of tissue-free spores, the time required for processing is about 8 h. Harvests of the plankton centrifuge procedure occupy many millilitres, and the yield of spores is mixed with a much greater volume of host tissue fragments. However, because the centrifuge method is simpler and requires but several hours to perform, it has found decided favor with many fish health specialists and diagnosticians. Accordingly, we sought to increase the sensitivity of spore detection of the plankton centrifuge method and to obtain spores that could be serologically identified by the fluorescent antibody technique (Markiw and Wolf 1978) with little background interference from autofluorescing tissue residues. We compared pepsin, pronase, and trypsin for their ability to reduce tissue residues and thereby increase the concentration of spores in the sample.

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Materials and Methods

PRELIMINARY TRIALS

Trout infected with whirling disease were dispatched, held at 50°C for 5–10 min, and defleshed, and their skeletal elements blended then processed with a plankton centrifuge. Resulting spore populations were quantified by microscopic count; the material was then digested for 30 min with pepsin, trypsin, or pronase, and spores were again concentrated by centrifugation and quantified. Pepsin was used at a concentration of 0.5%, at pH 1.8–2.0 and 37°C. Trypsin and pronase were each used at several concentrations in the range of 0.05–0.25%, at pH 7.2–7.5 and 8.0–8.5 and 24 ± 2°C.

TABLE 1. Effects of trypsinization in releasing and concentrating *Myxosoma cerebralis* spores obtained by the plankton centrifuge method.

Material	Volume (mL)	Volume of aqueous suspension (mL)	Spore count		Effective concentration of spores[a]
			No. per mL	Total	
Plankton centrifuge residues (60 fingerlings)	13	150	20 420	3 063 000	—
Trypsinization pellet	2.5	20	202 500	4 050 000	10-fold
Plankton centrifuge residues (60 yearlings)	12	648	50 000	32 400 000	—
Trypsinization pellet	3	60	608 540	36 512 400	12-fold

[a]Derivation: $\frac{\text{Spores per mL of resuspended digestion pellet}}{\text{Spores per mL of plankton centrifuge residues}}$.

DEFINITIVE TRIALS

Two trials were conducted, each with 60 intact heads (neither heated nor defleshed) of brook trout (*Salvelinus fontinalis*) — one group from fingerlings and the other from yearlings. Plankton centrifuge harvests from each trial were pooled and resuspended in 100 or more volumes of water, the spores were quantified, and the harvests treated with 10–20 volumes of 0.25% trypsin in Rinaldini's solution[1] at pH 7.2–7.5 for 30 min at room temperature. Digestion was stopped by adding an aqueous solution of bovine serum albumin to a final concentration of 1%. The mixture was centrifuged at 1200 × *g* for 10 min, the resulting pellet resuspended in 20 volumes of water — an empirically determined dilution that facilitated spore counting — and the average spore count again determined.

Results

PRELIMINARY TRIALS

The preliminary trials showed that when heated and defleshed cranial and axial skeletal elements were reduced in a blender some of the spores were physically damaged, and subsequent digestion with pepsin at 37°C for 30 min was additionally destructive. Trypsin or pronase were satisfactorily gentle in the near-physiological range of pH 7.2–7.5, but digestion with either enzyme at pH 8.0–8.5 damaged many spores. Digestion with either trypsin or pronase resulted in a loss of about half the spores, but because tissue residues were removed and the volume was reduced, there was an effective 25-fold concentration with trypsin and 29-fold concentration with pronase.

DEFINITIVE TRIALS

The use of trypsin on plankton centrifuge harvests of either fingerling or yearling trout infected with whirling disease released an average of 22% (range, 13–32%) additional spores and permitted a 10- to 12-fold concentration of spores over that in the original harvest (Table 1).

[1]Composition: NaCl, 8.0 g; KCl, 0.25 g; $C_6H_5O_7 \cdot 2H_2O$ (sodium citrate) 1.0 g; $NaH_2PO_4 \cdot H_2O$, 0.05 g; $NaHCO_3$, 1.0 g; glucose, 1.0 g; and water to 1000 mL (Rinaldini 1959).

Discussion

Results from the preliminary trials and the definitive work were in general agreement; trypsin or pronase effectively digested the tissue residues that contribute the bulk of plankton centrifuge harvests and released additional spores. Spores were significantly concentrated and sensitivity of detection was increased. We are not certain why spores were lost in the preliminary trials. We speculate that the heating step used to aid in defleshing increased the vulnerability of the spores to blending and to subsequent digestion. When heating and defleshing were omitted from the definitive work, spore numbers increased 32% in the harvest from fingerlings and 13% in that from yearlings.

The American Fisheries Society's *Blue Book* specifies that, in disease situations (diagnostic procedures by the plankton centrifuge method), material to be triturated be processed in 5% formalin as a means of killing spores and thus minimizing the chance of accidental dissemination of whirling disease. However, formalin should not be used when digestions are to be done, because tissue and enzymes become chemically denatured and digestion is prevented.

We stress that tryptic digestion of plankton centrifuge harvests is not intended as a routine additional step. Trypsinization is strongly suggested as a precautionary procedure for increasing the sensitivity of detection if spores are not found in plankton centrifuge harvests. Trypsinization greatly reduces the obstruction of autofluorescing background tissue and significantly increases spore concentration.

In operating the plankton centrifuge, we have found it advantageous to make a single harvest of the materials sedimented in the cup to save the time and effort required for multiple small harvests. In addition, the sedimented material can be resuspended in reduced volumes of water and allowed to settle for 2–3 min and the supernatant liquid searched for spores.

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Myxosoma cerebralis (Myxozoa: Myxosporea) Etiologic Agent of Salmonid Whirling Disease Requires Tubificid Worm (Annelida: Oligochaeta) in its Life Cycle

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ABSTRACT. Studies of the life cycle of *Myxosoma cerebralis* showed that development of infectivity did not occur endogenously but that the spore "aging" process required participation of an aquatic tubificid oligochaete. Data suggestive of such involvement were derived from trials in which spores were "aged" in an array of inert, sterilized, pasteurized, or natural aquatic substrates and from examination of aquatic soils from trout hatcheries in which whirling disease was epizootic. The role of the aquatic oligochaete was confirmed two ways. First, signs of whirling disease developed, and *M. cerebralis* spores were produced in young rainbow trout (*Salmo gairdneri*) that had been fed oligochaetes harvested from pond soil taken from two hatcheries where whirling disease was epizootic. Second, when containers of pasteurized soil were populated with four genera of oligochaetes—*Aeolosoma, Dero, Stylaria,* or *Tubifex*—from a biological supply house, or with tubificid worms from trout hatcheries free of whirling disease, and then seeded with *M. cerebralis* spores and "aged" for 4 months, whirling disease occurred only in trout held with *Tubifex* and with hatchery tubificids.

MYXOSOMA cerebralis (Hofer), the etiologic agent of whirling disease (WD) of trout and salmon, has been known since the early 1900s; but notwithstanding considerable research during the intervening years, the life cycle has remained largely unknown. As is the case with other myxosporeans, information on the life cycle of *M. cerebralis* is essentially confined to development of the parasite within the fish host. Not even the size and shape of the infective stage are known, and all that happens outside the fish—fully half the life cycle duration—has been an enigma.

Unlike many other protozoal diseases of fish, WD is not transmitted directly from fish to fish; in fact, no one has been able to infect fish with freshly isolated spores (2). Instead, spores must be "aged in mud or water" for several months in order to yield infectivity or become infectious. A little known fact is that the discovery of the critically essential but uncharacterized "aging" process should be credited to A. V. Uspenskaya. Her discovery was made during 1954 and 1955, but use of the aging procedure elsewhere occurred only after she described her method in personal communications to parasitologists during the 1960s. For the record, some details of the initial discovery were reported by Uspenskaya in 1978 (6). The experimental aging process was routinely used by others after Hoffman & Putz (3) published in English and included in their report the fact that cut-up heads of infected fish, added to tanks containing mud as well as being supplied with running water, produced infectivity.

The literature on WD and *M. cerebralis* is voluminous, and because the infection is an important problem in fish husbandry, the condition is usually discussed in standard texts on fish diseases. The most detailed coverage of the disease, however, and the parasite is in the review by Halliday (1), who included nearly 150 references.

Relevant to the new work reported here, our long range plans were based on research needs and voids in knowledge that were discussed in a 1974 overview of WD (7). Our beginning efforts went into the development of a method of releasing, concentrating, and purifying spores from infected tissues. That procedure (4) provided a highly sensitive method of spore detection

[1] Authorship is alphabetical.

and, additionally, the antigens needed for preparation of antiserum. Rabbits were immunized and the resulting antiserum was conjugated with fluorescein isothiocyanate for serological identifications with direct fluorescent antibody technique (5). Applications were made in diagnostics, and the methods were used in searching for stages in the life cycle.

The purpose of the present paper is to document our finding that a tubificid oligochaete plays an essential role during the "aging" of spores and development of infectivity in *M. cerebralis*.

MATERIALS AND METHODS

Fish. Throughout our studies we used pathogen-free fry or small fingerling rainbow trout (*Salmo gairdneri*) as test animals for assays of infectivity. Except where noted, all experiments with test fish were conducted at 12.5°C, and fish were fed commercial trout rations.

Spores. Spores used to initiate all experiments were obtained in part from naturally infected rainbow trout or brook trout (*Salvelinus fontinalis*), but predominantly from small rainbow trout that had been infected experimentally by holding them in tanks containing pond or stream soils from trout hatcheries where WD was epizootic. Alternatively, trout were infected by holding them in tanks containing soil from a pond or a hatchery effluent stream that initially bore no infectivity but samples of which had been seeded in the laboratory with *M. cerebralis* spores and held for 3 or 4 months before susceptible trout were exposed.

Determinations of endogenous or exogenous infectivity (first trial). A first question we asked was whether development of infectivity by or from spores was an endogenous process or whether other organisms were involved. Accordingly, various test substrates were set up either under well water or spring water, and additionally under one of several gas atmospheres. Well water was not chlorinated, nor did it harbor invertebrates. The chemically similar spring water occasionally carries freshwater shrimps, miscellaneous aquatic insect larvae, and infrequent small amphibians. Sand purchased from a chemical supply house was used as one substrate and soil from the laboratory's pathogen-free spring as another. That soil harbors planarians, isopods, freshwater shrimps, snails, and a few aquatic macroannelids.

Substrates to a depth of 5 cm were placed in each of nine 7-liter capacity glass containers that were provided with flow-through well or spring water or kept under static spring water. Six containers had normal atmosphere; one was made anaerobic with the BBL Gas Pak System; another was kept under a flow of nitrogen, and yet another was provided with 100% oxygen. One container of spring soil and spring water was sterilized by autoclaving. One container of untreated spring soil was kept under normal atmosphere and supplied with spring water; it was seeded with spores and served as the positive control. As quantified by hemocytometer count, all containers but one were seeded with 6.1×10^6 spores of *M. cerebralis*; the anaerobic container, because it was smaller, was seeded with 1.5×10^6 spores. All containers were kept at 12.5°C and, after allowing spores to "age" for 4 months, water samples were collected for concentration and examination by fluorescent antibody technique with rabbit anti-*M. cerebralis* serum (5). Sixty susceptible trout fry were then placed in glass containers for 5 days—sufficient exposure time if infectivity were present—and held for 4 months. The fish were then examined for spores by the pepsin-trypsin-dextrose centrifugation method (4).

Determination of endogenous or exogenous infectivity (second trial). The second trial was patterned after the first but with modification. In lieu of sterilization, the spring soil was simply depopulated of invertebrates and vegetative microorganisms by pasteurization. Also, well water was used in only one container, and all containers but one (kept under nitrogen) were kept under normal atmosphere. The positive control consisted of a 180-liter fiberglass tank that held untreated spring soil, was provided with a small flow of spring water, and was seeded with crushed cartilage from WD-infected trout. Density of spores seeded was 7×10^6 per glass container, and those added to a glass wool substrate were disrupted in a tissue homogenizer to the extent of 50–60% as a means of freeing the sporoplasm. At the start, each container was stocked with several planarians, snails, and freshwater shrimps. The containers were held at 12.5–14.5°C for 4 months and then assayed for infectivity. Postulating possible waterborne infectivity at the time of assay, flowing spring water was supplied to each container, and 15 test fry were held in the effluent for 2 weeks. Thereafter, other fry were placed directly in the containers in contact with the substrates, held there for 77 days, and examined for spores 4 months after first exposure.

Determination of the size of infective forms. We investigated water or aquatic soils, or both, from two Pennsylvania trout hatcheries (here identified as P1 and P2) having earthen ponds and raceways in which WD was epizootic.

Eighty liters of pond water were transported from the P1 hatchery to the National Fish Health Research Laboratory and sequentially passed through screens with mesh sizes of 200, 100, 50, and 25 μm, and then by pressure filtration through membranes (293-mm dia.) with mean pore diameters of 14 and 3 μm. Screens and membranes were then individually washed in separate containers holding 9 liters of pathogen-free spring water, and each container was aerated and stocked with 60 susceptible trout fry. The fry were exposed for 3 days then transferred to 1.5-liter containers supplied with flowing spring water, held for 4 months, and then assayed for spores.

Pond soil was also transported from the P1 hatchery, and about 7 liters of a slurry were poured through a 4000-μm mesh screen and then by gentle abrasion passed through screens of 520, 200, 100, 50, and 25 μm. As in the processing of pond water, the screens were washed in containers holding 9 liters of continuously aerated spring water; then the containers were stocked with susceptible fry for 4 days. Containers were covered to prevent aerosol contamination. Fry were removed to 1.5-liter containers supplied with flowing spring water, held for 4 months, then assayed for spores.

Soil samples taken from a stream at the P2 hatchery were transported to the laboratory, where a slurry was made and serially passed through screens with mesh sizes of 4000 to 25 μm; however, in lieu of the abrasion used earlier, the screens were gently shaken. Materials retained on the screens were sampled for examination, and the great bulk was transferred to containers holding 9 liters of spring water. Nine liters of the 25-μm filtrate were placed in a separate container. Containers were each aerated and stocked with 50 susceptible fry for 2 weeks; the fry were then transferred to 1.5-liter containers supplied with flowing spring water, held for 4.5 months, and assayed for spores.

Testing the role of oligochaetes in the life cycle. Stocks of aquatic oligochaetes, marketed as the genera *Aeolosoma, Dero, Stylaria,* and *Tubifex,* were obtained from a commercial supplier (Carolina Biological) and planted in four 7-liter containers containing soil from ponds and an effluent stream of the Ridge Hatchery (West Virginia). The hatchery had no history of WD, and previous research had shown that the soil and its invertebrate fauna bore no evidence of WD infectivity (8). As an additional precaution, the soil was pasteurized before the oligochaetes were stocked. A fifth container of pasteurized soil was

TABLE I. *Comparative number of* Myxosoma cerebralis *spores that developed in rainbow trout fry exposed to graded fractions of pond soil from a facility harboring whirling disease.*

Screen mesh size (μm)	Spores/fish
4000	Accidental loss
520	5300
200	70,600
100	230,300
50	558,000
25	21,000
25 μm filtrate	None

repopulated with oligochaetes from the Ridge Hatchery. Spores of *M. cerebralis* were added to the five containers, which were then supplied with a small flow of spring water. Small amounts of granular fish food were added occasionally to sustain the oligochaetes. Three negative controls were used—stocks of tubificids held without the addition of *M. cerebralis*.[2] The tubificids came from: (1) the commercial source (*Tubifex*); (2) from Leetown, West Virginia; and (3) from Ridge, West Virginia. The positive control consisted of tubificids from the P2 hatchery where WD was epizootic. All containers were held at 12.5°C for 4 months before 25 susceptible rainbow trout fry were placed in each. After an additional 3 months, five fry from each container were assayed for *M. cerebralis* spores, and a second assay was carried out on fish surviving for 4 months.

RESULTS AND DISCUSSION

The first attempt to determine if endogenous or exogenous factors were operative in producing infectivity from spores of *M. cerebralis* showed that neither the positive control nor any of the eight test environments produced infectivity. No evidence appeared of an endogenous process, and a modified repetition of the work was begun. Failure of the positive control to yield infectivity was at the time unexplained.

The second trial yielded results similar to those obtained from the first; intact or ruptured spores in all of the inert or pasteurized or clean spring soil substrates failed to produce infectivity. The lack of infectivity in the positive control was again puzzling.

Assay of the 80-liter water sample from the P1 hatchery showed no evidence of infectivity. In contrast, tests of the 7 liters of P1 soil slurry showed infectivity, but only in materials retained on the 4000- and 520-μm screens, not in fractions of smaller sizes—those that had been abraded.

Examination of materials sampled from the 4000- and 520-μm screens showed a few isopods, freshwater shrimps, larval insect forms, and annelids of several sizes. Annelids were the most numerous fauna, and when crushed and subjected to fluorescent antibody, some small tubificids showed focal areas of reactivity to anti-*M. cerebralis* serum.

Next, living small tubificids were selected and force-fed to small trout. The trout in time developed signs of WD and eventually produced *M. cerebralis* spores.

Fixed tubificids were also sectioned histologically and the sections processed with direct fluorescent antibody technique. Aggregates of small reactive bodies were found internally, but we were unable to correlate the findings with conventional light microscopy.

A similar trial with pooled samples taken from the P2 hatchery showed infectivity in the fractions retained on mesh sizes of 520 through 25 μm; however, a gradient was evident in the numbers of spores that were produced in the infected fish. The greatest number of spores was produced in fish exposed to materials on the 50-μm mesh size (Table I).

Microscopic examination of fresh and preserved materials from the 200- through 50-μm screens showed no tubificids or recognizable fragments thereof; however, as was found in tubificids from the P1 hatchery, when histologic sections of tubificids were examined with fluorescent antibody, reactivity was found within the body. Tubificids from the Ridge (West Virginia) trout hatchery (without WD) showed no such reactivity. When living, intact tubificids from the P2 hatchery soil were made available as food to susceptible fry, the fish developed WD.

Additional features of the infectivity were obtained from investigation of the susceptibility of young trout to materials in a tank holding pooled soil samples from the P2 hatchery. The tank was supplied with a small but continuous flow of pathogen-free spring water and the effluent passed by gravity through 200-μm mesh screen. All exposed trout contracted WD and developed spores. At another time, two cages holding young trout were placed in the tank—one cage in contact with the soil and the other suspended in water. Clinical signs of WD appeared first among fish in contact with the soil, but also appeared several days later among those held above the bottom; fish of both groups developed *M. cerebralis* spores.

The results support an interpretation that infectivity is harbored in soil, but that it can also be waterborne, though to a lesser intensity. Provisional incrimination of a tubificid being involved and actually necessary in the life cycle of *M. cerebralis* was obtained from definitive trials with four genera of oligochaetes held in pasteurized soil that had been seeded with *M. cerebralis* spores. Signs of WD and development of serologically identified *M. cerebralis* spores occurred only among young trout held in the presence of annelids marketed as the genus *Tubifex*; the genera *Aeolosoma*, *Dero*, and *Stylaria* proved incapable of

TABLE II. *Occurrence of whirling disease in rainbow trout fry exposed to oligochaetes with and without* Myxosoma cerebralis *spores.*

Genus or type of oligochaete, and source[a]	Spores of *M. cerebralis*	Whirling disease and confirmation
Aeolosoma, commercial	Yes	−
Dero, commercial	Yes	−
Stylaria, commercial	Yes	−
Tubifex, commercial^{NC}	No	−
Tubifex, commercial	Yes	+
Tubificid, L., West Virginia^{NC}	No	−
Tubificid, Pennsylvania^{PC}	No	+
Tubificid, R., West Virginia^{NC}	No	−
Tubificid, R., West Virginia	Yes	+

[a] Abbreviations: NC = negative control; PC = positive control; L = Leetown; R = Ridge.

[2] Prudence dictates that we use the general term tubificid (from the family Tubificidae), because stocks of aquatic oligochaetes that we collected at trout hatcheries and used in our studies were mixed populations that consisted of several genera. Samples of two populations were submitted to two different consultants for identification, and a uniform finding of multiple genera was reported. D. Kathman of E.V.S. Consultants Ltd., Marine Technology Centre, Sidney, B.C., Canada, and J. K. Hiltunen, U.S. Fish and Wildlife Service, Great Lakes Fishery Laboratory, Ann Arbor, Michigan, both identified *Tubifex tubifex* in the two samples. Many of the oligochaetes were immature, however, and could have been either *T. tubifex*, or *Hyodrilus templetoni*; the immature specimens of the two cannot be distinguished. Both consultants identified *Limnodrilus* in the two samples. D. Kathman classified them as immature *L. hoffmeisteri* and further identifed *Quistadrilus multisetosus* as present but least abundant in both samples.

yielding infectivity (Table II). Whirling disease occurred in the presence of *Tubifex* worms from the commercial source and in the presence of tubificids from pond soil from the Ridge (West Virginia) hatchery having no history of WD, but which had been seeded with *M. cerebralis* spores. Three negative controls—*Tubifex* worms and tubificids from two sources free of WD and held in the absence of spores—did not produce infectivity. Also, the positive control—Tubificids from the P2 hatchery but stocked in pasteurized soil—showed the expected transmission of WD.

Although determinations of genus—*Tubifex, Limnodrilus*, or *Ilyodrilus*—were not made, our results establish tubificid oligochaetes as an essential participant in the life cycle of *M. cerebralis* outside the fish host. Thus, it becomes evident that postulated life cycles involving only fish are no longer tenable. Likewise, what has been termed "aging" cannot be effected if the substrate is simply "mud or water"; the substrate must harbor one or more of the aforementioned tubificid oligochaetes.

The requirement for tubificid oligochaetes helps explain the lack of infectivity in the intended positive controls used in our two early experiments. In those experiments we sought evidence for endogenous or exogenous infectivity and used soil from the laboratory's clean spring site as the principal substrate. Retrospectively, we examined that soil but were unable to find tubificid worms. Those experiments also did not include periodic additions of fish food to the test containers—a measure we have since learned to do in order to maintain populations of tubificids. Accordingly, even if some tubificid oligochaetes had been present in the original clean spring soil, there was little to sustain them during the 4-month holding time. Tubificid worms are abundant in organically polluted or enriched environments, such as effluent streams or basins of fish hatcheries with their typical load of waste products and uneaten food residues.

The need for an invertebrate intermediate is consistent with previously known features of *M. cerebralis*: namely that fresh spores are not infectious for fish and that WD occurs in problem proportions in earthen ponds and raceways. Providing that the water supply is free of infectivity, WD does not occur in clean concrete raceways.

Requirement of tubificid oligochaetes in the life cycle of *M. cerebralis* suggests that other myxosporeans—particularly those that cause problems in fish husbandry (e.g., *Ceratomyxa shasta* and *Henneguya exilis*)—could similarly require an aquatic annelid.

It is now possible to investigate in detail the events that take place within the oligochaete. Inasmuch as WD was transmitted by feeding oligochaetes to trout fry, the inference is that the worms per se harbor infectivity. They might, however, also release an infective stage, for susceptible fish kept in the water column above an infective soil also contracted the disease; moreover, our inability to recognize peak infectivity in the materials retained on the 50-μm screen is puzzling.

We speculate that the recognized high susceptibility of young salmonids might not be a physiological phenomenon; instead, it might be due to feeding habits of small fish. It seems reasonable to believe that trout fry would graze on small annelids to a greater extent than would large fish.

The findings that tubificid worms are involved in the life cycle of WD presents a dilemma to agencies concerned with dissemination and control of the infection. On the one hand, testing of chemicals that are selectively lethal for annelids but nontoxic to trout is a possibility that could lead to effective eradication measures. On the other hand, a considerable industry is based on harvesting tubificids for distribution and marketing as tropical fish food. Where tubificids are produced commercially at salmonid facilities that have WD, the potential exists for accidental spread to areas not now known to have the disease. It becomes essential that the methods used to preserve the annelids be examined and, if indicated, that the final product be assayed for possible infectivity.

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McKinney, J.M and A.D. Bradford. 1970. Measuring respiration rates of *Myxosoma cerebralis* spores. The Progressive Fish-Culturist. 32: 120.

MEASURING RESPIRATION RATES OF MYXOSOMA CEREBRALIS SPORES

As a precursor to planned studies of the lethality of various treatments to the spores of Myxosoma cerebralis, it was necessary to develop methods for concentrating and measuring the respiration of the spores.

We successfully concentrated these spores by homogenizing the cranial area of infected trout and then centrifuging the homogenate. Respiring elements other than spores--such as bacteria, tissue, and water molds--were destroyed by a modified Tyndallization procedure which consisted of discontinuous heating (maximum, 65° C.; minimum, 20° C.). Sterility tests before and after Tyndallization verified the effectiveness of the procedure.

After the Tyndallization, the sample of spores was transferred to a reaction chamber of a YSI, Model 53, biological oxygen monitoring system. All of the data obtained from this system were recorded upon a Sargent, Model SRL, recorder. The oxygen monitoring system detected respiration after the Tyndallization; and because the spores of M. cerebralis were the only live organisms known to remain in the sample, all remaining respiration was attributed to this organism.

Research on whirling disease is being supported by funds issued under the Federal Aid in Fish Restoration (Dingell-Johnson) Act.

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Modes of Transmission of Whirling Disease of Trout

A REVIEW of the circumstances surrounding numerous whirling disease epizootics suggests that the pathogen (*Myxosoma cerebralis*) is most commonly transmitted through transplacement of infected fish[1]. Alternatively, it has been demonstrated that the spore phase of the parasite can be spread by currents throughout a water system. It is also widely accepted that equipment used in the propagation of infected trout is likely to become contaminated and the subsequent transplacement results in transmission of the disease.

Apart from these widely recognized modes of transmission, no other possibilities have been considered seriously[1]. But Schäperclaus[2] found myxosporidian spores in the faeces of kingfishers at an infected hatchery and believed that the disease could be spread in this manner. His observations are especially relevant in view of the recent appearance of the disease in northern Scotland[3]. He did not conduct further tests of this hypothesis and its validity remains in question. The ability to locate spores in the faeces and demonstrate their viability was imperative in our evaluation of the hypothesis.

For this purpose a spore isolation technique was developed. Isolation was accomplished by macerating the materials to be examined and subsequently passing them through a sintered glass filter with a porosity of 30–60 μm. This allowed the spore (10 μm) to pass through but cleared the macerate of much of the other material (broken cartilage and so on). The spores in the filtrate were further concentrated by centrifugation; they settled as the precipitate at low speeds (10,000*g*). We have used this technique to locate a few spores in large volumes of materials. As well as making possible faecal analysis, the spore isolation technique provides a more efficient means of diagnosing the disease in mildly infected fish (Fig. 1).

The great blue heron (*Ardea herodias*) was selected as the test avian species because of its predatory habits, the range of size of its fish prey, the volume of fish eaten per day, and its local and migratory range. Four great blue herons were collected and maintained under the authorization of state and federal scientific collecting permits.

Fig. 1. Spores of *Myxosoma cerebralis* in the precipitate phase of the spore isolation technique. Wet mount by phase contrast microscopy (×400).

Two fish, infected with whirling disease and weighing approximately one pound each, were fed each bird daily. The resultant faeces were collected and examined. In order to further narrow the amount of faeces to be examined, safranin stain was injected into the cranium of each fish and the resultant faeces were easily recognized by the pinkish colour.

Spores were separated by our isolation technique. Light microscopy revealed that the spore phase of the parasite had passed through the digestive tract of the bird, apparently unaltered (Fig. 2).

Viable spores extrude their filaments when in alkaline conditions (*p*H 10–12)[1] (Fig. 3), and so spores within the heron faeces were placed in 2 per cent KOH and the polar filaments were extruded (Fig. 4). Although the extrusion of filaments is not conclusive evidence of viability, it gives support to that assumption.

Fig. 2. *Myxosoma cerebralis* spore in the faeces of a great blue heron. This apparently unaltered spore had passed through the bird's digestive tract. Wet mount by phase contrast microscopy (×500).

Fig. 3. *Myxosoma cerebralis* spore with filaments extruded. Wet mount by phase contrast microscopy (×500).

Fig. 4. *Myxosoma cerebralis* spores in faeces of great blue heron. The filaments extruded when exposed to 2 per cent KOH (*p*H 10–12). Wet mount by phase contrast microscopy (×500).

These results suggest that avian vectors of transmission should be considered for myxosporidian diseases.

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SHORT COMMUNICATION

Use of the plankton centrifuge to diagnose and monitor prevalence of myxobolid infections in fathead minnows, *Pimephales promelas* Rafinesque

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The plankton centrifuge technique (PCT) of O'Grodnick (1975) is one of two procedures accepted for diagnosis of salmonid whirling disease (*Myxobolus = Myxosoma cerebralis*) by the American Fisheries Society (McDaniel 1979). This technique requires minimal time and laboratory equipment compared to other spore detection methods (O'Grodnick 1975; Markiw & Wolf 1980). Reports of O'Grodnick (1975) and Halliday (1976) indicate spore morphology is not altered by plankton centrifugation and fresh spores may be harvested in large numbers from infected tissues. Virtually all uses of the PCT have been in the examination of salmonid hosts suspected, or known to harbour spores of *M. cerebralis*, yet the technique holds potential for use in population studies of other myxozoans in feral fishes.

It is difficult to assess accurately infection prevalence and intensity of myxozoans. Spores in microscopic cysts may often go undetected in wet mount surveys; diagnosis by histologic sectioning is time-consuming. Accordingly, we set out to field test the PCT as a rapid method for diagnosis and monitoring prevalence of several myxozoan species in a feral host population. We have compared the PCT with routine wet mount diagnosis and have attempted to devise a practical procedure involving the PCT for myxozoan population studies.

The fathead minnow, *Pimephales promelas* Rafinesque, was selected as a test host. Minnows were collected by seining from a small stream, Pillsbury Creek, draining cropland in Dickinson County, north-west Iowa, U.S.A. Adult minnows (standard length 4·5–5 cm) were collected during June–August 1981. The PCT used in this study was modified from the original protocol of O'Grodnick (1975) as follows: (1) fish tissue was blended in 100 ml of saline, (2) blended material was filtered without vacuum through six layers of cheesecloth supported in a porcelain filter funnel by a 5·5-cm diameter 0·75 mm mesh nylon screen. A single rinse (approximately 50 ml saline) was poured through the filter. Filtrate plus 50 ml of flask rinse was poured directly into the plankton centrifuge running at high speed. Centrifugation was considered complete with 3–5 ml of suspension remaining in the centrifuge cup. Material was removed from the sides of the cup by rubber spatula and added to the suspension. Myxozoans were diagnosed by microscopic examination of two drops of the centrifuge suspension. Total time con-

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sumed in processing each fish tissue sample, including cleaning and reassembling the centrifuge, was 15–20 min. No attempts were made to count spores in the samples.

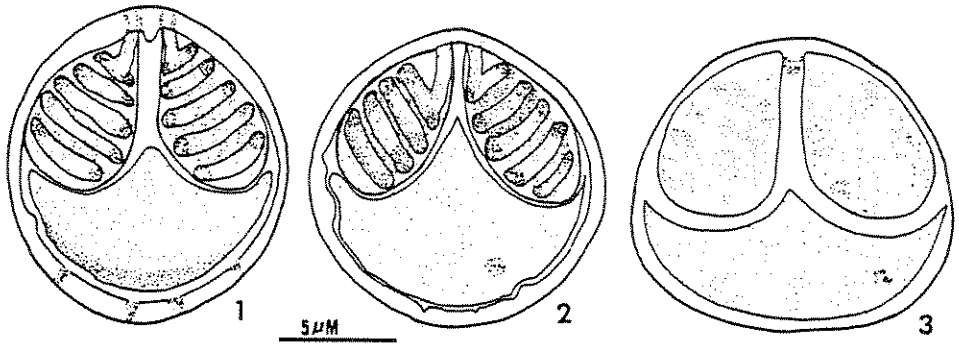
An initial survey was made by processing heads (including gills and pectoral girdles) of 25 minnows together through the PCT to estimate numbers and types of myxozoans in the population. Large numbers of three types of *Myxobolus* spores and a few spores of a *Unicauda* species were seen in this first sample, indicating the minnow population harboured suitable numbers and types of myxozoans for field testing the PCT and that these types were distinguishable after plankton centrifugation. A microscopic survey of cephalic and pharyngeal tissues and organs, skin and skeletal muscle in 25 other fathead minnows determined cyst characteristics and locations of the three *Myxobolus* types. For purposes of this study, and as reference for future taxonomic analyses, brief descriptions of spores and cysts, sites of infection and comparisons with known species are presented. Spore dimensions (measured in μm in saline wet mounts of material recovered during the microscopic survey) are recorded in Table 1.

Table 1. Spore dimensions (μm) and tissues infected by myxobolids in fathead minnows.

	Fresh spore dimensions. Range (mean)			
	Length	Breadth	Thickness	Infection site
Myxobolus sp. 1	11–15·5 (13·5) $n=30$	11–15 (12·5) $n=30$	10	Meninx primitiva, brain tissue
Myxobolus sp. 2	11·5–14·5 (12·9) $n=15$	9–12·5 (11·0) $n=15$	—	Musculature
Myxobolus sp. 3	16–20·5 (18·9) $n=18$	10–12·5 (11·0) $n=15$	9·5–10 (9·8) $n=3$	Gills, buccal and pharyngeal epithelium
	Capsular length	Breadth	Total length	
Unicauda sp.	12·5–16 (13·9) $n=6$	11–12·5 (11·4) $n=6$	34–48 (33·7) $n=6$	Undetermined

Myxobolus sp. 1. Cysts occurred within and immediately beneath the meninx primitiva and various sites within the brain. Cysts on the brain surface were often globose and visible to the naked eye as white opacities. Smaller cysts were found within brain tissue and clusters of spores were common within brain ventricles. Spores in front view were typically ovoid (slightly more narrowed anteriorly than posteriorly) (Fig. 1). Spheroid and ellipsoidal spores (Figs 2 & 3) were less common but regularly seen. Various valve opacities and indentations appeared in the posterior half of most spores. Staining with Lugol's iodine produced variable results. A few stained spores showed a distinct spheroid or crescentic iodinophilous vacuole.

Spores of this *Myxobolus* closely resembled many species in the genus, yet of the species known to infect nervous tissue, none produce spores of this type. Cysts of *M. neurophila* Guilford from yellow perch, *Perca flavescens* (Mitchill) and *M. subtecalis* Bond from *Fundulus heteroclitus* (L.) have similar appearance and location but their



Figures 1–3. Free-hand drawings of spores of Myxobolidae from fathead minnows. *Myxobolus* sp. from brain and meninx primitiva.

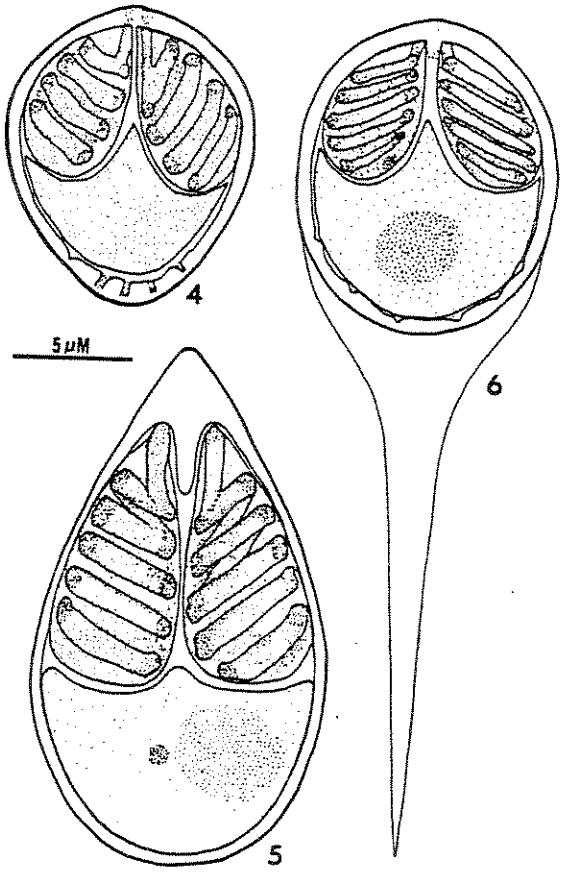
spores are distinctly different (Guilford 1963; Bond 1938). Taxonomy, histopathology and ultrastructure of this unique myxobolid will be described in a future report.

Myxobolus sp. 2 occurred as minute cysts within and between skeletal muscle fibres. Cysts contained spores of three distinct shapes. Most numerous spores were ovoid in front view with maximum breadth anterior to the transverse midline (Fig. 4); ovoid spores similar in size and shape to those of *Myxobolus* sp. 1 (Fig. 1) were less common but regularly seen in muscle infections. Several ovoid spores showed a short (2–3 μm) caudal extension of the valves. The sporoplasm stained variously with Lugol's iodine; most spores showed a diffuse positive reaction; a few showed a distinct iodinophilous vacuole in the posterior third of the sporoplasm. Spore morphology and variability of *Myxobolus* sp. 2 closely resembled that of *M. muelleri* Bütschlii common in various tissues in many cyprinid hosts in Eurasia and North America (Mitchell 1970).

A third *Myxobolus* species produced small (up to 0·8 mm) white spherical cysts on gill filaments and large (up to 2 mm) white globose cysts in pharyngeal and buccal epithelium. Spores (Fig. 5) were relatively large (Table 1) and pyriform with elongate cnidocysts. A distinct iodinophilous vacuole was visible in fresh and Lugol's stained spores. Many gill-inhabiting *Myxobolus* species produce spores virtually identical to this pyriform type. *Myxobolus* sp. 3 may be conspecific with *M. angustus* Kudo infecting gills of the bullhead minnow, *Pimephales vigilax* (Baird & Girard), in the midwestern United States (Kudo 1934).

Spores of a fourth myxobolid, *Unicauda* sp, were seen only in plankton centrifuge harvests of minnow heads and whole minnows. Caudal processes of many spores were partially broken after plankton centrifugation. Spores (Fig. 6, Table 1) closely resembled those of *U. crassicauda* (Kudo) described from fins and integument of the stoneroller, *Campostoma anomalum* (Rafinesque), from Illinois, U.S.A. (Kudo 1934). Minchew (1981) described a similar species, *U. magna*, from fin tissues of two fathead minnows in Pennsylvania.

After spore types were determined, prevalence estimates of the three *Myxobolus* forms in fathead minnows were made by microscopic survey and by the PCT. In this



Figures 4–6. Free-hand drawings of spores of Myxobolidae from fathead minnows. Figure 4 shows spore of *Myxobolus* from musculature and Fig. 5 that from the gills. Figure 6 is of *Unicauda* spore.

phase of the study, whole brains and gill arches were excised and scanned under a dissecting microscope for cysts. Smears of cysts were examined for spores with a compound microscope. Two wet mount smears were made of brain and gill tissue found uninfected and these were systematically examined under a compound microscope. Muscle infections were diagnosed by microscopic examination of two wet mount squashes prepared from a skinned, saline-washed right epaxial muscle strip (approximately $1 \times 0{\cdot}5$ cm). After microscopic examination each brain, complete set of gill arches and each muscle sample was processed separately by the PCT. The muscle-inhabiting *Myxobolus* was found in all fish examined by both methods. Ten out of 15 minnows were infected with *Myxobolus* sp. 1 by the PCT compared with six of 15 using wet mount examinations. *Myxobolus* sp. 3 was found in six of 10 fish using the PCT and five of 10 using wet mounts.

Finally 10 whole minnows were processed individually by the PCT. Spores of *Myxobolus* sp. 1 diagnosed by ovoid spores as in Fig. 4 and *Myxobolus* sp. 2 (diagnosed

by spheroid and ellipsoid spores, Figs 2 & 3) appeared in harvests of all 10 minnows. Spores of *Unicauda* sp. and *Myxobolus* sp. 3 were found in five and nine of these fish respectively. The higher prevalences indicated for the brain and gill *Myxobolus* after whole-fish processing may reflect occurrence of these myxobolids in other tissues. In the microscopic survey *Myxobolus* sp. 3 was found in pharyngeal and buccal epithelium as well as in gills. Bond (1938) found *M. subtecalis* in connective tissue throughout the viscera and in the fins in *F. heteroclitus*, although its main site of infection is the brain. The similarity among some spores of the brain and muscle myxobolids in the fathead minnow made diagnosis of these two forms difficult in whole-minnow harvests. Without detailed microscopic study of spore shape and variability these two myxobolids could not have been separately diagnosed in the whole-fish samples. The marked similarity in some spores seen in the two sites provides doubt that they are separate species.

This study suggests the PCT is applicable to studies of population dynamics of histozoic myxobolid infections in feral fish. The technique may be used initially to determine general infection levels and variety of spore types in a host population. The PCT seems more sensitive and less time-consuming than the routine microscopic survey. Whole organs or entire fish may be processed and examined rather than small samples. After infections are found by the PCT, spore and cyst characteristics and sites of infection may be ascertained by systematic microscopic survey. Prevalence may then be monitored by the PCT. Although we have made no attempt to monitor intensity of these myxobolid infections, from studies with *M. cerebralis* (O'Grodnick 1975, 1979) it seems the PCT could also provide valid estimates of this population parameter.

Acknowledgments

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EGG TRANSMISSION OF WHIRLING DISEASE

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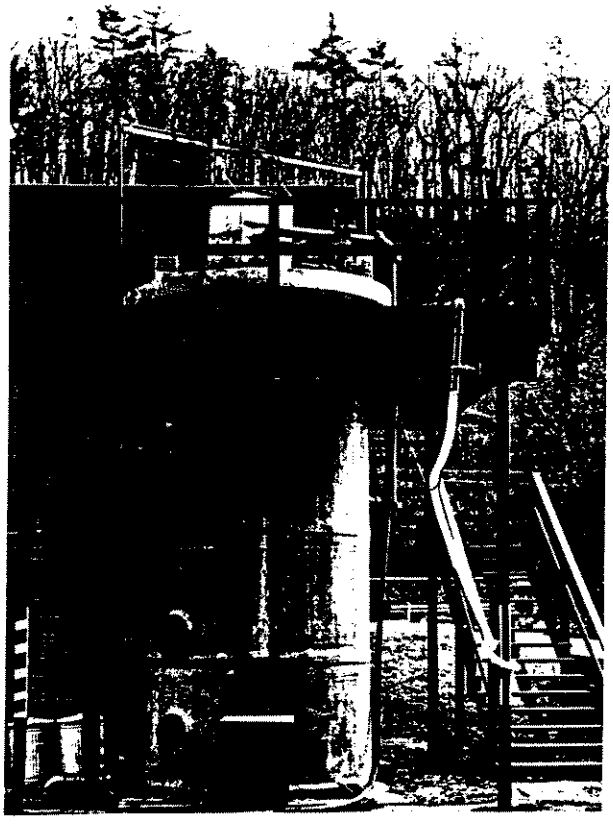
Rainbow trout (*Salmo gairdneri*) infected with whirling disease are known to transfer the disease when transported to other hatcheries. Fertilized eggs from infected trout, however, seem to be free of the infective agent of whirling disease. We carried out a 3-year large-scale experiment at the Pennsylvania Fish Commission's Benner Spring Fish Research Station to determine whether whirling disease-free fish could be raised from diseased-parent brood stock.

Large lots of rainbow trout eggs were taken from infected parents, hatched, and raised in vertical units using whirling disease spore-free spring water. Sixty fingerlings from each lot, the number necessary for the statistical estimate of a minimum of 5% incidence of disease [*3*], were examined for spores at 5 months of age.

METHODS

Rainbow trout brood stock infected with whirling disease were held for spawning purposes in separate concrete holding ponds. They were spawned during the fall of 1971, 1972, and 1973. A statistically significant number of eggs were taken each year; the fry were hatched and raised in whirling disease-free spring water at 51° F. During 1971, 50,000 rainbow trout were raised to fingerling size in the experimental silo (see figure). All the trout were progeny of infected parents verified by the detection of spores. In 1972, infected golden rainbow (*Salmo gairdneri*, golden color phase) trout were spawned and 5,000 fry were mixed with normal production fish in the silo. After 5 months, 60 golden trout were separated and examined for spores. In 1973, 25,000 rainbow trout were raised in 55-gallon vertical units using spore-free water. These fry were the progeny of 3-year-old infected brood stock.



The experimental silo uses spore-free spring water for whirling disease research.

At the end of the testing period each year, 60 rainbow trout fingerlings were sacrificed.

The fish heads were run through a spore concentration method developed at Benner Spring [2], and examined for the spores of whirling disease; this method has proven successful in the discovery of low levels of spores in rainbow trout exhibiting no symptoms of whirling disease.

RESULTS AND DISCUSSION

In the 3-year experimental period, no signs of whirling disease were observed and no spores were found in test fish. Presumably, freshly shed spores would have to age at least 4 months before becoming infective [1] and any spores adhering to eggs would be flushed away before or after egg hatching. Unless the effluent was used where trout fry were being reared the following year, it is not likely that whirling disease would become established. Further, because the spores have never been reported from the ovaries or eggs, it is possible that no spores or very few would be shed during the spawning procedure. Because young fry are very susceptible to whirling disease [4], this long-term experiment is considered valid and it indicates that transmission of whirling disease via fertilized eggs is highly unlikely. Using care to avoid contaminated water, mud, etc. during the spawning of valuable strains of fish, we believe that whirling disease transmission can be prevented.

This study was supported by funds issued under the Federal Aid in Fish Restoration (Dingell-Johnson Act).

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WHIRLING DISEASE *Myxosoma cerebralis* SPORE CONCENTRATION USING THE CONTINUOUS PLANKTON CENTRIFUGE

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Abstract: A method for concentrating and detecting the spores of the cause of whirling disease (*Myxosoma cerebralis*) of salmonids is described. The method involves homogenization of head skeletons, screening out tissue shreds, and concentrating with a continuous plankton centrifuge.

INTRODUCTION

The detection of whirling disease spores in carrier fish has always presented the biologist with diagnostic problems.[2] Since new disease regulations may require the diagnosis of whirling disease in large lots of trout,[3] practical methods for concentrating spores should be available to the fishery pathobiologist.

At the Benner Spring Fish Research Station a procedure using the continuous plankton centrifuge[1] (Figure 1) has been developed to concentrate whirling disease spores from fish in research experiments and production lots. This method has been used for 4 years and has given satisfactory results in the quantitative studies carried out in our whirling disease research projects. Rainbow trout, for example, which do not exhibit signs of the disease, have been shown by this method to harbor relatively large numbers of spores.

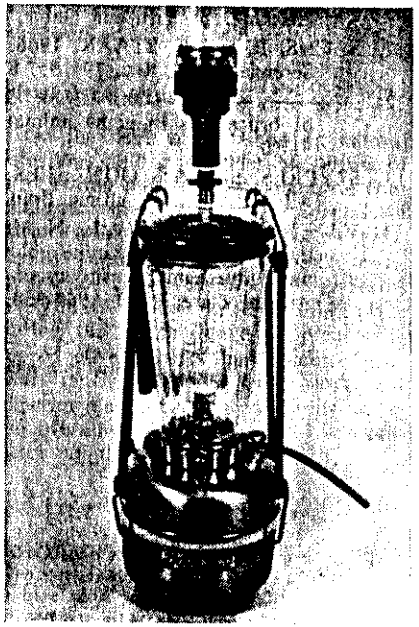

FIGURE 1. The plankton centrifuge removes the excess water from the filtrate through the centrifugal force created by the revolving drum. This excess water is carried away through a rubber tube situated below the drum. Most solid particles are deposited on the wall of the revolving drum in the first centrifuge run.

MATERIALS AND METHODS

The materials needed are: Waring blender, blender bottle (175 ml or larger), membrane filter apparatus, wire mesh prefilter screen—mesh size 725 mm,[2] continuous plankton centrifuge, separatory funnel and ring stand, 27 ml specimen bottle, hemocytometer.

The fish are decapitated near the base of the skull. Larger fish are best handled by heating the heads at 45 C for 2-3 min and then removing as much soft tissue as possible. Large pieces may be split or cut into smaller pieces.

[1] Plankton centrifuge manufactured by G. M. Mfg. and Instrument Corporation, P.O. Box 326, Nanuet, New York 10954.

[2] For more critical work one may screen the homogenate through graded mesh-size screens, e.g., 520 - 260 - 120 nm mesh.

STEPS

1. Take five to ten heads of 8-15 cm fish or one to five heads from larger fish and blend for 5 min in 175-200 ml of water. If spores are intended for infection experiments, chlorinated water should be avoided.
2. Remove the blended material and vacuum filter the entire volume through a membrane bacteriological filter using a wire mesh prefilter. The filter may clog and should be cleaned with water. The rinse water becomes part of the filtrate to be saved. The procedure removes fish scales and other coarse material which is discarded.
3. Place the entire amount of filtrate in a separatory funnel discharging into the plankton centrifuge. Set a low flow from the separatory funnel into the centrifuge and run centrifuge at high speed.
4. Centrifuge until all water is removed. The residue adhering to the walls of the revolving drum will contain spores and some fish tissue. Scrape residue from the walls of the plankton centrifuge revolving drum with a rubber policeman. Be sure to get all material.
5. Place the residue and suspended material from the drum into the 27 ml bottle and fill to the top with distilled water. Place the cap on the bottle and shake until the material is uniformly suspended (2 min).
6. Place a small amount of suspension under a coverslip of the hemocytometer if a spore count is desired.[4] Count the spores under four 1 mm^2 squares. We use four chambers, counting a total of 16 squares.

   Formula:

$$\frac{\text{number of spores}}{1\text{ ml}} = \frac{\text{total number of spores counted} \times 10^4}{\text{number of 1 mm}^2\text{ areas counted}}$$

   27 ml (Suspension Volume) x number of spores in 1 ml = total number of spores.
7. Equipment should be scrubbed thoroughly, soaked in half-strength household bleach (NaOCl) for 10 min and rinsed thoroughly after each use.

PLANKTON CENTRIFUGE EFFECTIVENESS

Using this technique we have recovered 3.4 x 10^1 to 9 x 10^6 from our pooled samples during the past 4 years. G. L. Hoffman recovered 4.2 x 10^5 to 4.3 x 10^6 spores per fish from heavily infected fish using this technique.[3] The late W. Tidd also obtained good results with the plankton centrifuge.

To confirm the effectiveness of the plankton centrifuge in spore concentrating, a test was devised to determine the relative loss of spores in the water which is removed from the centrifuge. Five heavily infected rainbow trout heads were run through the spore concentration procedure which consisted of three trial runs, reusing the same suspension.

[3] Hoffman, G. L. 1974. Personal communication. Eastern Fish Disease Laboratory, Kearneysville, West Virginia 25430.

Spores from the first run were concentrated and the number per ml estimated. The water from the first run was collected and recentrifuged, and the number of spores per ml of suspension was estimated for the second time. The water from the second run was collected, recentrifuged, and again the spores per ml of suspension was estimated. By this time the spore count was very low and further concentration was believed unnecessary.

The estimated spore number from each run was added and total spore count obtained (Table 1).

To determine the absolute loss of spores after the first centrifuge run, a seed population of 10,250,000 spores was added at step three of the procedure. An estimated recovery of 8,370,000 spores was achieved, which indicates an 83% efficiency of recovery.

TABLE 1. Spores recovered from the same suspension in three runs.

	Estimated Spores Per ml	Percentage of Total Spores Recovered
Run #1	458,750	96.2
Run #2	17,500	3.7
Run #3	630	0.1
TOTAL	476,880	

DISCUSSION

Because the spores are trapped in skeletal structures and granulomas they are difficult to detect in carrier fish. Recent studies on spore concentration methods include blending, filter paper filtration and centrifugation,[4,8] and fish digestion.[1,4,5,7] The plankton centrifuge concentrates whirling disease spores effectively for diagnostic purposes. It has the advantage of speed and simplicity and the final suspension is fairly debris-free. Relatively few spores are lost after the first centrifuge run, thus a reasonable quantitative estimate can be made and the disease level of the population determined.

Acknowledgment

The author wishes to thank Dr. G. L. Hoffman, Eastern Fish Disease Laboratory, for his aid in the testing of the method and in the preparation of the manuscript.

Research on whirling disease is being supported by funds issued under the Federal Aid in Fish Restoration (Dingell-Johnson) Act.

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MFR PAPER 1341

Susceptibility Studies of Various Salmonids to Whirling Disease: Histological Staining and Spore Concentration Procedures

JOSEPH O'GRODNICK

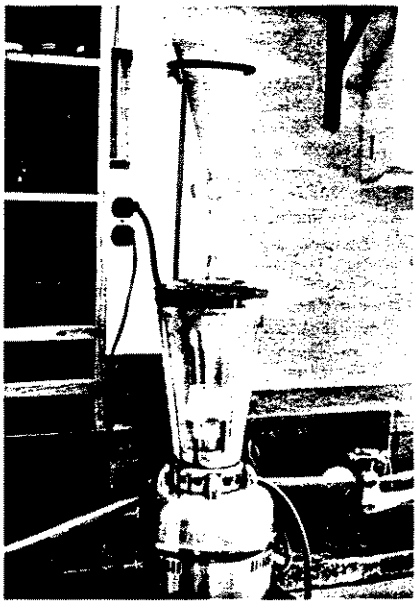

Figure 1.—The plankton centrifuge. The centrifuge removes water through the centrifugal force created by the revolving drum. The excess water is carried away through a rubber tube situated below the drum. Solid particles are deposited on the wall of the revolving drum.

Since 1968, research on the transmission, life history, and control of whirling disease in trout has been conducted by the Pennsylvania Fish Commission. Whirling disease is caused by a myxosporidian parasite, *Myxosoma cerebralis*, which invades and destroys cartilage of susceptible salmonid species. The parasite was introduced into the United States from Europe and has been established as a persistent hatchery disease in several areas.

Affected fish exhibit rapid, tail chasing behavior from the disintegration of the cartilaginous support skeleton of the organs of equilibrium. Also, severe crippling may result from destruction of the spinal skeleton if fish are exposed as fry. "Blacktail," which is caused by pressure on the nerves that control the caudal pigment cells, may be seen in some fish.

In this research, two methods were used to monitor the development of the whirling disease parasite in various salmonid species: 1) examination of histological sections, and 2) quantitative estimates of spores determined by the use of the plankton centrifuge procedure (Fig. 1). (This method involves homogenization of head skeletons, screening out tissue shreds, and concentrating with a continuous plankton centrifuge.

The life cycle of *Myxosoma cerebralis* was monitored in rainbow trout (highly susceptible species) and brown trout (highly resistant species) by the examination of histological sections. Tissues were prepared from infected fish at given intervals, averaging 3 days from initial exposure to the development of spores at 120 days. The following staining methods were used in the study: 1) Hematoxylin and Eosin, 2) Mallory Heidenhain (Casson-Modification), 3) Wright's stain, and 4) Ziehl-Neelson (acid fast staining for spores).

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Figure 2.—Trophozoites of *Myxosoma cerebralis* developing in rainbow trout cartilage 35 days postexposure. Mallory Heidenhain (Casson-Modification); 225×.

Figure 3.—Mature spores of *Myxosoma cerebralis* in rainbow trout cartilage. Wright's stain. 225×.

The study revealed the following sequence of events in rainbow trout tissue. The trophozoite stage (Fig. 2) was first observed at 20 days postexposure. No definite parasitic stage was observed prior to invasion of the cartilage at 20 days. There was rapid proliferation of the multinucleated trophozoites by numerous divisions for 70 days—90 days from initial exposure. At 90 days, the trophozoites began the transformation to the spore stage of the life cycle (pansporoblast stage). A connective tissue network began to surround the developing spores, and mature spores (Fig. 3) were seen at 120 days. The fish in this study were reared at 11°C water temperature.

The brown trout study revealed a similar development of *Myxosoma cerebralis*, but very few parasitic lesions were observed. There appeared to be some disintegration of active trophozoites 60-90 days after initial exposure. There were also many tissue eosinophils present in surrounding connective tissue of infected brown trout. These cells were also observed in control fish tissue sections. Spores developed in smaller pockets in brown trout cartilage and were difficult to find in histological sections.

A study of the relative susceptibility of various salmonids to whirling disease was conducted during 1975 and 1976. In 1975, fingerlings of four different salmonids were exposed to whirling disease for a 60-day period and then reared in uncontaminated water until spores developed at 160 days. Samples were run in five pools of five fish each (25 heads) for the plankton centrifuge technique and five fish (25 slides) for the histologic section technique. Table 1 contains the results of the 1975 study.

In 1976, salmonid fry were exposed for 3 days to whirling disease and then reared in spore-free water until spores developed at 160 days. Samples were run as in 1975. Table 2 contains the results of the 1976 study.

Table 1.—Susceptibility of four species of salmonid fingerlings exposed to whirling disease for 60 days as assessed by histologic and plankton centrifuge techniques.

Species	Histology: no. of positive slides (5 fish/25 slides)	Plankton centrifuge: average no. of spores per head (25 heads)
Rainbow trout	Not done	404,800
Brook trout	3	47,336
Brown trout	Not done	784
Coho salmon	0	0

Table 2.—Susceptibility of seven species of salmonid fry exposed to whirling disease for 3 days as assessed by histologic and plankton centrifuge techniques.

Species	Histology: no. of positive slides (5 fish/25 slides)	Plankton centrifuge: average no. of spores per head (25 heads)
Rainbow trout	100	1,619,156
Brook trout	56	552,150
Brown trout	8	6,075
Lake trout	0	0
Kokanee salmon	100	116,775
Chinook salmon	20	60,775
Coho salmon	0	[1]0

[1]Accidental loss, only one pool run.

The development of *Myxosoma cerebralis* varied extensively in the salmonids studied. Under controlled conditions, brown trout, lake trout, and coho salmon (which exhibited little or no evidence of spores) did not develop characteristic whirling disease signs. Rainbow trout, brook trout, kokanee salmon, and chinook salmon did develop whirling disease, exhibiting clinical signs of disease. Since whirling disease can cause substantial losses in some hatcheries, resistant species may be reared to sustain production in areas where *Myxosoma cerebralis* is endemic.

Recent studies have revealed that species other than salmonids may be carriers of the whirling disease parasite. We have found similar sporozoan parasites in cartilage of suckers and in brain tissue of creek chubs.

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Transactions of the American Fisheries Society 108:187–190, 1979

Susceptibility of Various Salmonids to Whirling Disease (*Myxosoma cerebralis*)

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Abstract

In four experiments with fry and fingerling salmonids during 3 years, rainbow trout (*Salmo gairdneri*) were most susceptible to whirling disease (*Myxosoma cerebralis*) followed, in decreasing order of susceptibility, by sockeye salmon (*Oncorhynchus nerka*), brook trout (*Salvelinus fontinalis*), chinook salmon (*Oncorhynchus tshawytscha*), brown trout (*Salmo trutta*), and coho salmon (*Oncorhynchus kisutch*). Lake trout (*Salvelinus namaycush*) were completely refractory. Absence of infection was determined by absence of spores and signs in exposed young fish. Refractory or least susceptible species of salmonids should be reared in enzootic whirling disease areas.

The spread of whirling disease caused by *Myxosoma cerebralis* (Protozoa: Myxosporida) in the United States has caused concern for state and federal agencies which are responsible for the regulation of fish cultural operations and for the impact of these operations on the natural environment. When whirling disease becomes established in a hatchery, the parasite can also become established in the wild salmonids of receiving streams (Yoder 1972). Once *Myxosoma cerebralis* becomes an enzootic parasite, eradication of whirling disease in hatcheries with open water supplies is virtually impossible. Complete destruction of fish stocks and disinfection of raceways, or abandonment of fish rearing facilities, have been recent practices when whirling disease has been verified in cultured fish. Neither practice has been entirely acceptable to fish culturists involved with the problem. Alternate means of management which are environmentally and economically sound should be made available when whirling disease is encountered.

Spores of *M. cerebralis* can develop in many salmonids (Hoffman and Putz 1969; Hoffman 1976) but quantitative comparisons of development of whirling disease under controlled conditions are rare. We have found no clinical signs of whirling disease and very low spore numbers during production of brown trout, even when this species was reared in highly contaminated water. The present study was conducted to determine the relative susceptibility to whirling disease of other salmonids normally reared at the Pennsylvania Fish Commission's hatcheries. Salmonids known to be resistant to *M. cerebralis* could be cultured in contaminated hatcheries without the development of clinical signs of whirling disease, or the production losses associated with the disease.

Methods

The 3-year study was carried out at the Benner Spring Fish Research Station, Bellefonte, Pennsylvania. Salmonid fry or fingerlings were held and exposed to whirling disease in a contaminated stream or in a hatchery under experimental conditions. The test fish were evaluated for whirling disease by several methods.

(1) Development of clinical signs of disease were noted. These signs included circular swimming motions (whirling), body deformities, and a blackening of fish tails due to nerve destruction of pigment control centers.

(2) Trophozoite development in cartilage tissue was assayed. Five fish were sacrificed from each test group 60 days after exposure. Five sagittal sections were prepared from each fish head (total, 25 tissue sections) and stained by the Mallory Heidenhain (Casson modification) staining procedure (Simpson 1950). The percentage of positive tissue sections was used as an indicator of relative susceptibility for each species.

(3) The number of *M. cerebralis* spores developed 160 days after the fish were exposed was determined. Spores were isolated by the following method. Five fish heads were pooled and homogenized in water in a Waring blender. The homogenate was filtered and concen-

trated in a continuous plankton centrifuge (O'Grodnick 1975). Microscopic examination of the concentrates with the aid of a clinical hemacytometer counting chamber gave an estimate of the number of spores per pool.

Because of varying degrees of susceptibility of each salmonid species to incidental bacterial and viral diseases, mortality of test fish was not used as an indicator for whirling disease susceptibility even though rainbow trout and sockeye salmon displayed classic signs of whirling disease and high mortalities.

The experimental design for each year follows.

1975

Brook trout, brown trout, rainbow trout, and coho salmon were exposed to *M. cerebralis* in a highly contaminated water supply: Cedar Run, Clinton County, Pennsylvania. The fish were exposed for 60 days and then reared in spore-free hatchery water until sacrificed at 160 days postexposure.

The brook, brown, and rainbow trout were Pennsylvania Fish Commission production fish exposed on May 8 (brown and rainbow trout), or November 7 brook trout). The coho salmon were provided by Michigan Department of Natural Resources and exposed July 9. Because of fish cultural activities on Cedar Run, I assumed that the level of contamination did not vary significantly during the year. Water temperature of the spring-fed stream averaged 11 C.

1976

Fry of seven species were exposed for 3 days in Cedar Run, 3 weeks after start of feeding (swim-up), during January–March. Rainbow, brook, and brown trout were Pennsylvania Fish Commission production fish. Coho and chinook salmon were received from Michigan Department of Natural Resources. Lake trout were received from the New York Department of Environmental Conservation, sockeye salmon were progeny of wild fish from Upper Woods Pond, northeastern Pennsylvania.

1977

In one study, rainbow, brown, and brook trout and coho salmon were exposed for 3 days in Cedar Run, as in 1976. In a second study, rainbow trout, brook trout, and coho salmon were exposed for at least 7 months under production conditions at the United States Fish and Wildlife Service's Tylersville National Fish Hatchery, which has a low incidence of whirling disease. Brook and rainbow trout were Bowden, West Virginia strains. Coho salmon were received from Michigan.

Results

In the 3-year susceptibility study (Table 1) rainbow trout were always most susceptible to whirling disease. Lake trout were always refractory and coho salmon usually were refractory. Brook trout, chinook salmon, and sockeye salmon were intermediate in susceptibility. Brown trout were always carriers of *M. cerebralis* spores. Clinical signs of whirling disease developed in only four species: rainbow trout, brook trout, chinook salmon, and sockeye salmon. Clinical signs were seen in rainbow and brook trout even in "low-incidence" hatchery waters.

The intensity of whirling disease development was related to the species of fish and size of susceptible fish when exposed, and to the level of contamination. These test results verify findings of Hoffman and Putz (1969) and extend the range of susceptible species.

Discussion

In this study the spores of *M. cerebralis* developed in all salmonids except lake trout, although the level of infection varied greatly. Similar patterns of species susceptibility to *Ceratomyxa shasta* were found by Zinn et al. (1977), and strain susceptibility differences were also apparent in their study. I cannot explain why lake trout were susceptible in Hoffman and Putz's 1969 work, but were refractory in my experiments; perhaps there was a strain difference. This species should be retested.

Myxobolus neurobius was found in the central nervous system of wild brook trout in the Tylersville hatchery water source. The larger spore and iodinophilous vacuole make this species easily distinguishable from the smaller spore of *M. cerebralis*. According to G. L. Hoffman (United States Fish and Wildlife Service, personal communication), no pathogenicity has ever been attributed to *M. neurobius*.

Because *M. cerebralis* has already become established in certain geographical areas, policies of eradication are not practical and abandon-

TABLE 1.—*Susceptibility of salmonids to infection with* Myxosoma cerebralis.

Species	Number of fish	Mean total length at end of test (cm)	Exposure period (days)	Signs of whirling disease 60 days post-exposure[a]	% of 25 tissue slides with tropho-zoites	1,000's of spores per 5 fish, 160 days after exposure or at termination of test	
						Mean	Range
1975 tests—stream exposure							
Rainbow trout	25	14.0	60	+++		2,025	1,539–2,592
Brook trout	25	12.6	60	+		237	176–286
Brown trout	25	14.4	60	0		3	0–17
Coho salmon	25	8.7	60	0		0	0
1976 tests—stream exposure							
Rainbow trout	20	6.4	3	++++	100	8,096	7,036–9,349
Brook trout	25	6.7	3	++	56	2,761	1,991–3,561
Sockeye salmon	25	5.5	3	++++	100	584	337–961
Chinook salmon	20	5.9	3	++	20	300	186–388
Brown trout	25	6.9	3	0	8	31	0–101
Coho salmon	5	5.7	3	0	0	0	0
Lake trout	15	7.0	3	0	0	0	0
1977 tests—stream exposure							
Rainbow trout	120	10.7	3	++		4,435	2,126–7,593
Coho salmon	60	10.8	3	0		10	0–67
Brown trout	60	8.1	3	0		3	0–34
Lake trout	60	9.8	3	0		0	0
1977 tests—hatchery exposure							
Rainbow trout	60	15.9	352	+		169	67–405
Brook trout	60	17.7	221	+++		68	34–135
Coho salmon	60	13.7	216	0		0	0

[a] Signs of whirling disease:
0 = no signs of whirling;
+ = less than 5% whirling;
++ = 5–20% whirling;
+++ = 20–50% whirling;
++++ = majority of fish whirling.

ment of existing facilities is not economically justifiable. An acceptable management alternative may be the rearing of proven-resistant salmonids in contaminated hatcheries. State agencies could arrange production schedules so that resistant species which do not develop clinical whirling disease are substituted for susceptible rainbow trout or brook trout. Coho salmon, brown trout, and lake trout, which we have found to be very resistant to whirling disease, can be reared in contaminated hatcheries. The number of spores developed by these species is quite low and it would be unlikely that whirling disease would be established in large water bodies when these fish are stocked. Also, receiving streams for hatchery effluent would recruit fewer spores from very resistant production fish and contamination would be reduced.

Since the value of the coho salmon and lake trout to the Great Lakes fishery is well documented, a careful evaluation of the policies regarding rearing these resistant species in known *M. cerebralis*-contaminated waters should be made.

Acknowledgements

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Fish Sporozoa: Extraction of Antigens from *Myxosoma cerebralis* Spores Which Mimic Tissue Antigens of Rainbow Trout (*Salmo gairdneri*)

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PAULEY, G. B. 1974. Fish sporozoa: extraction of antigens from *Myxosoma cerebralis* spores which mimic tissue antigens of rainbow trout (*Salmo gairdneri*). J. Fish. Res. Board Can. 31: 1481–1484.

This is the first report of an antigenic preparation from the spore stage of any histozoic sporozoan parasite. The method for successful extraction of the antigens is presented. This antigenic preparation is active in rabbits, but not salmonid fish. The detection of asymptomatic carrier fish using the spore antigens was not successful and evidence is presented to show that the parasite is successful because its antigens mimic those of trout. Immunoelectrophoresis indicates the parasite and trout antigens are different proteins.

PAULEY, G. B. 1974. Fish sporozoa: extraction of antigens from *Myxosoma cerebralis* spores which mimic tissue antigens of rainbow trout (*Salmo gairdneri*). J. Fish. Res. Board Can. 31: 1481–1484.

Cet article rapporte pour la première fois la préparation d'antigènes à partir de spores d'un sporozoaire histozoïque parasite, de quelque espèce que ce soit. L'auteur décrit la méthode utilisée pour extraire les antigènes avec succès. Cette préparation est active chez des lapins, mais non chez des salmonidés. Il a été impossible de détecter des poissons porteurs asymptomatiques au moyen d'antigènes de spores, et l'auteur démontre que le parasite réussit bien parce que ses antigènes imitent ceux de la truite. L'immunoélectrophorèse indique que les antigènes du parasite et ceux de la truite sont des protéines différentes.

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THE sporozoan *Myxosoma cerebralis*, causative agent of "whirling disease," attacks the cartilage of young salmonid fish (Hoffman et al. 1969; Hoffman 1970). Infected fish either die or become grossly misshapen with gaped jaws, deformed heads, and twisted bodies due to erosion of much of the cartilage and weakened skeletons (Hoffman et al. 1969). This parasite exhibits a wide geographical distribution (Elson 1969; Hoffman 1970; Roberts and Elson 1970; Yasutake and Wolf 1970) and is a serious threat to the salmonid resource. Current field methods of detecting infections in asymptomatic carrier fish are cumbersome (Landolt 1973) and depend upon visual observation of *M. cerebralis* spores by light microscopy (MacLean 1971; Halliday 1973; Markiw and Wolf 1974).

Because several immunological tests used for the diagnosis of parasitic diseases in humans (Fife 1971) appeared to have application as rapid, sensitive, and positive methods of detecting asymptomatic carrier fish, a method for extracting antigens from *M. cerebralis* spores for serological work was actively sought and is presented in this paper. Chen et al. (1974) developed a rapid and accurate test for the detection of asymptomatic carrier fish infected with kidney disease. After developing a rabbit antiserum against *M. cerebralis* spore antigens, the method of P. K. Chen, G. L. Bullock, H. M. Stuckey, and A. C. Bullock (unpublished data) was used with the hope of detecting asymptomatic whirling disease in rainbow trout (*Salmo gairdneri*). Surprisingly, however, the tests employed indicate that *M. cerebralis* possesses antigens that are immunologically similar to those found in the tissue of rainbow trout.

Materials and Methods

Spores were obtained from infected rainbow trout by the pepsin–trypsin digest method of Markiw and Wolf (1974). To insure complete removal of all *S. gairdneri* host tissue from the spores, ether extraction was performed as outlined by Landolt (1973). The

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combination of these two procedures gave clean spores free from any host tissue or bacterial contamination.

Although a variety of methods have been used to lyse protozoans and free their internal antigenic components, most investigators use some method that involves either freezing and thawing or sonication. Initially, several variations of the freeze–thaw and sonication method used to obtain antigens from amoebae and trypanosomes were tried (Goldman and Siddiqui 1965). To sonicate *M. cerebralis* spores, Sonifier Cell Disrupter (Model W185D)[2] with a microtip was used for 3-min intervals at 70 W. Glass powder or Alundum was used as an abrasive material to facilitate disruption of the spores. The spore solution was cooled continuously in an ice-water bath.

Proteins were successfully obtained from *M. cerebralis* spores by a method similar to that used for isolating plant proteins by Abrams (1972). Approximately 5 million spores in 2 ml Hanks' Balanced Salt Solution (BSS) were frozen and thawed 8 times in a dry-ice and acetone bath. Glass beads, 0.5 mm in diameter, were added in a ratio of 1:4, beads in liquid, and the mixture was placed in a cold mortar for crushing with a pestle. The spores were alternately frozen and thawed while being crushed. Periodic microscopic examination on a Neubauer hemocytometer showed that approximately 1 h of crushing was needed to rupture all the spores. The ground material was washed with 10 ml Hanks' BSS and the mixture was centrifuged at 1000 $\times g$ for 10 min at 4 C. The sedimented pellet obtained was resuspended in 10 ml Hanks' BSS and again centrifuged. The sedimented pellet was discarded. The supernatant fluids from each washing were pooled and concentrated to 5 ml (1 ml/million spores) by pressure dialysis to remove water and small ions. This was accomplished at 4 C with approximately 20 psi (14,062 kg/m^2) N_2 and an Amicon Ultrafiltration Cell that was fitted with a PM 10 Diaflow Ultrafilter having a molecular weight exclusion limit of 10,000 Daltons[3]. This concentrate was subjected to electrophoresis at 4 C on 7% acrylamide gel columns as outlined by Davis (1964). Gels were stained for total proteins with Coomassie brilliant blue R, 5% in 10% trichloracetic acid, for a minimum of 6 h, and differentiated in 7% acetic acid.

Antiserum to *M. cerebralis* was produced in New Zealand white rabbits by intramuscular injection of a total of 3×10^6 crushed spores (1×10^6 spores/ml BSS) per rabbit mixed with Freund's complete adjuvant (FCA) in a ratio of 1:1. Five rainbow trout, held at 12 C, received a total of 1×10^6 spores in FCA; half intramuscularly near the dorsal fin and half intraperitoneally. This injection protocol was followed two subsequent times, each 10 days apart. Blood was taken 10 days following the last injection. Rabbit blood was taken by ear bleeding. Trout were anesthetized with M.S. 222 (tricaine methanesulfonate) and then bled by caudal puncture using a nonheparinized Vacutainer®. After the first bleeding the trout were bled subsequently every 30 days for 4 mo. The straw-colored serum was separated from the clots after 2 h at room temperature for use in serological tests.

In an attempt to see how sensitive anti-*M. cerebralis* serum would be in detecting whirling disease, uninfected fish cartilage (5 g quantities) was seeded with various numbers of spores: 2×10^6, 1×10^6, 1×10^5, 1×10^4, 1×10^3, or 1×10^2. Glass beads, 0.5 mm in diameter, were added to the cartilage in a ratio of 1:4, beads in tissue, plus 5 to 10 ml Hanks' BSS. This mixture was then alternately frozen and thawed while being crushed in a mortar with a pestle until all spores were ruptured, as described above. Hanks' BSS was added to make a total volume of 50 ml. This mixture was centrifuged at 1000 $\times g$ for 10 min at 4 C. The supernatant fluid was removed and concentrated to 10 ml for serological testing. Uninfected fish cartilage without any spores, but treated exactly the same, served as a control.

Ouchterlony immunodiffusion tests were carried out in 60-mm plastic flat bottom plates, containing 5 ml of a medium consisting of 1.0% washed Noble agar (Difco) and 1:10,000 Merthiolate®. The well pattern consisted of a central well and six peripheral ones, made with an Autogel[4] punch assembly. The plates were incubated in a moist chamber for 24 h. Immunoelectrophoresis was carried out with Gelman[5] immunoelectrophoresis apparatus. A 1.0% mixture of washed Noble agar in 0.05 ionic strength barbital buffer at pH 8.2 was the supporting medium for electrophoretic separation of *M. cerebralis* and *S. gairdneri* antigens. Electrophoresis of the antigens was for 1 h at 200 V using the same barbital buffer as in the agar. Precipitation during immunodiffusion and immunoelectrophoresis was allowed to proceed in a moist chamber at room temperature for 24 h.

Results

Even in the presence of abrasives and with prolonged cooling, the protracted sonification times of 45 min to 1 h required to break the spore capsules severely altered the proteins, and only one weak protein band was visible in acrylamide gels (Fig. 1). However, the rupture of spores by the modified method of Abrams (1972) yielded four very strong protein bands and four weaker bands after electrophoresis in acrylamide gels (Fig. 1).

No immune response was detected by immunodiffusion in rainbow trout injected intramuscularly with spore sonicate in adjuvant. Only a weak response was observed in one of six rabbits injected

[2]Heat Systems — Ultrasonics, Inc., Plainview, Long Island, N.Y., USA.

[3]Amicon Corporation, Lexington, Mass. 02173, USA.

[4]Grafar Corporation, 12613 Woodrow Wilson, Detroit, Mich. 48238, USA.

[5]Gelman Instrument Company, P.O. Box 1448, Ann Arbor, Mich. 48106, USA.

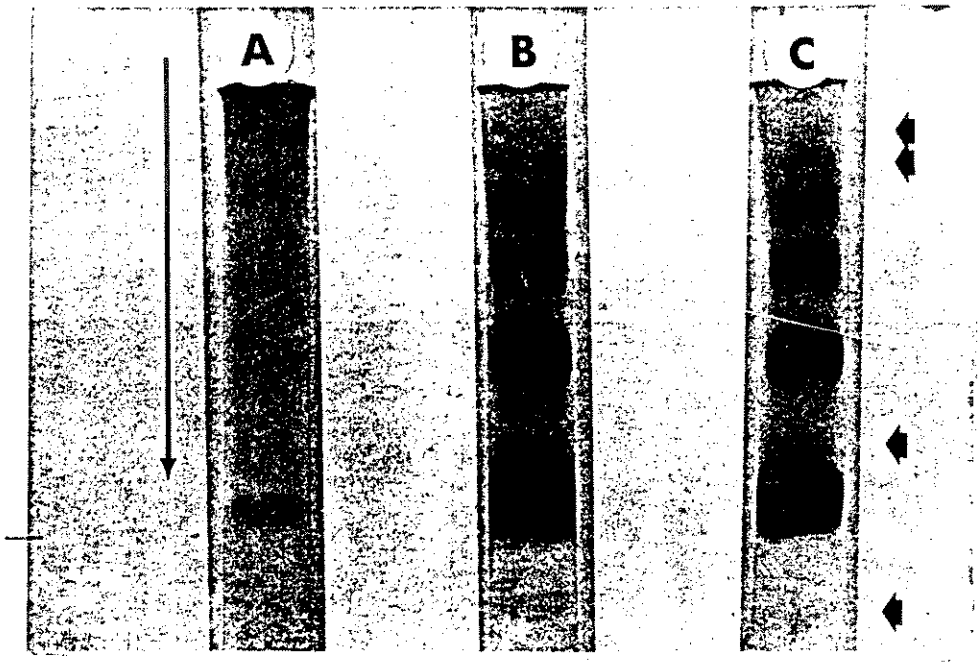


FIG. 1. Acrylamide gels showing proteins extracted from *Myxosoma cerebralis* spores. A, sonicated spores; B and C, crushed spores. Small arrows indicate position of four minor bands. Large arrow indicates direction of protein migration through the gel.

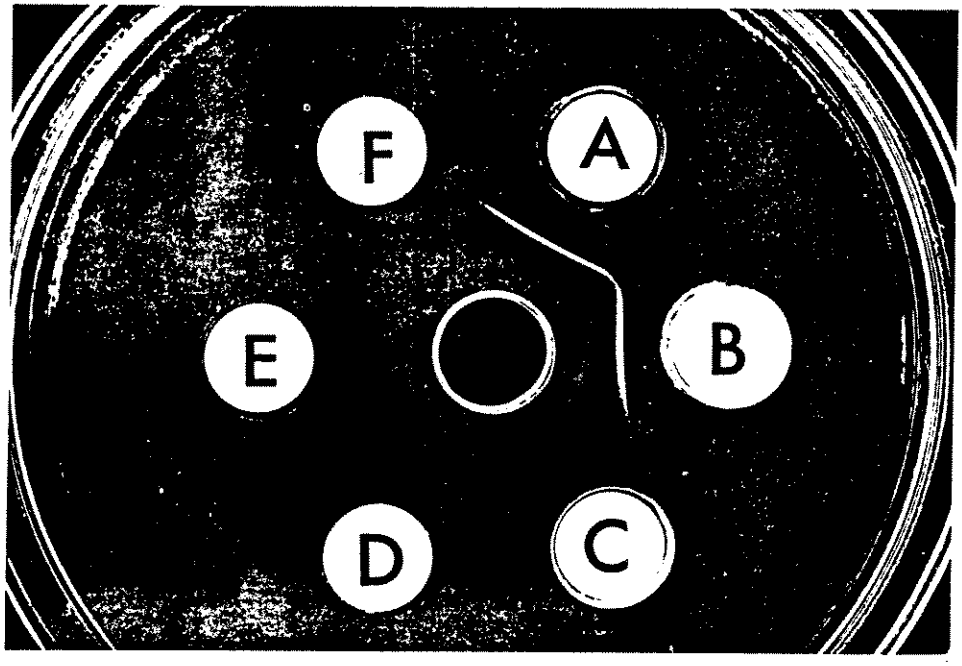
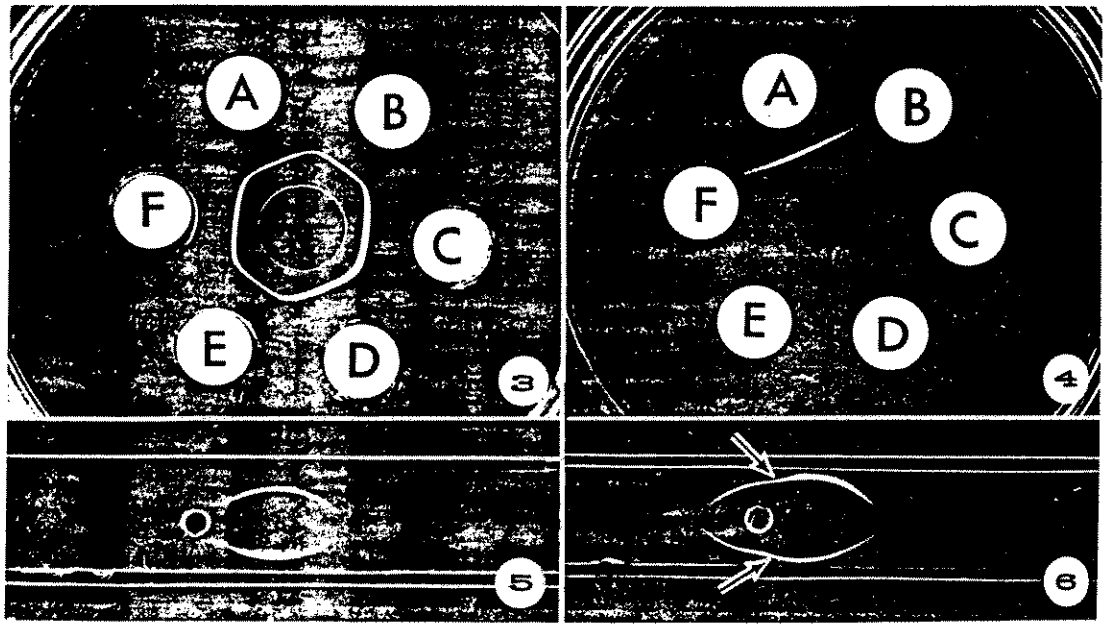


FIG. 2. Serological reactions between *M. cerebralis* antigens (center well) and rabbit antisera by the Ouchterlony gel diffusion technique. A and B, antisera against spores crushed by modified method of Abrams (1972); C and D, antisera against spore sonicate; E and F, antisera against whole spores.



FIGURES 3–6

FIG. 3. Serological reactions between rabbit anti-*M. cerebralis* (center well) and *M. cerebralis* antigens and rainbow trout (*Salmo gairdneri*) antigens by Ouchterlony gel diffusion technique. A and B, concentrated spore antigens containing 3×10^6 spores/ml and 6×10^6 spores/ml, respectively; C, D, and E, normal trout cartilage seeded with 2×10^6 spores, 1×10^5 spores, and 1×10^3 spores, respectively; F, normal trout cartilage. Note lines of complete identity; FIG. 4. Serological reactions between *M. cerebralis* antigens (center well) and rabbit antiserum (A) and fish serum (B–F). Note lack of reaction with any fish serum imunized 5 mo; FIG. 5. Immunoelecotrphoresis of *M. cerebralis* antigen. Outer troughs contain rabbit anti-*M. cerebralis*. Anode is to the right. Note alpha-1 migration pattern; FIG. 6. Immunoclectrophoresis of rainbow trout cartilage antigens. Outer troughs contain rabbit anti-*M. cerebralis*. Anode is to the right. Note alpha-2 migration pattern and the probability of two arcs, which are electrophoretically different but immunologically the same and confluent at arrows.

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intramuscularly with sonicated spores in adjuvant (Fig. 2). The crushed spores of *M. cerebralis* yielded an antigenic preparation for rabbits which was superior to that obtained by sonification (Fig. 2). Although antigens have been prepared from various stages of many different sporozoans, this is the first report of an antigenic preparation from the spore stage of any histozoic sporozoan.

Rabbits formed precipitating antibodies against crushed *M. cerebralis* spores after 1 mo (Fig. 3). This antiserum reacted very strongly with normal fish cartilage seeded with various numbers of spores as well as control fish tissue. These all exhibited lines of complete identity by immunodiffusion (Fig. 3). However, after 5 mo, rainbow trout were unable to form precipitating antibodies against *M. cerebralis* (Fig. 4).

Immunoelectrophoresis of crushed *M. cerebralis* spore proteins showed one arc that migrated toward the anode in the alpha-1 region (Fig. 5) which was completely in front of the origin well (Kabat 1968). Normal fish tissue antigens which cross-reacted with rabbit anti-*M. cerebralis* showed two arcs possessing complete immunological identity (Fig. 6). Both of these arcs appeared to fall in the alpha-2 region (Fig. 6) which was primarily in front of the origin well but extended behind it somewhat toward the cathode (Kabat 1968) (Fig. 6).

Discussion

Lom (1969) has indicated that myxosporidians evoke little if any host response in fish due to the general inability of fish to produce an inflammatory response. However, it is well established that trout are capable of inflammatory reactions similar to those of mammals (Weinreb 1959; Finn and Nielson 1971). The cross-reaction of rabbit anti-*M. cerebralis* with *S. gairdneri* tissue antigens (Fig. 3) indicates that an antigenic component of the parasite has been able to mimic normal trout tissue antigens, as demonstrated by their lines of identity upon immunodiffusion. This would account for *M. cerebralis* invading the host with impunity (Hoffman et al. 1969), as trout would not respond immunologically to "self-antigens."

Crushed antigens from the closely related sporozoan *M. cartilaginis* (Hoffman et al. 1965; Lom and Hoffman 1971) did not cross-react with the rabbit anti-*M. cerebralis*, indicating an immunological difference between these parasites. This eliminates the possibility that *M. cerebralis* rabbit antiserum gives nonspecific precipitin bands with numerous antigens.

Additional evidence that *M. cerebralis* antigens are not recognized as foreign by rainbow trout is the inability of any immunized fish to form precipitating antibodies against the spore antigens (Fig. 4). Even though rainbow trout are capable of producing precipitating antibodies against bacterial antigens in 28 days (Anderson and Klontz 1970), no precipitin lines were observed between *M. cerebralis* antigens and trout serum taken 5 mo after immunization. The maximum precipitin titer of 1:64 observed in fish after 49 days (Anderson and Klontz 1970) was as high as the maximum titer of 1:64 that we observed in rabbits against *M. cerebralis* antigens. It is well known that the ambient temperature affects the rate of antibody production in poikilothermic vertebrates (Avtalion 1969; Tait 1969). Therefore, the temperature difference of 12 C in this experiment and that of 16–18 C used by Anderson and Klontz (1970) might account for the lack of precipitating antibody in rainbow trout against *M. cerebralis*. However, two studies have shown that trout held at 11–12 C will develop high agglutinating titers against bacteria in 3–4 mo (Krantz et al. 1963; G. B. Pauley and G. L. Bullock unpublished data). This slower rate of antibody production should not account for the complete lack of precipitating antibody in fish challenged with *M. cerebralis*.

Immunoelectrophoresis of the *M. cerebralis* antigen (Fig. 5) and the fish antigen (Fig. 6) shows that they are different proteins even though they are recognized immunologically by rabbits as the same antigen. This is demonstrated by their different electrophoretic mobilities, with *M. cerebralis* migrating as a single protein in the alpha-1 region and normal fish tissue migrating as two proteins in the alpha-2 region.

Parasitic antigens which are similar enough to those of a host to be recognized as self-antigens by the host would be extremely favorable for the parasite's survival, as it would help suppress both the general defenses (inflammation) and specific defenses (antibody production) of the host. This would account for the extremely successful spread of *M. cerebralis* throughout the salmonid fishes of the world. It may also account for the general success of Myxosporidia as parasites in fish.

Unfortunately, my results indicate that Ouchterlony immunodiffusion tests will not be useful in detecting whirling disease in asymptomatic carrier fish. Fife (1971) indicated that immunoelectrophoresis has considerable potential as a method for diagnosing parasitic diseases. The results obtained here with *M. cerebralis* corroborate this statement and further tests should be performed to evaluate the use of immunoelectrophoresis as a method of diagnosing asymptomatic whirling disease.

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TECHNIQUES FOR EXTRACTING AND QUANTITATIVELY STUDYING THE SPORE STAGE OF THE PROTOZOAN PARASITE *Myxosoma Cerebralis*

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VERIFICATION OF WHIRLING DISEASE can only be made by demonstrating a developmental stage of *Myxosoma cerebralis* (Hofer, 1903). The usual procedure is to follow the method outlined by Hoffman, Dunbar, and Bradford (1962). This consists in macerating the skull of a fish in a mortar with water, stirring, and thoroughly examining two or more drops for spores at high-dry magnification (440×). Our laboratory over the past 2 years has found that this method is satisfactory when many spores are present, but when infections in natural or experimental fish are light, the spores are difficult to detect. Fishery biologists at Bozeman Hatchery have reported difficulties in critically evaluating large slide-samples of fish (personal communication). Since the demonstration of a single spore is sufficient to diagnose the presence of the disease in a fish sample, a more precise method for spore detection is needed.

In addition to difficulties in spore detection, researchers have failed to produce uniform results in laboratory induced infections. In more than 26 attempts over an 8-year experimentation period at the Eastern Fish Disease Laboratory, trout were infected in only seven aquariums (Hoffman and Putz, 1970). W. M. Tidd and R. A. Tubb (unpublished data) were unable to duplicate infection experiments with rainbow trout fry. Inability to quantify spore dosages used for challenging trout fry may be a contributing factor to failures of experimental infections. To date, little quantitative work has been done with *Myxosoma cerebralis* spores.

The objectives of our study were: (1) to develop a reliable method for extracting and concentrating *Myxosoma cerebralis* spores for the detection of whirling disease, (2) to develop a technique for estimating concentrations of spores from infected fish to quantify dosages used to inoculate experimental aquariums, and (3) to survey selected fish populations to determine ranges of spore numbers held in surviving fish and variance of spore numbers carried within infected populations.

MATERIALS AND METHODS

One source of *Myxosoma cerebralis* spores was naturally infected rainbow trout. The fish were 29.3 to 34.3 centimeters in length and had been frozen for 1 year. Spores were also obtained from experimentally infected rainbow trout, 4.7 to 11.4 centimeters, from the Eastern Fish Disease Laboratory at Leetown, W. Va.

The following procedure was used to extract and concentrate spores from large, naturally infected trout. The fish were decapitated at the base of the vertebral column, and the eyes, skin, gills, and lower jaw were removed. The remaining bones of the skull were disarticulated, cleaned of flesh, and washed in distilled water. The bones were macerated at high speed in a blender to release the spores. Sufficient distilled water was added to submerse the rotary blades of the blender. The bones were macerated effectively in 2 minutes. After initial blending, the liquid was decanted, additional

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distilled water added, and the remaining bone fragments reground for 2 minutes. This decanting and regrinding process was repeated as often as five times depending on how thoroughly the bone was ground.

Decanted liquid from the blending process was filtered through a double thickness of high quality cheesecloth to remove larger bone particles. The resulting filtrate was suction-filtered through coarse filter paper, Reeves Angel grade 202, in a Buchner funnel to remove finer skeletal debris. When severe clogging was noted, the filter paper was thoroughly washed with distilled water and replaced with a fresh piece. The final filtrate volume varied from 500 to 1,000 cubic centimeters.

Spores in the filtrate were concentrated by centrifuging at 15,000 revolutions per minute. The liquid containing the spores was centrifuged twice at a rate of 1 liter in 5 to 8 minutes. The catch was rubbed off the sides of the centrifuge bowl with a rubber policeman, and liquid and spores were transferred with a disposable pipette to a 10-cc volumetric flask. The centrifuge bowl was thoroughly washed with distilled water and the wash water added to the flask. The liquid in the flask was brought up to 10 cc by adding distilled water. It was from this 10 cc that samples were drawn for making spore counts.

The volumetric flask was shaken for 5 minutes before the first sample was withdrawn and 1 minute for the remaining samples. Each sample was entered into the counting chamber of a Neubaurer ruled Bright-Line hemacytometer. Spores were counted in 72, 1-square-millimeter areas at a magnification of 430×. Eight samples were taken to provide this number of counting areas. When counting spores in any given square, only those spores lying within or touching its lower or left hand border were counted.

The ruled surface of the hemacytometer is 0.1 mm below the cover glass. The volume over a 1-mm^2 area is 0.1 mm^3 or 1×10^{-4} cc. The average number of spores counted over a 1-mm^2 area multiplied by 10^4 results in an estimation of the number of spores in a 1-cc volume. The following expression can be used to calculate the number of spores in 1 cc when using a counting chamber with a depth of 0.1 mm:

$$\frac{\text{Number of spores}}{1\text{ cc}} = \frac{\text{Total number of spores counted} \times 1}{\text{Number of 1-mm}^2\text{ areas counted}}$$

Because all of the spores of one head were concentrated in a 10-cc volume, the number of spores in a head was obtained by multiplying the concentration per cubic centimeter by 10.

The following modifications were made for extraction and concentration of spores from the smaller, experimentally infected trout. Instead of using a blender, the bones of the heads were thoroughly macerated in a mortar. A similar decanting and regrinding process was followed using 10-cc volumes of distilled water. A 5-cc volumetric flask was substituted for a 10-cc flask. Accordingly, the total number of spores per fish equaled the spore concentration per cubic centimeter times 5.

RESULTS

Extraction and Concentration of Spores

The preceding method of spore extraction and concentration improved the ease and probability of detecting spores. Filtering produced a spore suspension almost completely free of skeletal debris and oil droplets. The semitransparent background of the hemacytometer placed spores into bold relief with polar capsules and sporoplasm vividly distinguishable. Concentrating spores in smaller samples for examination with a plankton centrifuge increased chances for detection.

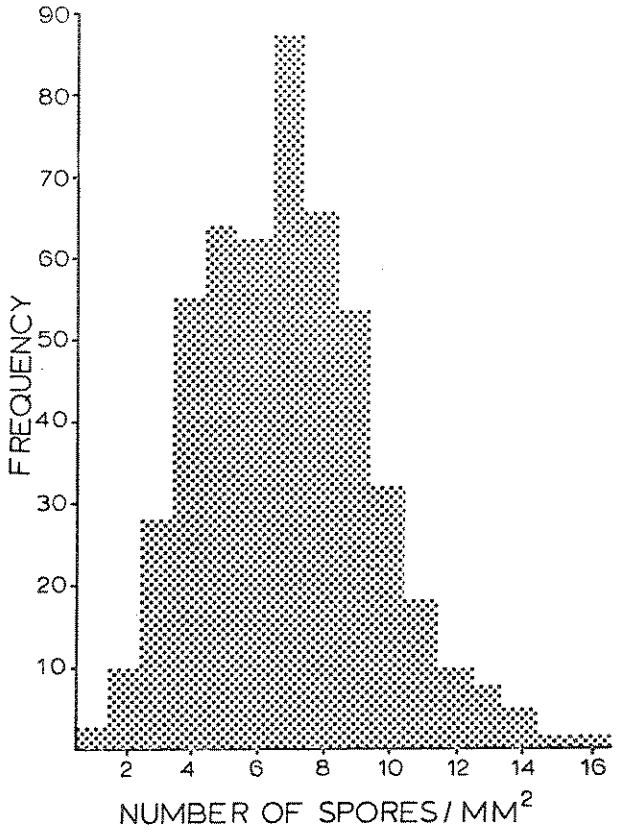
Spore concentrations were further increased by pooling fish for examination. Spore estimations were made on the heads of 30 experimental rainbow trout from the same population. Ten heads were processed individually and the remaining 20 were combined in two lots of 10 each. In 10-cc extractions from the 10 individual heads, the average spore concentration was 5,390 spores/cc. Concentrations for the lots of 10 fish were 51,400 spores/cc and 49,400 spores/cc. Increasing the number of fish from one to ten increased the spore concentration by approximately ten.

Validity of Spore Counting Technique

Spores were extracted and counted from the head of a 29.3-centimeter rainbow trout designated as Rainbow 1. Counts were made in 500, 1-mm² areas to obtain a suitable estimate of the standard deviation of the population. Results of those counts (see graph) show a normal distribution with a standard deviation of 2.58. This deviation was used to calculate the maximum error. At the 95-percent level of confidence this was found to be 0.608. Calculations from the 500 observations estimated the concentration of the 10-cc spore suspension to be 6.88×10^4 spores/cc.

As a demonstration of the consistency of the spore counting technique, seven sample groups (A to G) were taken from what remained of the spore suspension from Rainbow 1. Each sample group consisted of 72 spore counts of 1-mm² areas. Table 1 shows the concentrations which were calculated from those counts. A one-way analysis of variance was performed which indicated that the sampling technique provided representative samples.

Similar procedures were followed with the spore extraction of Rainbow 2, a 7.6-centimeter rainbow trout. Calculations from a 500-count sample estimated the spore concentration in a 10-cc suspension to be 1.82×10^3 spores/cc. Statistical tests demonstrated the validity of the spore counting technique at this lower concentration level.



Frequency distribution of spores present in 1-square-millimeter areas from 500 counts of a rainbow trout.

Survey of Infected Fish Populations

A survey was made of the spore numbers carried in surviving fish from three infected populations. Population I consisted of rainbow trout from a private propagating facility. Fish of populations II and III were experimentally infected rainbow trout from the Eastern Fish Disease Laboratory. Population II was exposed as swim-up fry to spores from the ground heads of 12 diseased fish that were 2 years and 7 months old. Population III was exposed to spores from 100 fish carcasses from a lightly infected lot 6 months old. All fish of populations II and III were 5-months post infection and had been frozen from 2 to 4 weeks. Table 2 shows results of these estimations.

Table 1.—*Estimated concentrations and sample statistics for sample groups A to G*

Group	Spores/cc	Sample mean	Sample variance	Standard deviation	Standard error
A	70,417	7.0417	6.7729	2.6025	0.3067
B	67,222	6.7222	8.3443	2.8886	0.3404
C	70,000	7.0000	6.3662	2.5231	0.2974
D	68,750	6.8750	7.0405	2.6534	0.3127
E	67,778	6.7778	6.9077	2.6282	0.3097
F	69,861	6.9861	5.4505	2.3346	0.2751
G	68,056	6.8056	6.0743	2.4646	0.2905

Table 2.—*Comparison of spore numbers from three infected populations*

Population	Size range of fish (centimeters)	Mean spore number per fish	Range of spore numbers
I	22.9—34.3	1.6933×10^6	2.900×10^5—6.487×10^6
II	4.7— 7.6	3.243×10^4	9.50×10^3—7.800×10^4
III	8.9—11.4	5.390×10^4	2.30×10^4—1.18×10^5

Results disclosed that extremely high numbers of spores can be harbored by fish which survive whirling disease infections. These data also revealed that a wide range of host susceptibility probably exists within populations. It was assumed that fish within each population were exposed to infection under similar conditions. Since the means and ranges of spore numbers per fish varied between the three populations we conclude that the degree of infection varied between populations.

DISCUSSION

Like others (Uspenskaya, 1957; Hoffman, 1962) we have observed that some trout may act as carriers of *Myxosoma cerebralis*. Such fish carry an extremely light spore load. It is important to detect these by spore examination, since carriers among hatchery-reared fish, if distributed, could serve as a new source of whirling disease dissemination. By using our method of filtering and concentrating pooled samples, the probability of spore detection can be greatly enhanced. Also, our method of spore estimation will enable investigators to replicate experiments with known spore estimates of *Myxosoma cerebralis*. Investigations to determine the threshold concentration of spores necessary to infect rainbow trout of different ages are currently underway.

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EARLIEST SUSCEPTIBLE AGE OF RAINBOW TROUT TO WHIRLING DISEASE

To determine the earliest age at which rainbow trout are susceptible to whirling disease (causative agent, Myxosoma cerebralis), 5,000 eyed eggs were purchased and divided into lots of 3,500 and 1,500. As a control, the smaller lot was kept in a stainless-steel trough in the hatchery and supplied with spore-free running spring water. The larger lot was placed in a live-box in the Leetown Run where whirling disease had been confirmed experimentally. Every day for 6 days until the hatching, 200 eggs were pipetted from the live-box, placed in small plastic aquaria in the hatchery, and supplied with spore-free spring water. Every day for 6 days after hatching, 300 sac fry were pipetted from the live-box and similarly placed in plastic aquaria in the hatchery and supplied with spore-free spring water. The remainder of the fish were left in the live-box as a second control group.

Three months after the initial day of the experiment, fish from the two control groups and from each of the aquaria were examined grossly and microscopically for whirling disease. None of the fish that had been exposed only as eggs became infected with the disease, but those that had been exposed as sac fry for as little as 3 days showed the gross symptoms and spores.

We do not know why 1- and 2-day-old sac fry showed neither gross symptoms nor spores. Possibly the infection is an accumulative process requiring a 2- to 3-day exposure.

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THE VETERINARY RECORD
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An Outbreak of Whirling Disease in Rainbow Trout

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Introduction

INFECTION of the head and spinal cartilages of salmonid fishes with the parasite *Myxosoma* (*Lentospora*) *cerebralis* was first recognised in Central Europe (Hoffman *et al.*, 1962) and has been a serious cause of mortality in fish farms on the Continent for many years. It is now also recognised in North America and on the island of Sakhalin (U.S.S.R.) in the Sea of Japan, which were, until recently, free from the disease.

Infection with *M. cerebralis* produces the condition clinically known as "Whirling Disease," and this disease was first confirmed in Scotland by Elson (1969). The present report describes the symptoms and pathological findings of an outbreak of the disease which occurred in a trout farm geographically distant from the original outbreaks (Elson, 1969), which indicates that the condition may now be widely distributed throughout Scotland.

Clinical Signs

Several young fish (four to six months old) from a batch of rainbow trout (*Salmo gairdnerii*) in a small trout farm, were observed to be having difficulty in approaching food at feeding times. Although setting off in the direction of the floating food crumbs, they moved in a tail-chasing, spiral way towards it, and eventually sank to the bottom of the tank exhausted. Between feeding times, these fish were lethargic and tended to settle near the outlets of the tank.

When they were examined, they were seen to have blackened tails and backs, and two out of the 10 specimen fish killed had marked scoliotic curvatures of the spine, and one a bulging skull. Affected fish were not necessarily stunted, and two were among the largest in the tank.

Losses of fish, not observed to have been whirling, had been higher than anticipated over the previous two months.

Pathology

The heads of the 10 fish killed were fixed in mercuric-chloride-formalin, embedded in paraffin wax, and transverse sections were cut, at the level of the orbit, and stained by haemotoxylin and eosin and Giemsa's method.

The sections of six of the fish appeared histologically normal, a surprising observation in view of the degree of locomotor disturbance present, but the remaining fish all showed invasion of the cranial cartilages and auditory capsule by *Myxosoma* spores.

In some cases (Fig. 1) the spores were extremely numerous, and embedded in a mesh of necrotic cell debris, in others, very few spores were present and no obvious tissue reaction was present.

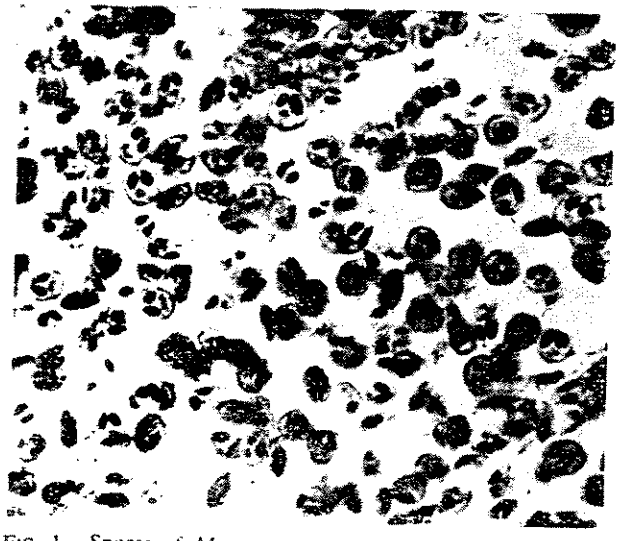

FIG. 1.—Spores of *Myxosoma cerebralis* in the cartilage of the head of rainbow trout. × 1200.

Discussion

The present case exemplifies the typical symptoms of Whirling Disease: characteristic whirling movements in young trout, with deformities and blackening of the back and tail owing to loss of nervous control of the posterior chromatophores. Histological examination has confirmed the diagnosis.

(Concluded at foot of col. 1 opposite)

An Outbreak of Whirling Disease in Rainbow Trout.—Concluded.

Whirling Disease may now be established in Scotland, and since the water from the holding tanks in this outbreak, and probably in others, flows directly into a loch and river system, it may well have established itself in indigenous species.

In America, where *Myxosoma* spores are thought to have been accidentally fed to fish (Hoffman *et al.*, 1962) it has spread over a wide area. Brown trout (*Salmo trutta*) and salmon (*Salmo salar*) have been shown to be less susceptible to the condition both in the wild, and under intensive conditions (Hoffman *et al.*, 1962), but epizootics of the disease could have severe effects in fish farms rearing table fish, since, for such farms, rainbow trout is a far more economic proposition (from a food conversion standpoint) than brown trout.

Acknowledgments.—We would like to thank Mr. J. T. B. Ellis for technical assistance and Mr. A. Finnie for the illustration.

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SCIENCE

Molecular Evidence That the Myxozoan Protists Are Metazoans

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Molecular Evidence That the Myxozoan Protists Are Metazoans

James F. Smothers,* Carol D. von Dohlen, Laurens H. Smith Jr., Richard D. Spall†

The evolutionary origins of the protistan phylum, Myxozoa, have long been questioned. Although these obligate parasites are like protozoans in many features, several aspects of their ontogeny and morphology have implied a closer relationship to metazoan lineages. Phylogenetic analyses of 18S ribosomal RNA sequences from myxozoans and other eukaryotes, with the use of parsimony, distance, and maximum-likelihood methods, support the hypothesis that myxozoans are closely related to the bilateral animals. These results suggest that the Myxozoa, long considered an assemblage of protozoans, should be considered a metazoan phylum.

The reconstruction of animal phylogeny has engaged scientists for over a century (*1*). With the advent of molecular characters, hypothesized relationships among some taxa have become solidified, whereas others remain contentious (*2*). Most biologists would agree, however, on the set of taxa that constitutes the Metazoa, or multicellular animals. Not since discovery of the Loricifera in the last decade has a phylum been formally added to this taxonomic group (*3*). Here, we present evidence that the Myxozoa, a phylum generally placed in the kingdom Protista (Protoctista) (*4–6*), shares its most recent common ancestor with a metazoan, not a protozoan, lineage.

The phylum Myxozoa comprises over 1100 described species of oligocellular, obligate endoparasites and is thus one of the largest protistan assemblages (*4*). Members of the class Myxosporea (the majority of myxozoans) principally infect teleost fishes, and members of the Actinosporea mainly infect aquatic, oligochaete worms (*7*). Myxosporea are either coelozoic within hollow organs or histozoic in solid tissues and cause tissue displacements and destruction and sometimes death of their hosts. For example, infections by the European parasite *Myxobolus cerebralis* in North American salmonid fish can cause serious losses to aquaculture industries. The avirulence of most myxosporeans in their natural hosts, however, implies a long history of association (*8*).

The diagnostic feature of Myxozoa is the infective spore, a dispersal stage composed of distinct gametic (autogamic) cells and polar capsules with coiled, ejectable filaments, enveloped by valve-like somatic cells. Proliferative stages (trophozoites) are often multinuclear and may grow to macroscopic size (*8*). However, the complete life cycle is unknown, because myxozoans have been intractable to

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laboratory culture. Experimental transmission studies of both myxosporeans and actinosporeans suggest that the two parasites are possibly alternative forms of a single, complex life cycle: *M. cerebralis* infections in fish, for example, may be initiated by an actinosporean of tubificid worms (*9*).

The phylogenetic affiliations of Myxozoa have remained unclear since their discovery and recognition as a distinct taxonomic group in the 19th century (*10*). Though protistan in habits and size, myxozoans exhibit a degree of multicellularity and cell differentiation found in no other protozoans (*11*). On the basis of the common possession of spores with ejectable filaments and amoeboid cells, Myxozoa and Microsporea once shared the class or subphylum Cnidosporidia in the old phylum Protozoa. After discoveries about their morphology and life history, Microsporea were assigned to their own protistan phylum; molecular evidence confirmed that these minute, unicellular organisms are extremely ancient eukaryotes (*12*). The cellular complexities of myxozoans, in contrast, have provoked speculation that these parasites share a most recent common ancestor with metazoan taxa, not protists (*13*). For example, the structural and developmental similarities of polar capsules and cnidarian nematocysts, and the striking parallels between sporoblasts and larval Narcomedusae, have engendered hypotheses that myxozoans share a most recent common ancestor with the Cnidaria (*14*). Other workers have interpreted their cellular differentiation, and features such as the desmosome-like structures between valve cells, to suggest that myxozoans are a primitive (non-Cnidarian) animal lineage (*15*).

Given such uncertainty regarding myxozoan origins, we determined the sequences of small-subunit (18S) ribosomal RNAs (rRNAs) for five myxosporean species in three different genera (*16*) to resolve their phylogenetic position according to molecular evidence. We used parsimony and neighbor-joining methods to find the best supported tree for myxozoans and a selection of other eukaryotes (*17*) and then used parsimony and maximum-likelihood methods to test three alternative hypotheses: that myxozoans share their most recent common ancestor with (i) an alveolate or rhizopod protistan lineage, (ii) a cnidarian lineage, or (iii) a bilateral metazoan lineage.

The position of the myxozoans as a metazoan lineage was supported with 100% bootstrap confidence in both parsimony (*18*) and neighbor-joining analyses (*19*) (Fig. 1). The topology recovered in bootstrap parsimony analysis was the same as one of four shortest length trees inferred with maximum parsimony, all sites and transformations weighted equally [the four trees (length = 1586) differed only in the positions of the placozoan and ctenophoran]. The inclusion of myxozoans in the bilateral animal clade also held when transversions were weighted 10 to 1 over transitions. The relationships of nonmyxozoan taxa that were well supported in our analyses (bootstrap values >80%) generally corroborate previous morphological and molecular studies that included the same groups (*20–24*), whereas branching events with lower bootstrap support (<80%) involve taxa that show conflicting arrangements in other studies (*1, 2, 23, 24, 25*). It is possible that the set of taxa included, or the presence of long, unbranched lineages can affect, patterns recovered in phylogenetic analyses (*26*). The myxozoans are comparatively long-branched taxa, as are all the bilateral animals relative to other taxa in this study. For this reason, we analyzed subsets of the taxa shown here, as well as different alignments including other taxa. In all of these analyses, the status of the Myxozoa as a metazoan lineage did not change.

With both parsimony and maximum-likelihood methods (*27*), we evaluated different tree topologies that corresponded to the alternative hypotheses of myxozoan origins, as stated above. Under topologically constrained parsimony searches, 35 to 46 extra steps were required if myxozoans were forced to be a sister group to different protistan lineages, 22 extra steps were added if myxozoans were constrained as a sister group to the Cnidaria, and 16 to 29 extra steps were necessary if myxozoans were sisters to different bilateral animal lineages. Only five extra steps were added if myxozoans were a sister group to all bilateral animals. When similar topologies were evaluated with maximum likelihood, the tree in which myxo-

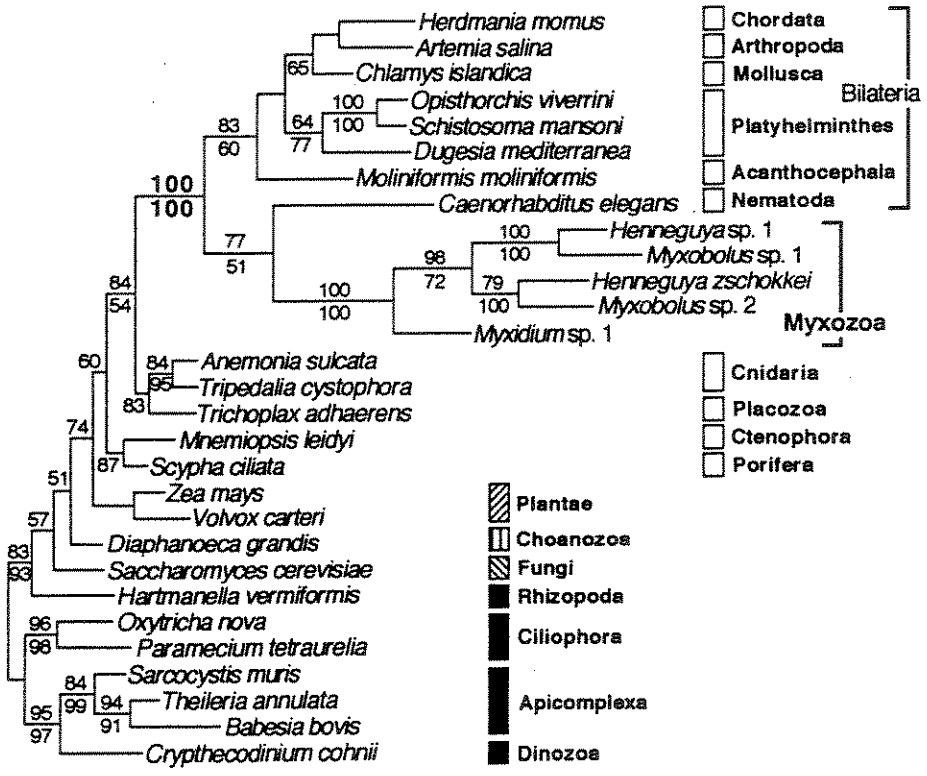

Fig. 1. Phylogenetic position of Myxosporea (Myxozoa) among representative eukaryote groups (*17*), as inferred from 18S ribosomal RNA sequences. Open bars indicate metazoan taxa; closed bars indicate protistan taxa. Four of the five Myxosporea (numbered) were previously uncharacterized species. The structure of the tree is the consensus of 500 parsimony bootstrap replicates (*18*). The percentage of parsimony bootstrap resamplings supporting a given branching event is shown above the branch, and the percentage of neighbor-joining (*19*) replicates supporting a branching event is shown below the branch (confidence levels greater than 50% are indicated). Branch lengths are proportional to the number of substitutions. The branch uniting the myxozoans and all bilateral animals has a decay index (*31*) of >5. Only sites that could be aligned without ambiguity were included (*32*). All analyses were performed as unrooted; here, the root is placed between the alveolates and the rhizopod (*24*). Maximum-likelihood methods (*27*) also supported the position of the myxozoans shown here.

zoans were a sister group to the Nematoda (Fig. 1) had the highest log-likelihood value and was significantly more likely than other topologies that placed the myxozoans as a sister group to individual protistan, cnidarian, or other animal lineages. However, the topology of Fig. 1 was not significantly different from an alternative topology that placed the myxozoans as a sister group to all bilateral animals.

These molecular data provide evidence, as others have suggested on the basis of morphological criteria, that myxozoans constitute a metazoan, not a protozoan, lineage. Further refinement of myxozoan relationships to other metazoan phyla may emerge when complete 18S rRNA sequences are available for additional myxozoan and metazoan taxa, or when information from other molecules is gathered. We found no support, however, for the hypothesis that myxozoans and cnidarians share a recent, common evolutionary history. Rather, myxozoan origins appear to date later in metazoan phylogeny, to the appearance of the bilateral animals. At present, we cannot distinguish whether myxozoans are members of the bilateral animal clade or a sister group to them. Under either scenario, the ancestors of extant myxozoans must have undergone extensive reductions in morphology and development. Unlike cnidarians and higher metazoans, myxozoans are not known to develop multiple differentiated tissues, eggs, and sperm or form a blastula (*15*, *28*), but the common ancestor of myxozoans, bilateral animals, and cnidarians most likely possessed these features. Myxozoans may be an extreme example of the pattern of degeneracy that is characteristic of parasite evolution.

Our results also have implications for myxozoan systematics. Classification of these parasites traditionally has been based on spore morphology (*29*), now known to be a plastic trait in some species (*30*). Uncertainty concerning the closest relatives of Myxozoa has precluded the use of outgroups to determine primitive versus derived traits; thus, hypotheses of relationships within the phylum necessarily have relied on host phylogeny. Our study shows that rRNA sequences contained sufficient information to resolve even intraclass relationships. In fact, the data imply that two genera (*Henneguya* and *Myxobolus*) are paraphyletic (Fig. 1). Further sequence data will be instrumental in the systematics of these widespread but little recognized organisms. Potential hosts of myxozoan parasites are vastly undersampled, and given the abundance of teleost fishes (more than 40,000 species), there are likely many undescribed species. Perhaps our results will lead to future studies of myxozoan life history, development, and evolution, as well as additional molecular work to further refine the systematic position of these organisms.

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17. Metazoan taxa were chosen to represent the breadth of phyla for which complete 18S rRNA sequences were available in GenEMBL. Protozoan taxa were chosen to represent the more recently derived groups, which have been proposed as close relatives of Myxozoa (*6*). Plants and the fungus were taxa that have been included in other published molecular phylogenies.
18. Parsimony analyses were conducted with the computer program PAUP (*PAUP: Phylogenetic Analysis Using Parsimony*, 3.1; D. L. Swofford, Illinois Natural History Survey, Champaign, IL). We performed 500 bootstrap replicates using the heuristic search strategy, with random addition and all sites and transformations weighted equally. Searches were performed with no outgroups specified, to avoid influencing the position of myxozoans.
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ATTEMPTS TO CONTROL WHIRLING DISEASE BY CONTINUOUS DRUG FEEDING

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Abstract: Six drugs were fed continuously to susceptible trout held in known *Myxosoma cerebralis* contaminated water in an attempt to prevent infection. Drug efficacy was judged by comparing numbers of spores which developed in medicated and non-medicated fish. Fish after having been fed furazolidone in two tests had fewer spores than the other groups.

INTRODUCTION

Whirling disease, caused by *Myxosoma cerebralis*, was first reported in Nevada in 1966 at the State Hatchery located in Verdi. The disease had existed in the State for many years prior to this time. Microscopic examination of heads from trout preserved in 1957 showed *Myxosoma* spores.[2] Many of the State's waters were planted with infected fish, because the disease had been present but undetected for many years. Included in these waters was the Carson River, which served as part of the water supply of the Lahontan National Fish Hatchery, Gardnerville, Nevada. During 1970, 1,362,914 cutthroat trout *(Salmo clarki henshawii)* and 47,057 rainbow trout *(Salmo gairdneri)* were destroyed by the U.S. Bureau of Sport Fisheries and Wildlife in two separate outbreaks of the disease at this hatchery.

Whirling disease is known to exist in State Hatcheries at Verdi and Ruby Valley, Nevada at the present time. Disinfection procedures have reduced the infection at Ruby Valley to one dirt pond which is fed by springs entering the bottom of the pond. The Verdi Hatchery has the disease in most of the rearing areas because it is necessary to use water from a known contaminated source, the Truckee River. Whirling disease, as seen in this area, is subclinical in nature. Less than 1% of the hatchery trout show the typical signs described for the disease.[2] Few deaths can be attributed directly to the disease in the hatchery. However, periodic examination of asymptomatic fish at the hatchery has shown the presence of spores in low numbers. Stream surveys have failed to show any effect on natural populations.

The use of drugs as a treatment for this disease has been tried previously in other areas of the world.[1,4] This paper reports attempts to prevent infection of susceptible fish held in known *Myxosoma* contaminated water by continuous feeding of several drugs. The tests were not intended to determine drug levels but only to indicate which drugs might be effective. Facilities were constructed at the Verdi Hatchery specifically for this study.

[1] This study was supported by Contract number 14-16-008-634, Bureau of Sport Fisheries and Wildlife. Contribution of the Nevada Agricultural Experiment Station, University of Nevada Journal Series #234.

[2] WOLF, H. 1970. Personal Communication. California Department of Fish and Game, 2111 Nimbus Rd., Rancho Cordova, Calif. 95670, U.S.A.

MATERIALS AND METHODS

Test fish were "bowcutt" trout (offspring from rainbow males and Lahontan cutthroat females). The fish had been raised in *Myxosoma*-free spring water and were 58 days old at the start of the study. Approximately 350 trout were placed in each of 9 troughs at the start of the test. Individual groups of fish received food medicated with either oxytetracycline, sulfamerazine, furazolidone, nicarbazin, amprolium or Merck experimental drug number 930. The last three drugs had been shown to be effective against protozoa in other animals. Two levels of each of the anti-protozoan drugs were fed. Drug intake was not maintained at a constant level, because of fluctuations in water temperature during the test. The drug levels fed to each group are given in Table 1. Drug levels indicate the range fed during the entire 12 months study. The levels of furazolidone, Merck 930, amprolium and nicarbazin in the food were assayed by the manufacturer of the drug. Levels of sulfamerazine and oxytetracycline were not assayed.

One trough with approximately 700 trout received non-medicated food during the test. Water temperature dropped to 1 C 4 months into the test. One half of the fish from each trough were transferred to 12 C well water for the remainder of the test in order to accelerate *Myxosoma* development (Group 1). The other half remained in the original troughs at Verdi (Group 2).

Drugs were evaluated by examining killed fish because none of the fish showed clinical signs of whirling disease. The head from each fish was removed and split lengthwise into halves. One half was preserved in Bouin's fixative for future tissue sectioning. The other was either examined immediately or preserved in 10% formalin in saline. Cartilage and connective tissues posterior to the eye and dorsal to the roof of the mouth were minced and ground with mortar and pestle for wet mount examination. Water was added and a slurry made for mounting on a glass slide with a coverslip, similar to methods described by Hoffman *et al.*[3] At least 100 microscope fields were examined at 400X magnification. The fish was considered infected with whirling disease if one or more spores was observed.

Records were kept on daily water temperatures, death losses, appetite, and weight gains during the 1 year test period.

RESULTS AND DISCUSSION

Problems were experienced with external protozoan infections, therefore, all fish were treated periodically with a 1:6000 formalin bath. The medicated foods were palatable, except those containing furazolidone, which were usually not completely eaten. The growth of fish in this group was approximately 50% less than the fish in the experimental control trough.

Spores were first noted in experimental control fish in group 1, 7½ months after the start of the study. Samples of these fish were taken periodically for another 45 days with the hope that the percentage of fish showing spores would increase. This did not occur. Therefore, 9 months after the start of the study, fish from each trough in group 1 were sacrificed and examined (Table 1). Furazolidone was effective in inhibiting spore formation although trophozoites and granulomas were present in tissue sections in some of the fish.

Spores did not appear in the Group 2 fish, which were held at colder water temperatures, until 11 months after the start of the test (Table 2). Furazolidone again reduced spore formation. This was indicated by the smaller percentage of fish showing spores and also by the lower average number of spores present.

This study indicates that in a natural stream situation with fluctuating water temperatures, the incubation period may be much longer than previously reported.[2]

These initial studies indicate that the drug furazolidone had an inhibitory effect on spore formation. Additional studies are in progress to determine if lower

levels of drug are effective. The general approach of drug feeding to eliminate or lessen the severity of whirling disease would benefit those trout raisers who are forced to use a contaminated water supply, a situation common in Europe. These benefits would not be seen in lessening the severity of the disease in Nevada where it is now subclinical. However, furazolidone could result in a substantial reduction in disease organisms being placed in the State's waters. This could aid in the elimination of the disease from this State over a number of years.

Acknowledgement

The authors wish to express their sincere appreciation to Thorlief Rangen, Buhl, Idaho, for donating the feed used in these tests and for mixing the drugs at the various levels. We also wish to thank Merck & Co., Rahway, N.J., for providing the amprolium, nicarbazin, and experimental drug MK 930.

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TABLE 1. Degree of **Myxosoma Cerebralis** Infection in Group 1 Trout.

Drug	DRUG FED									
	Merck 930 mg/kg body wt.		Amprolium mg/kg body wt.		Nicarbazin mg/kg body wt.		Oxytetracycline	Furazolidone	Sulfamerazine	Control
Levels	8-15	33-64	13-18	24-44	6-14	30-60	68-152	152-194	15-36	
Number of Heads Examined	20	16	20	22	20	21	19	63	20	76
Number of Heads With Spores Present and Percentage	4(20)	3(19)	3(15)	2(9)	3(15)	3(14)	2(11)	0(0)	5(25)	14(18)

TABLE 2. Degree of **Myxosoma Cerebralis** Infection in Group 2 Trout.

Drug	DRUG FED							
	Merck 930 mg/kg body wt.	Amprolium mg/kg body wt.		Nicarbazin mg/kg body wt.		Furazolidone mg/kg body wt.	Sulfamerazine mg/kg body wt.	Control
Levels	33-64	13-18	24-44	6-14	30-60	152-194	15-36	
Number of Heads Examined	20	20	20	20	20	46	20	30
Number of Heads With Spores Present and Percentage	14(70)	15(75)	18(90)	17(85)	18(90)	20(43)	17(85)	21(70)
Average Number of Spores in Infected Heads per 100 Microscope Fields	17	35	14	30	14	1.6	38	31

NOTE: Fish fed oxytetracycline and low level Merck 930 were killed by predators before the test was completed.

OPERCULAR CYST FORMATION IN TROUT INFECTED WITH *Myxosoma cerebralis*[1]

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Abstract: External opercular cysts were observed in cutthroat trout, *Salmo clarki henshawii* affected with whirling disease. Microscopic examination of the cysts revealed numerous spores of *Myxosoma cerebralis* in their lumen and walls. Rupture of these cysts may provide a method of whirling disease transmission from live infected fish.

INTRODUCTION

Myxosoma cerebralis is the cause of whirling disease in salmonids. The life cycle of the organism has not been completely established although the disease is worldwide and has been known for over 70 years.[1] Hoffman, *et al.*[2] and others theorized that transmission was accomplished by ingestion of spores liberated from cartilage after death of infected fish. Rydlo[4] and Uspenskaya[5] suggested that spores were liberated from living fish through the intestinal tract. This paper reports the results of observations made of external cysts located in the operculum of whirling disease-affected fish.

MATERIALS AND METHODS

Observations were made on Lahontan Cutthroat trout, *Salmo clarki henshawii* raised at the Nevada State Hatchery, Verdi, Nevada. The fish varied in age from 6 to 18 months and were raised in water contaminated with *Myxosoma cerebralis*. Very few showed symptoms of whirling disease but microscopic examinations of wet mounts of macerated head cartilage demonstrated myxosomal spores in approximately 90% of the fish.

Approximately 300 fish, randomly picked from rearing ponds were anesthetized and examined grossly on several occasions during the year. Fish with cysts were killed and photographed. The affected tissue was removed and preserved in Bouin's fixative for tissue sectioning. Wet mounts were prepared from material expressed from the cysts. The spores were measured and tested for an iodinophilic vacuole with Lugol's iodine.

RESULTS AND DISCUSSION

Approximately 1-5% of all fish examined had opaque white raised cysts 1-3 mm in diameter on the medial surface of the operculum. The cyst location varied along the length of the operculum (Fig. 1). Some fish had as many as three cysts on one side. The gross features on these cysts have similarities to those of *Myxosoma cartilaginis* in bluegills[3] (*Lepomis macrochirus*).

[1] Supported by Dingle-Johnson (Federal Aid in Sport Fish Restoration Act) funds, Nevada Project F-20-9.

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FIGURE 1. *M. cerebralis* cyst on medial aspect of operculum at base of gill arch. Operculum is viewed looking ventrally.

The inflammatory response produced by *M. cerebralis* was of the granulomatous type. The organism, in its trophozoite state, had invaded the cartilage of the operculum, resulting in necrosis and destruction of the cartilagenous cells (Fig. 2). Trophozoite forms were easily seen in cartilage cells. Within the cartilagenous matrix itself, there was no concomitent inflammatory reaction.

As the cartilage was broken down, inflammatory cells, predominantly mononuclear leukocytes and macrophages, had infiltrated the surrounding soft tissues and formed granulomas. These granulomas were composed predominantly of epithelioid and mononuclear cells, proliferating fibroblasts, and occasional multinucleate giant cells, (Fig. 5). Some had necrotic centers. Rarely, a myxosomal spore was found within a granuloma. Granulomas occurred in the soft tissues immediately adjacent to the cartilage of the gill and under the epidermis.

Opercular cysts formed in the subepidermal regions between the superficial squamous epithelium and the bone and cartilage of the operculum. The cyst wall was composed of proliferating fibroblasts, rapidly growing capillaries, and numerous mononuclear cells and macrophages, (Fig. 3). Rarely, acute inflammatory cells (neutrophils) were observed. Multinucleate giant cells were occasionally present in the cyst wall, but were uncommon. Special stains revealed numerous spore forms of *M. cerebralis*, within the cyst wall, (Fig. 4) and occasionally in a giant cell. The cyst wall had no epithelial lining. Instead, cells comprising the wall were seen to abut directly into the lumen.

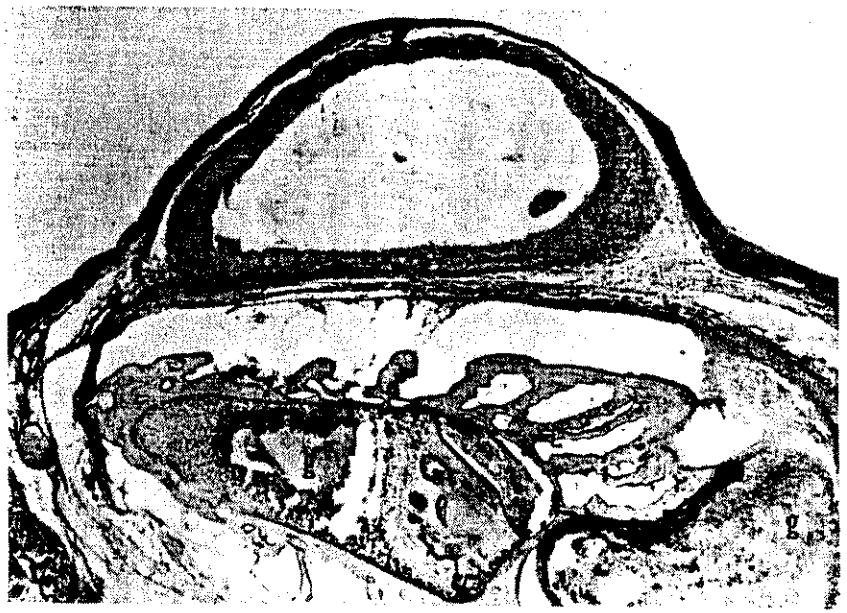

FIGURE 2. Low power photomicrograph of opercular cyst just underlying the surface epithelium. Beneath the cyst, the cartilage is involved by a granulomatous inflammatory reaction (g) caused by **M. cerebralis** H & E X25.

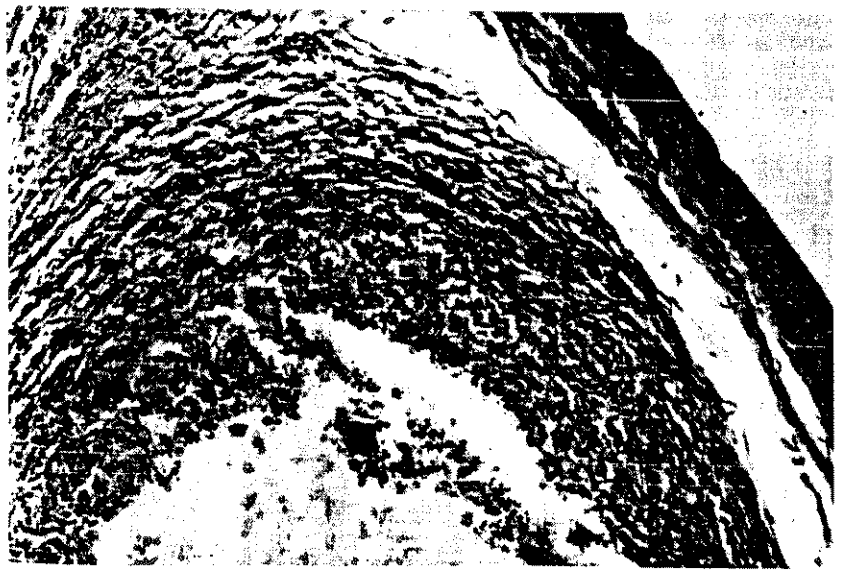

FIGURE 3. Higher power view of cyst wall showing its inflammatory nature and the absence of an epithelial lining. H & E, X200.

FIGURE 4. **M. cerebralis** spores in the cyst wall. Saffranin & Fast green, X400.

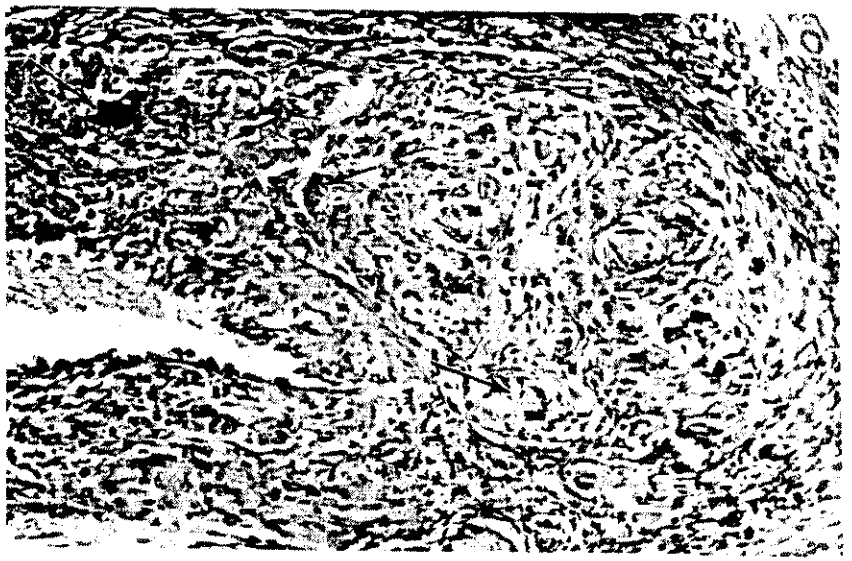

FIGURE 5. Photomicrograph of an area of cartilagenous destruction caused by **M. cerebralis**. Note the granulomatous nature of the inflammatory response. Multinucleated Langhan's type giant cells are present in the reaction (arrows). H & E, X250.

The lumen itself was partially filled with myxosomal spores and amorphous debris.

Fresh spores taken from head cartilage and opercular cysts averaged 8.8 μm by 8.15 μm and did not possess an iodinophilic vacuole. On this basis they were identified as *M. cerebralis*.

Rupture of these spore containing cysts into the environment could transmit whirling disease from live trout.

Acknowledgement

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Transmission of Salmonid Whirling Disease by Birds Fed Trout Infected with *Myxosoma cerebralis*

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SYNOPSIS. Mallard ducks, *Anas platyrhynchos* and a black crested night heron, *Nycticorax nycticorax* were fed trout infected with *Myxosoma cerebralis* (Hofer) in 2 separate experiments. Feces from the birds were deposited in troughs containing *M. cerebralis*-free mud as well as in 1 trough without mud. Spore suspensions were also added directly to mud in 1 trough and to another trough without mud. Susceptible rainbow trout, *Salmo gairdneri*, developed whirling disease in all troughs containing mud contaminated with *M. cerebralis* but remained free of infection when exposed to *M. cerebralis* in troughs without mud. This demonstrates the possibility of bird transmission of the organism causing whirling disease to previously non-contaminated waters.

Index Key Words: *Myxosoma cerebralis*; salmonid whirling disease; transmission by waterfowl.

WHIRLING disease caused by the myxosporidan *Myxosoma cerebralis* (Hofer) has been enzootic in Nevada since 1966. Part of the life cycle remains unknown, and successful experimental transmission has been accomplished only through the use of aged spores (1-3). A program is presently under way to rid 2 Nevada State Hatcheries of this disease. One of these (Gallagher Hatchery, Ruby Valley, Nevada) is located on a wildlife refuge. Whirling disease is present in rainbow trout, *Salmo gairdneri* (Richardson), and brown trout, *Salmo trutta* (Linnaeus), in the waters of the marsh area of the refuge as well as in a part of the rearing ponds of the hatchery. Ducks routinely fly between the marsh and the rearing ponds. All rearing ponds are made of concrete, but much of the hatchery water supply comes from springs which flow from open dirt ponds to the rearing ponds.

To eradicate the disease from the hatchery, it was necessary to know the role of migratory waterfowl. The purpose of the present study was to determine whether migratory waterfowl feeding on infected fish could transmit the disease.

MATERIALS AND METHODS

The mud used in the study was obtained from a stock watering spring, in which there had never been any fish. Water was supplied to 8′ × 14″ test troughs from a well used to supply our fish laboratory and also known to be free of *M. cerebralis*. Six troughs were used in the 1st test. Each trough had approximately 4 liters of mud in the upper ¼ held in place by a perforated partition. Susceptible fingerling rainbow trout were placed below the partition in the trough. Water flowed continuously over the mud and through the troughs, maintaining a depth of 5 inches. Cages were placed above the mud on 4 troughs. Mallard ducks, *Anas platyrhynchos* (Linnaeus), were placed in 2 cages (over trough Nos. 1 and 2) and a black crown night heron, *Nycticorax nycticorax* (Linnaeus), was alternated between the other 2 troughs (trough Nos. 3 and 4). All of the birds were fed for 2 weeks on small trout which had *M. cerebralis* spores present in their head cartilage. The ducks were removed from their cages and fed by forcing the infected fish down their gullets. The heron voluntarily ate the whole fish. Care was taken to eliminate the possibility of any fish tissue accidentally contaminating the trough. Feces from these birds fell directly into the trough mud below through perforations in the floor of the cages. Trough No. 5 was left without any additives to the mud as a negative control. Ground head material from infected fish was added to the mud in trough No. 6 as a positive control.

A 2nd test was performed using 5 troughs, 3 with *M. cerebralis*-free mud, as described above, and 2 with no mud. A black crested night heron was again rotated between trough number 1 with mud and trough number 2 without mud. Trout infected

TABLE 1. *Fingerling rainbow trout,* S. gairdneri, *exposed for 5½ months to mud containing feces from birds feeding on fish infected with* M. cerebralis *(Test No. 1).*

Trough No.	Trough Contents	No. trout with spores/ no. trout examined
1, 2	Mud with contaminated duck feces*	14/15, 15/15
3, 4	Mud with contaminated heron feces	17/17, 14/15
5	Noncontaminated mud (neg. control)	0/25
6	Mud with *M. cerebralis*; no feces (pos. control)	17/24

* Feces contaminated with *M. cerebralis* (Tables 1, 2).

* This investigation was supported by Commercial Fisheries Research and Development Act Funds Project 4-27-R-2.

† Contribution of the Nevada Agricultural Experiment Station, University of Nevada Journal Series No. 362.

TABLE 2. *Fingerling rainbow trout,* S. gairdneri, *exposed for 7 months to feces from birds fed on fish infected with* M. cerebralis.

Trough No.	Contents	No. trout with spores/ no. trout examined
1	Mud with contaminated heron feces*	20/20
2	Contaminated heron feces; no mud	0/20
3	Noncontaminated mud (neg. control)	0/20
4	Mud with *M. cerebralis*; no feces (pos. control)	20/20
5	*Myxosoma cerebralis*; no feces; no mud	0/20

* See note to Table 1.

with *M. cerebralis* were fed to the heron for a period of 2 weeks, and the feces collected in the troughs below. Trough No. 3 contained mud only as a negative control and *M. cerebralis*-infected ground head material was added to mud in trough No. 4 as a positive control. In addition, ground head material was placed above the partition in trough No. 5 with no mud present.

Test fish were judged to be infected with whirling disease when 1 or more spores was observed in wet mount preparations of ground head cartilage.

RESULTS

The test fish were examined starting at 3 months after each experiment was initiated. In test No. 1, spores first appeared after 5 months and the test was concluded after 5½ months. Spores were found in all groups, except for the negative control group. Infection was very heavy in the fish exposed to the contaminated bird feces which probably received more infective units, since no attempt was made to quantify the amount of spores fed to the birds (Table 1).

In test No. 2, spores did not appear for a much longer period. The experiment was terminated at 7 months when large numbers of spores were noted in the positive control group. Spores were present in test fish exposed to mud to which contaminated bird feces or spores had been added. The negative control group, and the 2 other groups which were in troughs without mud did not become infected (Table 2).

DISCUSSION

The results of the tests detailed in this report indicate that the birds which feed on infected fish can spread the infective agent to surrounding waters by transfer of fecal material. Deposition of feces in streams, dirt ponds, or springs could result in eventual infection of fish through contamination of the mud. Deposition of feces in cement ponds would probably not cause infection of hatchery fish since at this point *M. cerebralis* is not infective but requires maturation in mud. The necessity of maturation of spores in mud before they are infective agrees with previous work by Hoffman et al. (1-3). This is the first report in which it is shown that the spores are still viable after passage through birds and can eventually cause whirling disease.

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MODIFICATION OF *MYXOSOMA CEREBRALIS* SPORE EXTRACTION TECHNIQUE

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A TECHNIQUE FOR EXTRACTING AND estimating spore numbers of *Myxosoma cerebralis* from the heads of rainbow trout (*Salmo gairdneri*) was described by Prasher, Tidd and Tubb in the October 1971 issue of *Progressive Fish-Culturist*, vol. 33, no. 4, p. 193-196. Since then, considerable experience has been gained in extracting spores. Sectioning jaws and gills of infected fish has shown them to be sites of spore occurrence; both structures are now cleaned and included with the bones of the head in the extraction process. By repeated examination we have found that flesh next to bone contains spores. To include these spores in our estimates, we now remove most of the flesh from the head, leaving approximately 1 or 2 millimeters of muscle and connective tissue. When the bones are disarticulated and cleaned of this flesh, the wash water containing the debris is saved. It is strained through cheesecloth, and the remaining debris is carefully rinsed. The filtrate is added to that of the head. This filtrate is then suction-filtered, centrifuged, and spore numbers estimated as described in the original article.

Spores were extracted from seven separate lots of wash water used to clean the heads of infected rainbow trout. It was found that wash water contained as much as 12 percent of the total spores in the head. The data on these large heads are given below in table 1.

In addition to the modifications outlined above, we have found that in extracting spores for infection experiments we could harvest an additional number of spores in small fish by cleaning the entire spinal column and extracting spores from its filtrate and wash water. Tables 2 and 3 show the percentage of spore content of heads and spinal columns from 9 small fish (length 58-127 mm) and 9 large fish (length 275-400 mm). The small ones were infected experimentally at the Eastern Fish Disease Laboratory and the larger ones came from a natural infection.

Table 2 shows an average of 27 percent of the total spore load carried in the spinal column and adjacent tissues of small fish (range 55-127 mm) with a variation of 8 to 57 percent, while the large fish of table 3 (range 225-400 mm) carried only an average of approximately 1 percent with a smaller variation of 0 to 3.4 percent. In addition to this data we extracted spores from 59 other small fish from the same population. The heads of these fish were pooled in lots of 2-10. The spinal columns were also pooled in the same size groups. The average spore numbers in the heads were estimated at 68 percent as against 32 percent for the spinal columns. We also pooled data for 10 other large trout in which again the average spore number for the spinal column was slightly less than 1 percent.

If one wished to quantify the degree of infection of *Myxosoma cerebralis* by estimating spore numbers, we feel that all bones of the head, the spinal column of small fish, and the wash water used in cleaning these spore sites must be utilized. The wide range of spore numbers in the spinal column of small fish, 8 to 57 percent, makes the utilization of the column necessary if reliable comparisons are to be made between individual lots of small fish.

The fish for this study were received from the Eastern Fish Disease Laboratory and the Michigan Conservation Department.

NOTE.—This study was supported in part by the National Marine Fisheries Service Development Act Contract 4-68-R-3, the Ohio Division of Wildlife, and Ohio State University.

Table 1.—Spores of Myxosoma cerebralis *extracted from bones of the head and wash water*

Length of head (mm)	Spore content of head bones alone	Spore content in wash water from head bones *	Percent of total spores in wash water *
50	1.167×10^5	+	+
63	1.474×10^5	1.946×10^4	12
50	5.68×10^5	1.39×10^4	2
69	1.985×10^5	+	+
50	1.723×10^5	2.36×10^4	12
63	8.61×10^4	1.11×10^4	10
58	1.176×10^6	1.11×10^5	9

* + positive, but spore numbers were too low for a reliable estimate.

Table 2.—Estimated spore numbers in artificially infected small fish

Length (mm)	Spores per head	Percent of spores in head	Spores in spinal column	Percent of spores in spinal column
75	1.53×10^4	47	1.74×10^4	53
113	4.65×10^4	70	1.95×10^4	30
83	1.67×10^4	43	2.22×10^4	57
113	5.15×10^4	90	5.5×10^3	10
127	5.3×10^4	83	1.05×10^4	17
55	1.27×10^5	65	6.75×10^4	35
58	7.65×10^4	77	2.35×10^4	23
60	5.95×10^4	92	4.85×10^3	8
64	3.07×10^5	87	4.45×10^4	13
Average		73		27

Table 3.—Estimated spore numbers in naturally infected large fish

Length (mm)	Spores per head	Percent of spores in head	Spores in spinal column*	Percent of spores in spinal column
288	1.936×10^6	>99	+	<1
300	1.835×10^6	97	6.4×10^4	3.4
225	1.327×10^6	>99	+	<1
275	2.885×10^6	99.6	1.25×10^4	0.43
275	1.52×10^4	100	0	0
400	5.666×10^5	>99	+	<1
300	5.097×10^5	>99	+	<1
288	1.011×10^6	>99	+	<1
320	1.27×10^6	>99	+	<1
Average			>98.9	<1.1

* + positive, but spore numbers were too low for a reliable estimate.

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THE ECOLOGY AND SPREADING OF THE PATHOGEN OF TROUT WHIRLING DISEASE—MYXOSOMA CEREBRALIS (HOFER, 1903, PLEHN, 1905) IN THE FISH PONDS OF THE SOVIET UNION

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One of the most dangerous of the known parasitic diseases is the so-called "whirling disease" of the Sturgeon family which is caused by Myxosoma cerebralis. The pathogen invades the cartilaginous tissues of young trout, destroying the ground substance of the cartilage. Most often it affects the cartilage of the inner ear and also other parts of the skeleton: cartilage of the skull, spinal column, fins, etc. This peculiarity in the choice of place of residence by the parasite is manifested by very characteristic symptoms of the disease.

The most characteristic symptom is the disorder caused to the motor function. As a result of the destruction of the cartilaginous acoustic capsule the sense of balance is lost; the fish loses the ability to move normally and begins to swim excitedly in one direction, in small circles; then there is a period of rest, when it lies in an apparent state of exhaustion on the bottom. Apart from that, the destruction of the cartilage of the skeleton produces all kinds of deformities: curvatures of the spine in different directions, underdevelopment of the operculum, formation of a pug-like head, etc.

Another symptom of whirling disease is the extensive darkening of the tail part of the body. The darkened part is clearly marked from the rest of the body, which retains its natural color. This has to do with the disturbance of the control of the pigment function of the sympathetic nervous system.

The study of this disease began in Germany, where it is widespread and causes great damage to trout-breeding. The parasite which causes whirling disease was described for the first time in the year 1903 by Hofer and subsequently in greater detail, according to stages of development by Plehn (1905, 1924) and Schaperclaus (1931, 1932). Recent works (Heuschman, 1940, 1949, Schaperclaus, 1954, Tack, 1951, Luling, 1952) show that even at the present time the whirling disease forms a menace to trout-breeding in Germany, although to a lesser degree, since measures have evolved to combat it (Schaperclaus, 1954).

Apart from Germany, whirling disease is also prevalent in France (Vanco, 1952), Italy (Scolari, 1954), Czechoslovakia (Dyk, 1954), and Poland (Kocylowski, 1953). Until 1952 no instances of Myxosoma cerebralis disease of trout were noticed in the fisheries of the Soviet Union, although V. A. Dogel' (1932) observed in one of the basins of southern Finland wild trout with external signs of this disease. The appearance of whirling disease on a large scale, has first been registered in the Soviet Union in 1952 in the fisheries "Ropsha" (Leningrad Oblast'). Later, in 1954, it was also found that Myxosoma cerebralis struck salmon bred in Chernorechensk trout fishery (Abkhazian SSR).

The appearance of this disease in our country makes it necessary to study in detail the biology of its pathogen and makes it imperative to see how it will behave in new conditions in order that we should be able to introduce control measures suitable to the local requirements. It is interesting to compare the results of our observations of trout afflicted with whirling disease with the material of foreign authors who studied this question. During the years 1953 and 1954 we made systematic observations of trout in Ropshchskie ponds and made periodical dissections and fixations

of diseased trout. During this time 700 complete parasitological dissections and 225 fixations were made. Apart from that, many incomplete dissections were made with a view to discover spores, required for the experiments. In 1956 we made observations of the Chernorechensk trout fisheries, where we made 344 diasections and 105 fixations of wild and artificially bred salmons.

The pathogen of the whirling disease—Myxosoma cerebralis strictly adheres to a definite place of habitation and, as it has been shown by histological studies of diseased fish, the bed of infestation never extends beyond the cartilage tissues. According to literature, the emergence of spores to the exterior occurs only after the death of fish and the true number grows proportionally to the progress of decomposition of the cartilage. However, we have found spores of Myxosoma cerebralis in almost all organs and tissues of diseased trout; in cartilage, in brains, in muscles, in liver, in gallbladder, and even in the contents of the stomach. Apparently, from the destroyed cartilages spores find their way into lymphatic or blood vessels and are carried by the lymph or blood current to various organs, including the liver, wherefrom they pass, through the bile duct into the bowels. This fact permits us to assume, that certain quantities of spores get into water from the living fish.

Our data on this have been confirmed to some extent by data of foreign authors, although they have not given the problem its proper emphasis.

Hofer (1903), who described the pathogens of the whirling disease, found, for the first time, spores of this parasite only in the brains of trout, whence the name of the species—"cerebralis"—derives its origin. Then Plehn (1906) proved that this germ is a parasite of the skeletal system, and while pointing out that Hofer erred in localizing the pathogen proposed to name the species "chondrofagus" which has, however, not been accepted since according to zoological rule the first name given to an animal remains unaltered.

The results obtained by us allow us to maintain that the place of habitation of the parasite is, in fact, in the cartilaginous tissues. Yet, discovery of spores in the brain does not point to a careless investigation, but rather to the possibility that the spore is carried to other organs. This fact is important in solving the question of the manner in which the infestation spreads.

The spore penetrates the fish, as in the case of most pathogens, through the alimentary tract.

As has been shown by Plehn (1905) spores which were kept in the stomach of trout in bags of filter paper from 24 to 48 hours do not discharge polar filaments.

This has been confirmed by experiments made by us, when spores which remained in the stomach of trout for 56 hours underwent no changes. No polar filaments were discharged, neither under the influence of acidic solutions, nor in chemical gastric juice. On the basis of experiments carried out by Plehn, she arrives at a conclusion that polar filaments are discharged in the intestine of the fish, i. e., in alkaline conditions. Plehn did not check the direct effect of gastric juice on the spore but she points out that the discharge of polar filaments can be easily attained by putting spores in weak solutions of alkali (1% of potassium or sodium hydroxide) and in lime water.

Experiments made by us in this direction have also been successful. After putting spores in a 1—2% KOH solution, the polar capsules, as a rule, discharged polar filaments.

As our observations have shown, the effect of seasonal growth changes on the degree of infection of trout by Myxosoma cerebralis in "Ropsha" fishery follows the following pattern.

Symptoms of whirling disease in the new generation, in the ponds, were noticed at the end of June and beginning of July. The affected young fish performed the characteristic whirling movements. In some of them, darkening and curvature of the tail were already noticed in July.

In some of the young fish inability to feed and serious exhaustion was noticed, and was followed by early death.

In 1955, in "Ropsha," trout affected by whirling disease were already discovered in the nursery pond. In that year, owing to the unduly long and cold spring, the transfer of fry into ponds was somewhat delayed. In the first Mel'nichnyi ponds, from

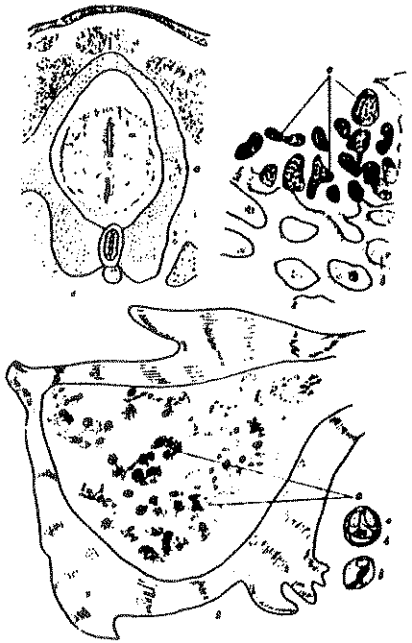

Figure 1. Cross sections of cartilage box of a young Salmo irideus trout, infected by the pathogen of the whirling disease – Myxosoma cerebralis (stained by haemalum and eosin)

a – cavity formed as a result of the destruction of cartilage by the parasite (low-power microscopic magnification)

Figure 2. Damage to the cartilage of the cranium, shown in Figure 1a. 1 – High-power magnification (stained by haemalum and eosin)

a – plasmodium of parasite; b – cartilage cells

Figure 3. Ossification of the gill arch formed around a section of cartilage destroyed by the parasite (stained according to the quartering method of Orcharova)

a – spores of Myxosoma cerebralis (at high-power magnification)

Figure 4. Spores of Myxosoma cerebralis in immersion in different planes

a – view from the side of the fold; b – view from the side of the suture

which water flowed into the nursery pond, a sufficient quantity of infectious material has been collected. In this period the parasite is in the plasmodial stage and therefore, on opening the diseased fish, the spore is not discovered, but in the microscopic sections one can see multinucleate plasmodia settled in the vacuoles formed as a result of the destruction of the cartilage.

Spores of Myxosoma cerebralis in trout of a new generation may be first discovered in the middle of August. In September–October a mass creation of spores begins, and spores can be found, not only in black-tailed, i.e., in fish with distinct outward symptoms of whirling disease, but also in trout with normal pigmentation. The amount of spores continues to increase and they can be found in all organs of the trout. From September to February, spores can be found in various tissues and organs. In the following summer they are found only in the cartilage. During the course of time spores are, apparently, eliminated from organs which are abundantly supplied with lymph and blood more quickly than from bone and cartilaginous tissues. While in autumn and winter spores are found, both in year-old fish with obvious symptoms of whirling disease as well as in fish seemingly healthy, in the next summer they are always found even in black-tailed trout of the same generation. Apparently, with the age of fish there is not only a gradual elimination of spores from various organs but, on ossification of the skeleton, also death of some of them. In the second summer of the life of the fish spores are found only in various parts of the skeleton, and they become fewer in two- and three-year-old fish, which, according to external signs, are obviously ill with the whirling disease.

Yet, although with the growth of trout there is a gradual cleansing of the organism of spores, fish two and three years old which passed whirling disease should be classified as carriers of the disease, since a certain small quantity of spores remains in them. In some instances, when the infection is very serious, the destruction of bone tissue also continues in more advanced age. Thus, in some trout affected by the illness, which had been separated in a nursery pond, a progressive curvature of the spine was observed, and in one of them a puglike deformation of the head became apparent only in the third year of its life.

Data on the dynamics of the seasonal growth of infection of trout with the whirling disease, obtained by us, coincides with data of foreign authors (Schaperclaus, 1931, Scolari, 1954). Some discrepancies in dates of appearance of spore are, apparently, due to the difference of the time of emergence of larva, and the transportation of young fish into ponds, which depend on climatic conditions in different regions of trout fisheries.

When making a comparative study of the material at our disposal, an obvious difference in dates of infection and sporulation between the northern and southern fisheries of the Union become apparent. In the Chernorechensk fishery, where hatching of trout begins much earlier, a large quantity of spores was already found in the one-year-old fish of 1954, in July, while in "Ropsha" they could be found in such quantities only in September.

Yet, the periods of collection of eggs, their incubation, the hatching of larvae in the Chernorechensk trout fishery are very long. The first larvae may already appear in January and hatching may continue till the end of May. Therefore, young fish, which begin to feed at different times, are not subjected at the same period to infection by Myxosoma cerebralis. This explains the fact why in this case the sporulation does not proceed simultaneously with that of Ropsha. While in some fish the parasite could have passed long ago into a dormant state, in others, on the other hand, which had a later opportunity of being infected, the pathogen is only in its early stage of development. In 1956, trout with obvious external symptoms of the whirling disease were discovered in nurseries already in the middle of March. Yet, in a number of one-year-old fish infected by the whirling disease which were examined in the beginning of July, no spores were discovered.

According to our own observations, as well as to information given in literature, the death of trout from the whirling disease occurs only at an early age. In 1953, the first dead fingerlings in "Ropsha" were noticed in July. In such young fish

one observes derangement in movement functions and an obvious abnormality of pigmentation. When they attempted to feed they had a strong attack of whirling.

In September, among the black-tailed one-year-old fish transferred to a nursery, specimens of whirling trout were observed, but the number of fish with this disease greatly diminished by winter and in November only few such specimens were observed. During the period from September to November sporulation occurs. Together with plasmodium stages, the one-year-old "Ropsha" trout has already quite a large quantity of spores. By the next summer all surviving fish behave normally.

The blackening of the tail may also disappear gradually. However, when the damage is more serious, it remains for a very long time and we have even seen it in three-year-old fish, weighing more than 360 g.

Deformities of the skeleton - a more serious damage - remain, thus affecting the fish for life. This has been recorded in one-year-old and even in older fish.

In trout which have survived, the disease causes retardation of growth and nutritional state in comparison to healthy fish.

The following figures may be cited in respect to weight of diseased and healthy trout, which have grown in the same conditions.

In 1953 the weight of diseased one-year-old fish did not exceed 8.5 g, while at the same time healthy fish reached 64 g. In 1954 diseased one-year-old fish weighed, on the average, 17 g, the healthy ones - 24 g. Three-year-old diseased fish weighed, on the average, 56.6 g, the health ones - 220 g.

The process of the disease passes through two stages:

The first, the acute stage of the disease, is when the parasite passes through the plasmodium stage. During this period the destruction of the basic tissues of cartilage takes place. It is manifested outwardly either by strong attacks of whirling disease or by the inability of the young to feed, and it dies of exhaustion. In many fish, blackening of the tail appears. This is the most dangerous period, during which death occurs.

During the second stage of the disease, when the parasite is in the state of sporulation, attacks of whirling and such external signs as blackening of the tail usually cease, and if the fish did not have serious deformities of the skeleton, it may appear normal. During this period, as a rule, no deaths occur, but fish which have survived form a source of contamination.

However, it is not always possible to make a clear delimitation of these two stages. Death may occur immediately after the severe stage. It may happen that the infection is not serious and the destruction of the skeleton is slight, if there exist in the cartilage plasmodium stages and no severe attacks of whirling are witnessed. In such cases where there is severe damage to the organ of balance, whirling movements are witnessed also in trout of an older age (for example, two-year-olds in which the parasite has produced spores a considerable time before). Such fish begin to make the whirling movements, as a rule, after they make quick or sudden movements when having been frightened or when in pursuit of food.

Schaeperclaus (1931) distinguishes four forms of disease:

1. Trout making whirling movements.

2. Early death of young fish without having performed the whirling movements. This form is of a severe character and is observed, according to the author's data, in June. Before death, the young fish do not feed and are very exhausted. Schaeperclaus assumes that the death of the young fish is connected with the damage done to the central nervous system as a result of the damage inflicted to the head cartilages.

3. This form is observed when the infection is comparatively mild or late and is of a chronic nature. It is characterized by the blackening of the tail of the diseased fish. The symptom comes as a result of the damage to the sympathetic nerve, which lies in the tail part in the immediate vicinity of the spinal cord.

4. This form is without external symptoms. It is also of a chronic nature and does not cause death; it is dangerous when fish are transported, since it is impossible in such a case to discover the diseased specimens.

In "Ropsha" fishery of the Leningrad Oblast', trout with whirling disease have been found in almost all ponds, and since most of the ponds of the fishery have

intercommunications, it can be assumed that all of them are infected with spores of Myxosoma cerebralis.

Apart from Salmo irideus, the pathogen strikes also Salmo trutta fario and Salmo salar, bred in the ponds of the fishery, brought from Taibola, and Baltic salmonids. The branch of "Ropsha" fishery in Gostilits is also infected by whirling disease because there is a constant exchange of fish between them. As already stated, the fisheries of Chernorechensk, the Abkhazian SSR, is also badly affected by the whirling disease.

It is difficult to decide what is the source of infection of the fisheries mentioned, but we should keep in mind two possible sources of spreading of the infection: either its import from fisheries which have already been infected or from wild fish. Salmo irideus have been imported into "Ropsha" fishery in the form of eggs, in the year 1947, from Western Germany, from the fishery Marsel'. As is known, trout fisheries in Western Germany suffer from whirling disease.

In his work, in the year 1931, Schaeperclaus points out the fact that spores can also be transferred together with eggs. If water is let into a nursery pond from a contaminated pond, then the possibility of spores attaching themselves to eggs should not be excluded. It is true that the same author, in a later work (1954), expresses doubt, relying on his experiments, as to whether importation of spores with eggs and stock is possible, but he does not consider this question as having been finally decided. Even if the importation of Myxosoma cerebralis into "Ropsha" in this manner has not been definitely proved, this possibility should be kept in mind.

At a conference on the question of renewal of fish stock, which took place in Moscow, in December 1954, V.A. Dogel', in his address on the part played by fish diseases on the renewal of fish stock, expressed an assumption that whirling disease might have been brought into "Ropsha" from the Karelian Isthmus, from which fish were imported into this fishery during the post-war years.

In respect to the Chernorechensk fishery, it may be said that there too the possibility that the parasite had been imported is not excluded. In 1951 spawners of Salmo irideus were brought into Chernorechensk fishery from the fishery of "Klyuchiki" of Kursk Oblast'. Into this same fishery, trout were brought in the year 1949 as a replenishing stock, from "Ropsha" before whirling disease had been discovered there.

Investigations made in 1956 have shown that wild Salmo trutta (labrax) and wild Salmo irideus which live in Chernaya River, are infected with the whirling disease pathogen and it happened thus that in the Chernorechensk fishery a natural bed of infection has formed. It is difficult to say whether this bed of infection existed before the Chernorechensk fishery had been established or that it formed after trout infected with whirling disease had been brought into it and possibly infected the wild fish. As it is, a permanent source of infection formed in this fishery.

The history of the three above-mentioned fisheries, while it does not exclude the possibility that the infection came from local wild fish, makes us believe that it is necessary to stop all transfers of trout from the mentioned fisheries and to check the state of trout in other fisheries into which fish have been transferred from "Ropsha" before quarantine measures were introduced.

An important question in the study of whirling disease is the rapidity with which the accumulation of infection is formed in ponds. In order to obtain precise data a long period of observation is required; however, we have already succeeded in acquiring some information.

In some ponds of "Ropsha", the accumulation of spores has been quite fast. For example, in Mel'nichnyi III pond, a small pond with fast-running water which began to function in 1952, Salmo irideus has been kept from the first year of its exploitation. In 1953 undersized trout from Malogorodsk pond, including some infected by whirling disease (more than 100 specimens with external symptoms of the disease) were transferred into this pond. Part of the diseased trout died during the summer, which intensified the infectiousness of the pond. In 1954, 1,300 fry of Salmo irideus were transferred into this pond. By autumn, 35.6 % died. Of those which survived, 50 % had external symptoms of whirling disease and among fish which had no such symptoms many had spores of the parasite. (All of the ten dis-

sected fish had spores.) Thus, two years were sufficient for accumulation of such an amount of spores, which were capable of infecting more than 50% the fish.

In other ponds the accumulation of spores proceeded more slowly. In 1954, the infection of trout did not exceed 0.2—0.8%, in 1955 – 0.5%.

Although in some ponds the initial accumulation of infection has been rapid, in "Ropsha" as a whole, the increasing process of the disease has been checked, and for a long time it did not assume the size of a catastrophic epizootic. This is due to the fact that the method of management of the fishery in "Ropsha" somewhat checked its progress. According to this method, the initial breeding of fingerlings in ponds of large areas, on natural food, limits the contact between the fingerlings and the spores. Further feeding of meat from grates also draws the fish away from searching for food at the bottom.

However, in 1956 whirling disease already reached an epizootic character. The spreading of infection in the ponds increased up to 60—100%. This sharp rise in infectiousness may be explained thus: the initial infection in the year 1956 apparently took place before fish were transferred into the ponds - in the nursery. This supposition is confirmed, for example, by the following fact. In pond Kipen' II, which was first exploited in the year 1955 and filled with fry in 1955 and 1956 from the same nursery pond, 96% of one-year-old fish were infected by September. As already stated, fry infected by whirling disease were discovered in the nursery pond in the year 1955. By 1956, the contamination by spores of pathogens in the Mel'nichnyi I pond, which contained, during the last years one-year-old fish, spawners, and the replenishing stock of Salmo irideus and served as a source of water supply to the nursery ponds, increased. Apart from that, in the year 1956, the fry of Salmo irideus of "Ropsha" was weakened by other causes, which also contributed to the development of Myxosoma cerebralis. Further infection of the young fish, proceeded undoubtedly in other ponds, where spores accumulated from year to year. All this brought about the outbreak of the disease.

Another development took place in the Chernaya River. There the fishery is built according to a different pattern. The water supply system is separate in all places. The young fish are bred in concrete basins. From the hatcheries fry are first transferred into nursery ponds, where they are kept until they weigh 3 g, then they are transferred into round basins, where they grow to 25 g, and after that into ponds. Before the basins are filled with fish they are cleaned and therefore there is no great accumulation of spores. Fish transferred into ponds were of an age when whirling disease does not form a danger to them. However, as has already been mentioned, the perpetual stock of contamination is the river - the source of water supply.

In a naturally formed body of water, where there is no crowding of fish, the infection seldom reaches such an intensity as found in conditions of artificial breeding.

In the year 1956, the general infection of wild fish was 5.7%. It should be noted that wild Salmo irideus is more easily affected than salmons of Chatnomorsil. The extensive infection of Salmo irideus reaches 12.5% while that of other salmons only 1.5%.

The intensity of infection of both species is very low and on dissection of the diseased fish only a few spores are discovered. None of the diseased fish which were examined, had been damaged by the disease.

According to the material of 1956, 16.6% of the fish of the size group 18—21 cm were affected by the pathogens of the whirling disease while only 1.3% of fish of the size group 10—17 cm were infected.

These fish groups belong either to different generations, or the hatching of the specimens, which constituted each of these groups, occurred during the same generation but at different times.

From what has been said, it follows that in the natural conditions of Chernaya River there occur in different years or in different seasons outbreaks of the disease. It is natural that such outbreaks should be expected to occur also in artifical reservoirs which receive their water supply from the river, but the modern preventive measures may protect the fishery from an epizootic.

From all that has been said the following conclusions may be drawn:

1. Whirling disease has appeared in the trout fisheries of the Soviet Union ("Ropsha", Chernaya River).

2. The threat of the spreading of the whirling disease in the Soviet Union makes it imperative to make a detailed study of this disease in our conditions and impose a strict supervision over trout fisheries.

3. The history of those fisheries, in which whirling disease has appeared, shows that it is absolutely necessary to stop transfers of trout, of all age groups, from the above-mentioned fisheries and to doubt the advisability of transferring eggs from an infected fishery, if the hatchery is fed from a pond which is contaminated by spores.

4. In the fishery of Chernorechensk, a natural bed of infection has formed. Wild fish in Chernaya River are infected by the pathogen of whirling disease.

5. The most pathogenic are the plasmodium stages of the parasite.

6. During the period of a mass sporulation the pathogenesis of Myxosoma cerebralis weakens, and destruction of the skeleton cases and the "black-tail" may disappear.

7. Spores of the parasites from the destroyed part of the skeleton may reach, through lymphatic and blood vessels, other tissues and organs, and are found, not only in cartilage, but also in muscle, brain, liver, gallbladder and intestinal lymen.

8. Some emission of spores from the alimentary tract may occur while the fish is still alive.

9. In the course of time a gradual cleansing of the spores takes place in the system of the trout. However, since this process proceeds at a different rate in different fish, fish of all age groups which have undergone whirling disease should be regarded as potential sources of infection.

10. Differences in dates of infection, sporulation, and rapidity of accumulation of the initial infection, are observed in different trout fisheries of the Soviet Union.

11. Apart from Salmo irideus, the agent of whirling disease has been discovered in Murmansk salmon, Salmo trutta fario and in Baltic and Black Sea salmonids.

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New Data on the Life Cycle and Biology of Myxosporidia

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With 24 Figures

Summary

Myxosporidia with different localization in fish organism were investigated using electron microscopy, cytophotometry and cytochemistry. As a result of our cytophotometric investigations, a new understanding of the life cycle of Myxosporidia as a cycle with alternation of haplophase and diplophase was reached. The study of coelozoic, histozoic and intracellular Myxosporidia has shown a great variability of their surface ultrastructures. The surface ultrastructure is closely connected with the mode of nutrition of Myxosporidia, in its turn depending on their localization inside fish organism.

All possible types of nutrition were observed in trophozoites of myxosporidian species investigated: extracellular digestion of food by means of enzymes secreted by the trophozoite; contact or membrane digestion followed by active transport and/or pinocytosis; phagocytosis followed by intracellular digestion inside food vacuole. These nutritional types occur in various combinations in different species of Myxosporidia.

By cytochemical methods we have demonstrated that the metabolism in Myxosporidia can follow both aerobic and anaerobic pathways. A new approach to the study of polar capsule discharge mechanism, using glycerinated spore models, has revealed the role of contractile systems in this mechanism.

Introduction

The investigation of Myxosporidia with the help of electron microscopy, cytophotometry and cytochemistry gives much for the understanding of their life cycle and biology and also allows to make the previous data based on light microscopy more precise. This can be perfectly illustrated by the first electron microscopic study by Grassé (1960) who proved the multicellular nature of the Myxosporidia.

Of major interest is to carry out by these methods the comparative study of Myxosporidia belonging to various taxonomic groups and of different localizations in the host organism. There are about twenty electron microscopic investigations at present where the ultrastructure of trophozoites and spores and also sporogenesis events are described (Grassé 1960; Cheisin, Schulman and Vinnichenko 1961; Lom and Puytorac 1965a, 1965b; Uspenskaya 1966, 1969, 1971, 1972; Schubert 1968; Lom 1969; Current 1979; Current and Janovy 1976, 1977, 1978; Current, Janovy and Knight 1979; Desser and Paterson 1978; Grassé et Lavette 1978). Most of these papers are concerned with a single species. Only one of them (Lom et Puytorac 1965b) includes data on several species of different localization in fish

organism, and in another work (CURRENT and JANOVY 1978) the ultrastructure of two forms of the same species localized on different parts of fish gills are compared.

A number of papers deal with cytochemistry of Myxosporidia (PETRUSCHEWSKY 1932; BOND 1940; CHAKRAVARTY, MAITY and RAY 1962; WALLIKER 1968; CHAUDHURY and CHAKRAVARTY 1970; USPENSKAYA 1966, 1978b, 1979b; PODLIPAEV 1972, 1974; PODLIPAEV and SCHULMAN 1978; DESSER and PATERSON 1978). However, most of them are concerned with iodinophilous vacuole contents. Up to now only we have studied the amount of DNA in the nuclei at different stages of the myxosporidian life cycle with the help of Feulgen cytophotometry (USPENSKAYA 1975, 1976b, 1978a, 1979a, 1981).

The investigations of the discharge mechanism of the polar filament of myxosporidian spores mostly deal with the effect of various reagents on such a discharge (KUDO 1918; LOM 1964, and others). We have attempted a new approach to the study of polar capsule discharge mechanism using the glycerinated spore model (USPENSKAYA 1976a, 1977).

Materials and Methods

Our material was obtained during several expeditions from both marine and freshwater fish. It included coelozoic species (*Sphaeromyxa elegini, S. hellandi, S. cottidarum, Myxidium gasterostei, M. perniciosum* from the gall bladder, *M. lieberkühni* from the urinary bladder), histozoic species (*Myxosoma cerebralis* from cartilage, *Myxobolus disparoides, Henneguya lobosa* from gills, *H. zschokkei* from intramuscular connective tissue), and intracellular species (*H. oviperda* from fish eggs, *Kudoa quadratum* from muscle fibers). Our aim was to investigate species of different localizations in fish organism. For electron microscopic studies we have used Palade fixative as well as 2% osmium tetroxide in cacodylate buffer, and 2% glutaraldehyde in phosphate buffer followed by postfixation in 1% OsO_4 solution. The samples were embedded in methacrylate, araldite, epon, or a mixture of epon and araldite. The sections were cut with glass knives using the LKB ultratome. Thin sections were stained in saturated aqueous solution of uranyl acetate followed by lead citrate and viewed and photographed with Tesla BS-500, JEM-5 or JEM-7 electron microscopes.

For cytophotometric measurements of the nuclear DNA, smears fixed in Carnoy's mixture were hydrolyzed in 1 N HCl at 60 °C for 8 min and stained with Schiff's reagent prepared according to the "cold Schiff" method (LILLIE 1965). Nuclei of Myxosporidia are stained more intensely by this method than by the standard one. To minimize the error connected with the difference in time of hydrolysis and staining, we placed all the stages to be compared on a single slide or, at most, on two slides which were processed simultanously. The Feulgen stained nuclei were photographed with a MUV-6 microscope in monochromatic green light (546 nm). The densities of the negatives were measured with a scanning microphotometer MF-4 combined with a computer. The nuclear area was measured by planimetry on tracings of the nuclei enlarged by a photomagnifier (final magnification, ×5,400). The DNA content was expressed in arbitrary units.

Several cytochemical methods were used. The glycogen was revealed by the PAS reaction, the controls being performed by α-amylase, saliva, cold and hot water treatments. Total protein was stained by the mercuric bromphenol blue technique; DNA, by the Feulgen nucleal reaction; DNA and RNA, using methyl green — pyronin or gallocyanin-chromalum techniques (control with ribonuclease). Neutral fat was stained with Sudan III, Sudan black B, or it was osmicated according to CHAMPY followed by Na_2S solution treatment. Mucopolysaccharides were revealed with the alcian blue technique; acid phosphatase, with GOMORYS method; alkaline phosphatase, with the technique of GOMORY and TAKAMATSU for light microscopy, and with that of HAGEN and BERGENSE for electron microscopy. Succinate dehydrogenase activity was revealed nisug tetranitro-

blue tetrazolium; ATP-ase activity, by the method of PADYKULA and HERMAN (PEARSE 1968; LILLIE 1965; LUPPA 1977). Glycerinated spore models were prepared according to the schedule developed for amoebae (ARRONET 1971).

Abbreviations used in Figures

B	— trophozoite body with strong phosphatase activity	MTM	— mitochondria of muscle cell
C	— central core of external tube	MTT	— mitochondria of trophozoite
CC	— cartilaginous cell	MV	— microvilli
CM	— cartilaginous matrix	N	— nuclei
CPC	— capsulogenic cell	P	— plaques in supramembrane layer
CV	— cavity inside cartilaginous matrix	PB	— polar body
EN	— envelope of *Esox lucius* egg.	PCH	— pinocytotic channel
GL	— granules of glycogen	PHP	— phagocytized protofibrils
IF	— infected muscle fiber	PHT	— trophozoite phagocytizing the cortilaginous cell
IV	— iodinophilous vacuole	PV	— pinocytotic vesicle
LPF	— lysed protofibrils	R	— "root" of trophozoite
MF	— muscle fiber	S	— spore
MP	— membrane of pansporoblast	SC	— sporoblast cell
MS	— membrane of sporoblast	SML	— supramembrane layer
MSP	— part of body with mature spores	T	— trophozoite
MT	— membrane of trophozoite	UF	— uninfected muscle fiber
MTP	— microtubules	VC	— valvogenic cell
MTBS	— microtubules of spindle	Y	— yolk

Results

1. Life cycle

As a result of our cytophotometric investigations of nuclear ploidy at different stages of development of Myxosporidia belonging to several taxonomic groups, a new understanding of their nuclear and life cycles was reached. The study was made with *Sphaeromyxa elegini* DOGIEL 1948 and *Myxidium perniciosum* DOGIEL 1957 [from the suborder Bipolaria TRIPATHI 1949 emend. SCHULMAN 1959, family Myxididae THELOHAN 1892], and *Henneguya zschokkei* (GURLEY 1894) [from the suborder Platysporea KUDO 1919 emend. SCHULMAN 1954, family Myxobolidae THELOHAN 1892].

Contrary to the recent ideas that Myxosporidia are diplonts with gametic reduction (NOBLE 1944; SCHULMAN 1966, 1972), we have made the conclusion that alternation of diploid and haploid phases occurs during their life cycle.

It is well known that the life cycle of Myxosporidia has two phases. One of them is a parasitic phase represented by trophozoites in the form of multinucleate polyenergidic plasmodia displaying nuclear differentiation (vegetative and generative nuclei) and reproducing asexually inside fish organs and tissues. According to DOGIEL (1963), this is the agglomeration phase. The second phase, represented by multicellular spores, is a resting one and occurs in water, serving for distribution and infection of new fish hosts. According to DOGIEL, this is the dispersion phase.

Our cytophotometric investigations of diplosporoblastic Myxosporidia (two spores forming inside each pansporoblast), such as *Sphaeromyxa elegini*, *Myxidium perni-*

Table 1. Data on nuclear ploidy of some myxosporidian species at different phases of life cycle (according to cytophotometry)[1])

Life cycle phases	Type of nuclei	Suborder Bipolaria, family Myxididae		Suborder Platysporea, family Myxobolidae
		Sphaeromyxa elegini	*Myxidium perniciosum*	*Henneguya zschokkei*
diploid, of agglomeration	I. Trophozoite nuclei:			
	1. vegetative:			
	in trophozoite cytoplasm	4 n	4 n	—
	in pansporoblast	2 n	—	—
	2. generative, from pansporoblast having:			
	2 nuclei	2 n	2 n	—
	4 nuclei	2 n	—	—
	8 nuclei	2 n	2 n	—
haploid, of dispersion	II. Spore nuclei:			
	of sporoplasm	n	n	n
	capsulogenic	n	n	n
	valvogenic	n	n	n

1) Data on mean DNA values in nuclei investigated see USPENSKAYA 1976b, 1979a, 1981.

ciosum, *Henneguya zschokkei* (USPENSKAYA 1975, 1976b, 1978a, 1979a, 1981), show that their vegetative nuclei are polyploid (4 n at average). The generative nuclei and their derivatives are diploid up to their last division during sporogenesis. This division gives rise to haploid nuclei of the spore-forming cells. All the cells of a spore (valvogenic, capsulogenic and sporoplasm) contain haploid nuclei (Table 1, Fig. 24). In diplosporoblastic forms which have spores with two polar capsules and two valves, these haploid cells do not divide any more, but undergo some differentiation and morphogenesis (formation of complex polar capsules, of differently shaped valves, etc.). So it is evident from our data that not only gametic cells (sporoplasm) but also a number of somatic cells become haploid and constitute a distinct haploid phase in the life cycle of Myxosporidia. In other words, the myxosporidian life cycle includes alternation of diploid and haploid phases, the former being prominent and the latter being rather reduced.

Among Protozoa, such a life cycle with alternation of haploid and diploid phases is characteristic of Foraminifera; outside the Protozoa, it is typical of the plants (alternation of a diploid sporophyte and a hyploid gametophyte. In higher plants the haplophase is strongly reduced and represented by the germ sac with only a few cells).

The myxosporidian meiosis must thus occupy an intermediate position in their life cycle, as compared with either gametic or zygotic reduction. Sexual process in

Myxosporidia is primitive autogamy or pedogamy which takes place at the moment when the sporoplasm is leaving the spore engulfed by the fish host. According to our data there is no sexual process preceding pansporoblast formation inside the plasmodium, since both residual and generative nuclei of two-celled pansporoblasts are diploid (Table 1).

2. Surface ultrastructure

Investigation of Myxosporidia which have different localizations in fish organism reveals significant variability of their surface ultrastructure. Trophozoites of coelozoic, histozoic and intracellular Myxosporidia are of diverse shape, size, and surface structure.

Histozoic and intracellular Myxosporidia. Among histozoic Myxosporidia there are, on the one hand, such forms as the small amoeba-like trophozoites of *Myxosoma cerebralis* with ray-like pseudopodia or lobopodia, parasitic in salmonids. These plasmodia actively move inside the cavities of the cartilaginous matrix, which are produced as a result of their parasitism (Figs. 1, 2; HOFER 1903; PLEHN 1924; USPENSKAYA 1955, 1957; HALLIDAY 1976, etc.).

On the other hand, among tissue and intracellular Myxosporidia we can find very large plasmodia with a definitely shaped body. Trophozoites of *Kudoa quadratum*, parasitic in muscle fibers of *Myoxocephallus scorpius*, can be regarded as intracellular parasites. The vegetative stage of this species is usually called "cyst" but in fact it is rather large (0.3×2 mm in our material) fusiform plasmodium covered by a single unit membrane. The surface of the trophozoite carries rare and short microvilli (Fig. 3, 4). The growing plasmodium is filling up the muscle cell, and the membrane of the muscle fiber becomes closely adjacent to the parasite's body and produces the false impression of the existence of a second plasmodial membrane. But under the electron microscope, the remnants of undigested muscle protofibrils are seen between the two membranes (Fig. 5).

Inward of the plasmodium membrane, a dense zone of cytoplasm with pinocytotic channels is situated, then a zone rich in rough endoplasmic reticulum, vacuoles with various inclusions, and mitochondria (Fig. 4). Deeper in the parasite's cytoplasm, generative cells, vegetative nuclei and stages of sporogenesis are situated. Only one spore is formed inside each sporoblast of this species. The cytoplasm around the mature spores is vacuolized, and completely formed spores lie inside large vacuoles (Fig. 6). The spores of this species have 4 polar capsules and 4 valves, so that 9 spore-forming cells are formed in each sporoblast. The density of the cytoplasm of generative cells and sporoblasts is higher than that of plasmodia. For this reason they were taken, under the light microscope, for small plasmodia occurring inside the "cyst" (SCHULMAN 1978; KOVALEVA and SCHULMAN 1978). Actually, only one large plasmodium in each muscle fiber can be observed in our material.

The plasmodium is always enveloped by the muscle fiber membrane. The whole development of *K. quadratum* throphozoite thus seems to take place inside a single muscle fiber, till the spore formation is completed. Only after the disintegration of the

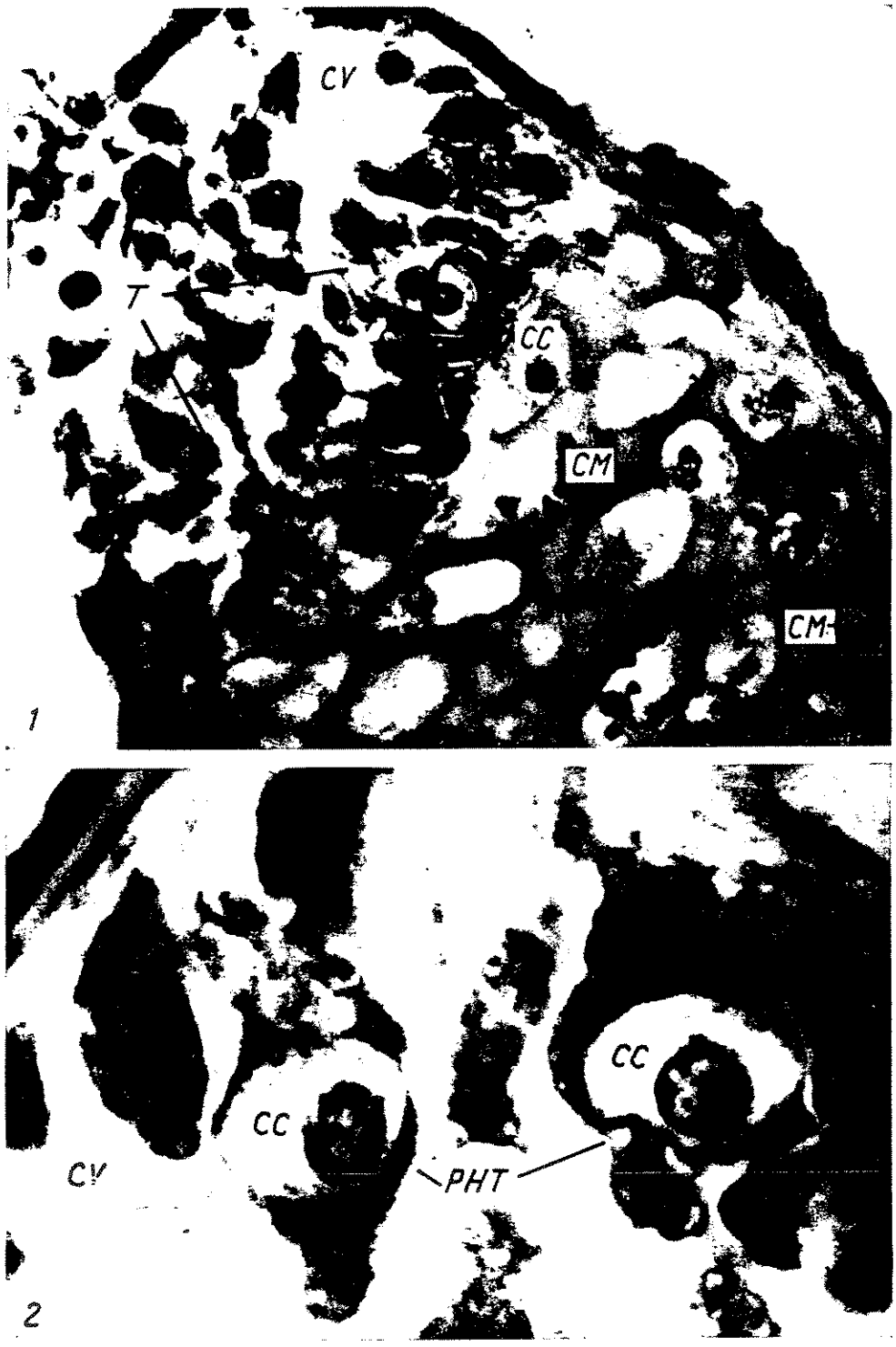
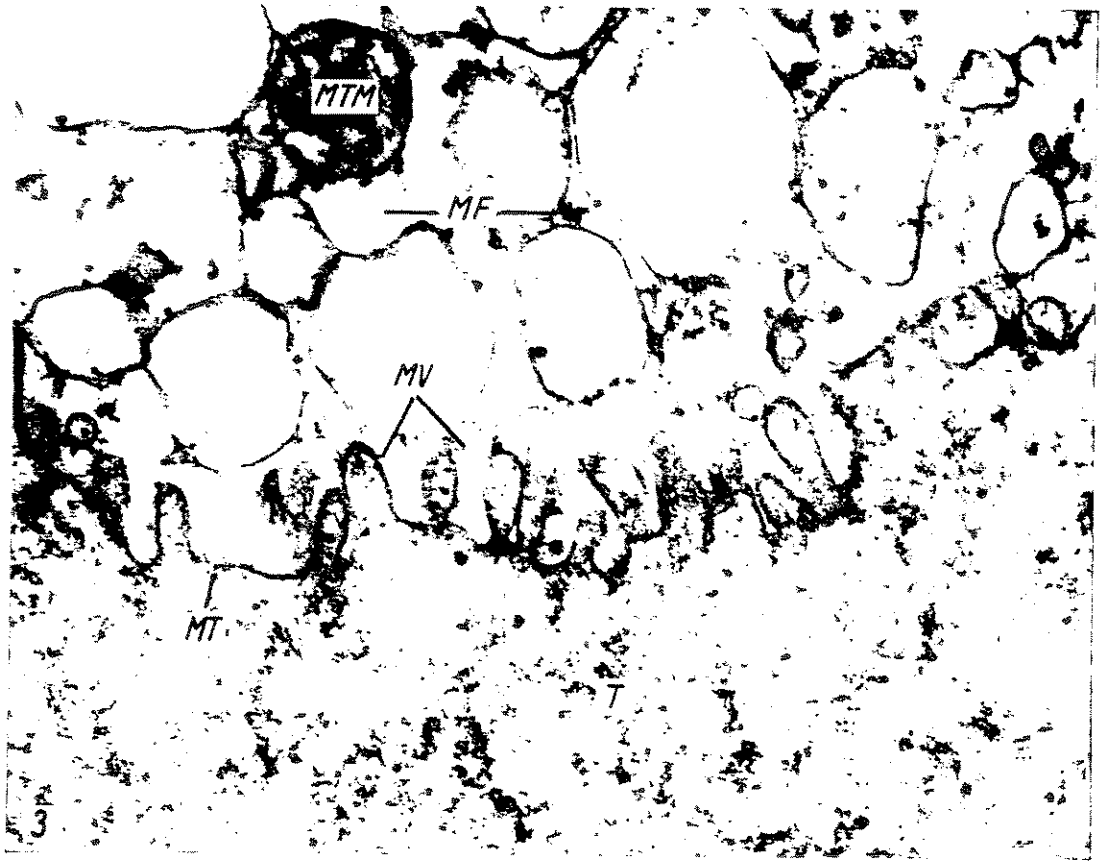
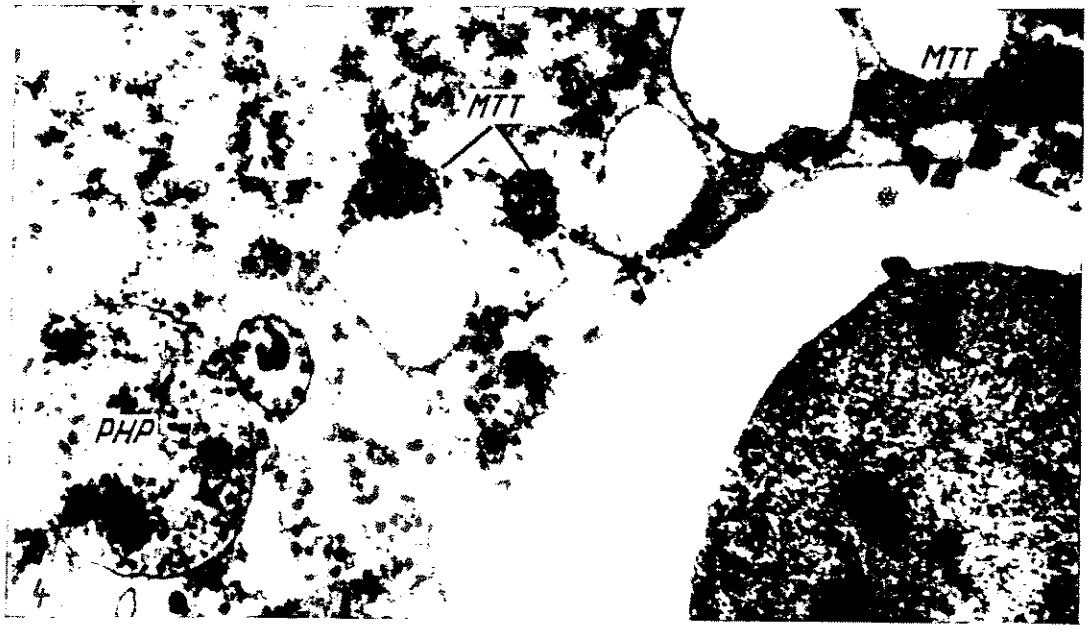


Fig. 1. Trophozoites of *Myxosoma cerebralis* in the cavity formed inside the cartilaginous matrix as a result of its lysus by the trophozoite enzymes. Light micrograph. MÜLLER's hemalaun — eosin staining. ×800.

Fig. 2. Trophozoites of *Myxosoma cerebralis* phagocytizing cartilaginous cells. Light micrograph. MÜLLER's hemalaun — eosin staining. ×2,400.





Figs. 3, 4. Ultrastructure of *Kudoa quadratum* trophozoite inside the muscle fiber of *Myoxocephalus scorpius*. Fig. 3 surface of the trophozoite (34,020); Fig. 4. cytoplasm of the trophozoite. (×34,020).

plasmodium which is overfilled with mature spores, the membrane of the muscle fiber can be disrupted and the spores get into the interfibrillar space. If the plasmodium is not so large as to press the surrounding tissue, the muscle fiber closely adjacent to the infected one appears to be normal (Fig. 7).

No histolysis of muscle tissue, which is considered to be a characteristic feature of multivalvulean parasitism (KOVALEVA and SCHULMAN 1978), was observed in the case of *Kudoa quadratum* both in living and in dead fish hosts.

Henneguya oviperda is a parasite of *Esox lucius* oocytes: as well as *Kudoa quadratum*, it is an intracellular parasite. The vegetative stage of *H. oviperda* is a large spherical plasmodium. As it was previously shown by SCHULMAN (1966), young plasmodia have microvilli at their surface. The surface of a mature plasmodium is smoother (Fig. 8) and comprises a single limiting membrane. Inward of this membrane, the same zones of cytoplasm as in other large myxosporidian plasmodia can be distinguished: the zone with pinocytotic channels; the zone rich in mitochondria; the zone with well developed rough endoplasmic reticulum, containing vegetative nuclei, generative cells, and sporogenesis stages. The central zone of the plasmodium is vacuolized and mature spores (two are formed in every pansporoblast) occur in large vacuoles.

Practically there is no oocyte in the infected fish egg; its place is occupied by the plasmodium, and only some yolk remains outside it. The egg envelopes (zona pellucida, follicular epithelium, basal membrane, thecal cells) are developed to a variable extent, depending on the time of penetration of the parasite into the egg. The parasite thus utilizes the resources which normally serve for oocyte nutrition. The fact that the plasmodium is feeding itself is proved by the pinocytotic activity of its surface.

Myxobolus disparoides was obtained from *Schizothorax intermedius* captured in Eastern Pamirs, river Mrugab (ASHUROVA 1973). The specimens investigated under the electron microscope were found in the terminal parts of primary gill filaments, where afferent vessels are connected with efferent ones. The end of the gill filament swells at the place where the parasite is localized, and normal positions of cells and capillaries of the secondary gill filaments are disturbed. Blood vessels are shifted peripherally and squeezed. The parasite is separated from the environment by only a thin layer of gill epithelium. From the proximal side of the filament, gill tissue cells, blood cells and fibrous material are close to the parasite body. Vegetative stages of this species are round-oval white plasmodia which are frequently rather large (2 to 5 mm). As in the above two species, the same zones of cytoplasm can be detected inside the plasmodium. This species is a diplosporoblastic one.

The surface ultrastructure of *M. disparoides* differs from that of the two species mentioned. Outside the unit membrane, the plasmodium has a supramembrane coat, $\sim$ 80 nm thick (Fig. 9), which is a layer of low electron density and uniform thickness, exactly repeating all curves of the plasmodium surface.

Into this electron-light layer, many electron dense oval plaques are immersed. The plaques are arranged into a regular pattern where every three of them, having different diameters and situated one under another at equal intervals, form small three-layer cones. The plaques are well seen in tangential sections (Fig. 10). In trans-

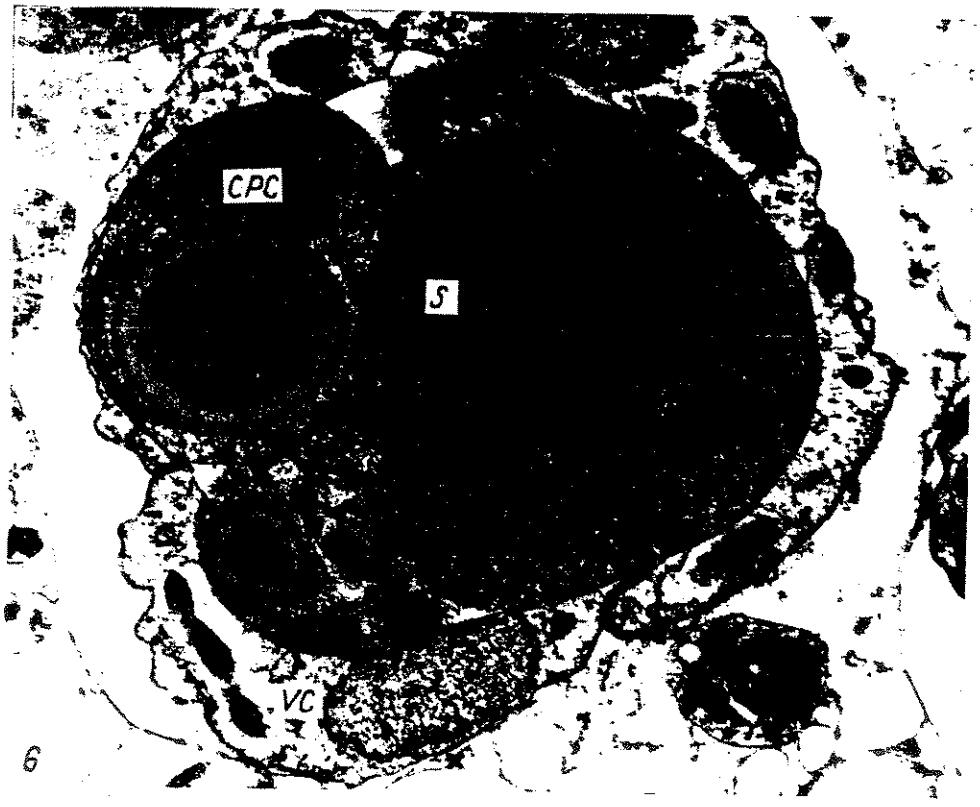


Fig. 5. Muscle protofibrils outside the trophozoite of *Kudoa quadratum*. ×25,110.

Fig. 6. Spore of *Kudoa quadratum* inside the vacuole. ×26,082.

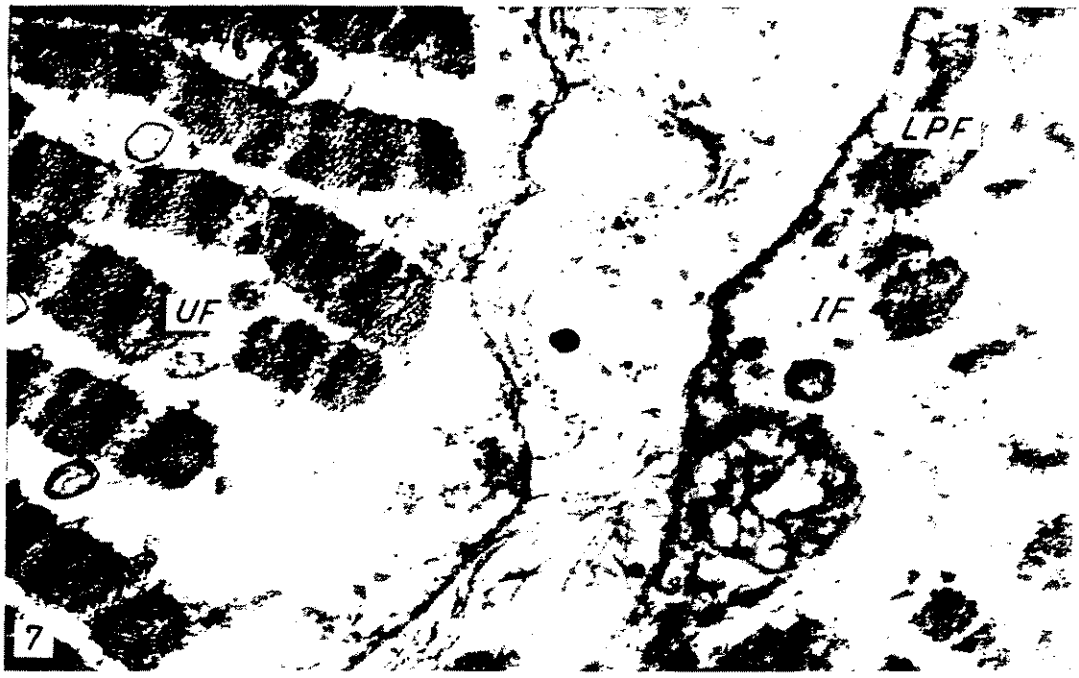
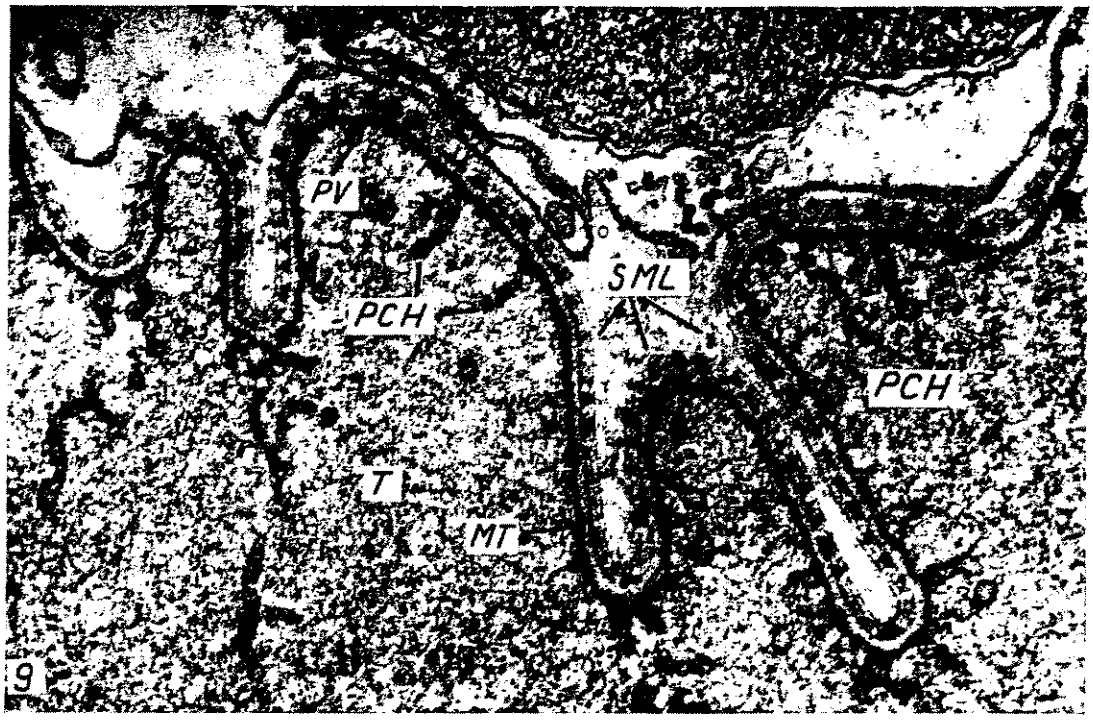


Fig. 7. Muscle fiber infected by *Kudoa quadratum* and the neighbouring uninfected one. ×25,110.

Fig. 8. Surface ultrastructure of *Henneguya oviperda* trophozoite. ×3,105.



Figs. 9, 10. Surface ultrastructure of *Myxobolus disparoides* trophozoite in transverse section (Fig. 9) and in tangential section (Fig. 10). ×36,000.

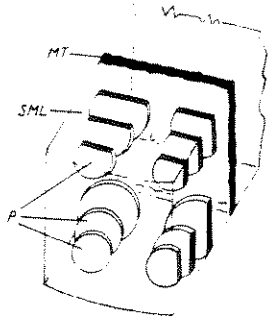


Fig. 11. Scheme of surface ultrastructure of *Myxobolus disparoides* trophozoite.

verse sections, the cones are seen as striped pyramids where dense lines alternate with light ones (Figs. 9. 10). A schematic picture of the supramembrane coat is given in Fig. 11. The coat seems to be elastic due to the alternation of electron-dense and electron-light parts.

Several suppositions as to the role of this coat can be made. On one hand, it can serve for protection of the plasmodium, making it rigid. This can be useful for species which are exposed to water pressure, being parasites of such a soft tissue as the branchial one (all the more that due to the activity of plasmodia the turgor of gill tissue is destroyed). On the other hand, it can play a certain part in nutrition. Undoubtedly it is permeable for nutrients and this is proved by the high pinocytotic activity of the plasmodial surface membrane. It may also play some role in osmoregulation. The surface coat may be compared with the glycocalyx of some amoebae, the more so that it is intensely stained with alcyan blue. The amoeba glycocalyx is supposed to regulate the ion flux (Page and Blakey 1979).

Coelozoic Myxosporidia. Among coelozoic Myxosporidia investigated, there are very large flat and oval plasmodia like those of *Myxidium gastreostei*, *M. perniciosum* and species of *Sphaeromyxa* from fish gall bladders. There are also smaller, elongated and branched plasmodia like those of *Myxidium lieberkühni* from the urinary bladder, and small amoeba-like plasmodia of *Chloromyxum truttae* from the gall bladder.

The surface of all these Myxosporidia is characterized by the presence of a more or less dense brush border. In the last two species, the microvilli are rather scarce. In *M. perniciosum* and *M. gasterostei* they are long and form a dense brush border resembling that in mammalian intestina (Fig. 12). In *Sphaeromyxa* species, the microvilli are branched and anastomose with each other, forming a more or less dense net. These are different ways to enlarge the absorbing surface of trophozoites (Grassé 1960; Uspenskaya 1966; Schulman 1966).

3. Mode of nutrition

The surface structure of myxosporidian vegetative stages is closely connected with the mode of their nutrition. All possible types of nutrition were observed in the species investigated. Small amoeba-like plasmodia of *Myxosoma cerebralis* phago-

cytize cartilage cells (USPENSKAYA 1979c) (Fig. 2). We also found phagocytosis in *Kudoa quadratum*. Vacuoles of the plasmodium cytoplasm can contain remnants of undigested muscle protofibrils (Fig. 4). The digestion takes place in this case inside the phagosomes and in food vacuoles by means of lysosomal enzymes.

Among the Myxosporidia investigated, extracellular digestion, corresponding to that in the intestinal lumen of higher animals, was also demonstrated. *Myxosoma cerebralis* is well known to perform enzymatic histolysis of the cartilage matrix (HOFER 1903; PLEHN 1924; HALLIDAY 1976; USPENSKAYA 1979c) (Fig. 1). It has been found that *Myxidium lieberkühni* dissolves the mucopolysaccharides of the intercellular spaces of pike urinary bladder epithelium by means of enzymes released by the "root" (USPENSKAYA 1966).

According to our data, *Kudoa quadratum* from the muscle fibers of *Myoxocephalus scorpius* enzymatically dissolves the surrounding protofibrils. We have shown by histochemical methods the existence of the contact or membrane digestion in Myxosporidia (USPENSKAYA 1966, 1979b), which is otherwise characteristic of the mammalian intestine (UGOLEV 1962, 1967, 1972). The membrane digestion is followed by active transport of nutrients. At both light and electron microscopic levels, the outer surface of plasmodium microvilli proved to contain acid and alcaline phosphatases (Figs. 13 to 15), enzymes which play the main part in membrane digestion (USPENSKAYA 1966, 1979b). During this type of digestion, splitting of macromolecules into monomers takes place in the brush border zone (UGOLEV 1962—1972). This zone is positively stained with alcian blue, which is characteristic of the glycocalyx of both higher animals and Protozoa. Acid phosphatase was found in *Myxidium lieberkühni* brush border (USPENSKAYA 1966). In floating plasmodia, this enzyme is revealed along their whole surface (Fig. 14), while attached forms have it only in the part of their body which is turned towards the urinary bladder lumen. The "roots" do not show any acid phosphatase activity (Fig. 15). The plasmodia whose cytoplasm is full of mature spores and which do not feed any more, have no phosphatase activity in their brush border, but the young buds of such plasmodia show high phosphatase activity. Thus, different parts of the plasmodium do not play one and the same role in nutrition processes during its life cycle (USPENSKAYA 1966). Alcaline phosphatase activity was demonstrated in the brush borders of *Myxidium gasterostei* and *Sphaeromyxa* species and on the surface of *Kudoa quadratum*.

The pinocytosis plays a great part in the utilization of food by Myxosporidia. In coelozoic Myxosporidia pinocytotic vacuoles are situated at the bases of the microvilli. In the intracellular parasite *Henneguya oviperda* (Fig. 8), the ectoplasm is penetrated by long pinocytotic channels, and pinocytotic vesicles pinch off from their proximal ends. Pinocytotic channels are well developed also in branchial parasites, such as *Henneguya lobosa* or *Myxobolus disparoides* (Fig. 9). The presence of a complex supramembrane coat in the latter species apparently does not prevent the penetration of nutrients to the cell membrane. It can be supposed that they pass through the electron light spaces of the supramembrane layer as through pores.

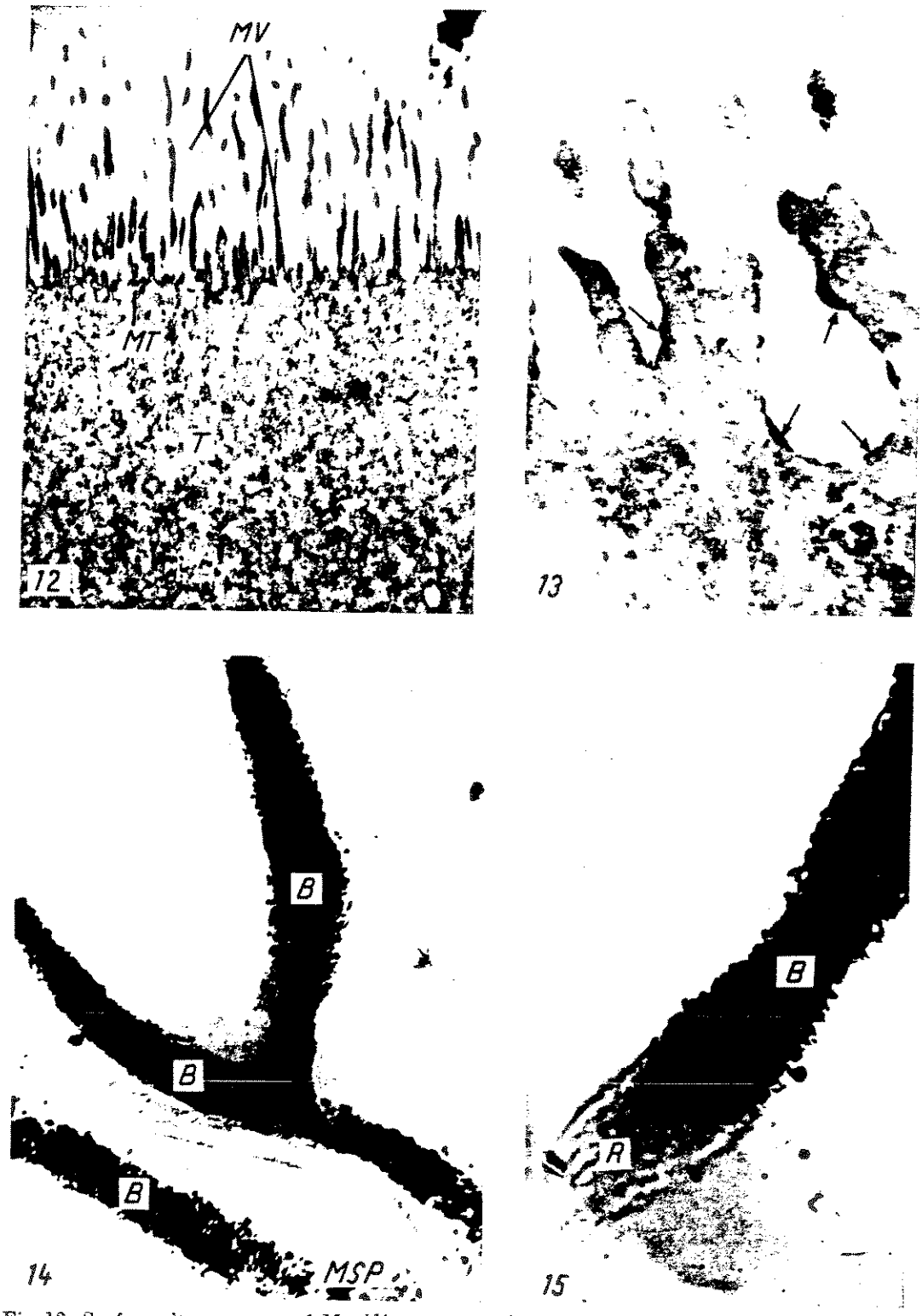


Fig. 12. Surface ultrastructure of *Myxidium gasterostei* trophozoite. ×18,000.
Fig. 13. Alkaline phosphatase at the outer surface of microvilli in *Sphaeromyxa* (arrows). ×54,000.
Figs. 14, 15. Distribution of acid phosphatase activity in the trophozoite of *Myxidium lieberkühni*. Light micrographs. ×540.

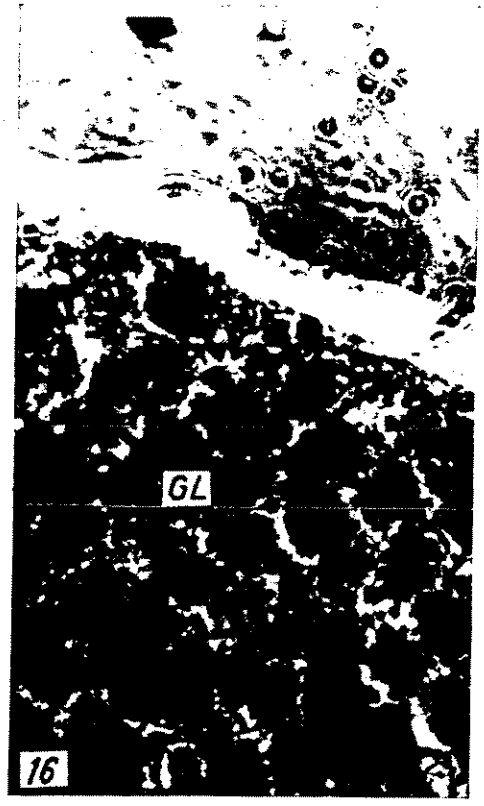


Fig. 16. Glycogen in the trophozoite of *Kudoa quadratum*. PAS reaction, light micrograph. ×1,000.

Fig. 17. Glycogen in the trophozoite of *Myxobolus disparoides*, obsreved only in the iodinophilous vacuoles (arrows). PAS reaction, light micrograph. ×750.

Fig. 18. Succinate dehydrogenase activity in *Myxidium lieberkühni* trophozoites. Light micrograph. ×800.

4. Metabolism

Cytochemical investigations were made with myxosporidian species enumerated above in "Materials and Methods" and living in different parts of the fish organism. Though these studies are far from being complete, they make it possible to distinguish between species with aerobic and anerobic types of metabolism. In field conditions we had no possibility to investigate their enzymatic activity. Only in some cases we managed to reveal succinate dehydrogenase activity indicating the existence of Krebs cycle in these animals (PEARSE 1968; STRAUB 1965; LEHNINGER 1972).

It has been shown for parasitic animals that one can judge about their type of metabolism by the amount of glycogen and neutral fat in their tissue or cells (BRAND 1952; MARKOV 1950; 1958, 1961; PALM 1967, 1968; GINETSINSKAYA 1968, and others). In oxybionts the amounts of these substances are much lower than in anoxybionts. We have tried to use this criterion to judge about the type of metabolism of Myxosporidia from different habitats.

Polysaccharides positive to PAS reaction, dissolved by saliva and α-amylase, poorly dissolved in cold water, and dissolved in hot water but slower than the glycogen of multicellular organisms, were observed in Maxosporidia. The Lugol mixture stains them reddish-brown. No biochemical study of this polysaccharide was carried out, but it seems to be a form of glycogen.

Using the PAS reaction and reactions for neutral fat (Sudan III, osmication after Champy + Na_2S treatment), we have shown that the cytoplasm of the small plasmodia of *Myxosoma cerebralis* contains relatively large amounts of glycogen and drops of neutral fat (which is apparently the so-called excretory fat, see BRAND 1952; PALM 1967; USPENSKAYA 1979c). Living in anaerobic conditions in the cartilaginous matrix they apparently utilize glycogen as the main source of energy. They obtain carbohydrates by lysis of the cartilaginous matrix rich in polysaccharides and by phagocyting cartilaginous cells. Thus, *Myxosoma cerebralis* can be regarded as an anaerobic organism (USPENSKAYA 1979c).

The anaerobic type of metabolism is also characteristic of *Kudoa quadratum* from muscle fibers of *Myoxocephalus scorpius*. The cytoplasm of their plasmodia is rich in glycogen (Fig. 16). Staining with Sudan III reveals numerous small drops of fat. Repeated attempts to reveal succinate dehydrogenase activity in *K. quadratum* were negative. This enzyme was not detected in plasmodia of *Myxobolus musculi*, parasitic in muscle tissue of *Abramis brama*. It seems that these myxosporidian species obtain their energy by glycolysis, using glycogen as the main source of energy. This is undoubtedly connected with the well-known peculiarity of their habitat, the muscle tissue, where almost anaerobic conditions arise when at work, and for which the main mode of energy production is anerobic transformation of phosphocreatine and carbohydrates, especially in the white muscle (STRAUB 1965; LEHNINGER 1972).

Unlike these three species, the parasites of gill filaments are aerobes. Succinate dehydrogenase activity is high in *Myxobolus disparoides* and *Henneguya lobosa* from the gill filaments of fish. These parasites are localized in the proximity of blood capillaries and separated from water by only a thin layer of gill epithelium. Accordingly

Meyers, T.U., J. Scala and E. Simmons. 1970. Modes of transmission of whirling disease of trout. Nature. 227: 622-623.

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Modes of Transmission of Whirling Disease of Trout

A REVIEW of the circumstances surrounding numerous whirling disease epizootics suggests that the pathogen (*Myxosoma cerebralis*) is most commonly transmitted through transplacement of infected fish[1]. Alternatively, it has been demonstrated that the spore phase of the parasite can be spread by currents throughout a water system. It is also widely accepted that equipment used in the propagation of infected trout is likely to become contaminated and the subsequent transplacement results in transmission of the disease.

Apart from these widely recognized modes of transmission, no other possibilities have been considered seriously[1]. But Schäperclaus[2] found myxosporidian spores in the faeces of kingfishers at an infected hatchery and believed that the disease could be spread in this manner. His observations are especially relevant in view of the recent appearance of the disease in northern Scotland[3]. He did not conduct further tests of this hypothesis and its validity remains in question. The ability to locate spores in the faeces and demonstrate their viability was imperative in our evaluation of the hypothesis.

For this purpose a spore isolation technique was developed. Isolation was accomplished by macerating the materials to be examined and subsequently passing them through a sintered glass filter with a porosity of 30–60 μm. This allowed the spore (10 μm) to pass through but cleared the macerate of much of the other material (broken cartilage and so on). The spores in the filtrate were further concentrated by centrifugation; they settled as the precipitate at low speeds (10,000*g*). We have used this technique to locate a few spores in large volumes of materials. As well as making possible faecal analysis, the spore isolation technique provides a more efficient means of diagnosing the disease in mildly infected fish (Fig. 1).

The great blue heron (*Ardea herodias*) was selected as the test avian species because of its predatory habits, the range of size of its fish prey, the volume of fish eaten per day, and its local and migratory range. Four great blue herons were collected and maintained under the authorization of state and federal scientific collecting permits.

Fig. 1. Spores of *Myxosoma cerebralis* in the precipitate phase of the spore isolation technique. Wet mount by phase contrast microscopy (×400).

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SHORT COMMUNICATION

Use of the plankton centrifuge to diagnose and monitor prevalence of myxobolid infections in fathead minnows, *Pimephales promelas* Rafinesque

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The plankton centrifuge technique (PCT) of O'Grodnick (1975) is one of two procedures accepted for diagnosis of salmonid whirling disease (*Myxobolus = Myxosoma cerebralis*) by the American Fisheries Society (McDaniel 1979). This technique requires minimal time and laboratory equipment compared to other spore detection methods (O'Grodnick 1975; Markiw & Wolf 1980). Reports of O'Grodnick (1975) and Halliday (1976) indicate spore morphology is not altered by plankton centrifugation and fresh spores may be harvested in large numbers from infected tissues. Virtually all uses of the PCT have been in the examination of salmonid hosts suspected, or known to harbour spores of *M. cerebralis*, yet the technique holds potential for use in population studies of other myxozoans in feral fishes.

It is difficult to assess accurately infection prevalence and intensity of myxozoans. Spores in microscopic cysts may often go undetected in wet mount surveys; diagnosis by histologic sectioning is time-consuming. Accordingly, we set out to field test the PCT as a rapid method for diagnosis and monitoring prevalence of several myxozoan species in a feral host population. We have compared the PCT with routine wet mount diagnosis and have attempted to devise a practical procedure involving the PCT for myxozoan population studies.

The fathead minnow, *Pimephales promelas* Rafinesque, was selected as a test host. Minnows were collected by seining from a small stream, Pillsbury Creek, draining cropland in Dickinson County, north-west Iowa, U.S.A. Adult minnows (standard length 4·5–5 cm) were collected during June–August 1981. The PCT used in this study was modified from the original protocol of O'Grodnick (1975) as follows: (1) fish tissue was blended in 100 ml of saline, (2) blended material was filtered without vacuum through six layers of cheesecloth supported in a porcelain filter funnel by a 5·5-cm diameter 0·75 mm mesh nylon screen. A single rinse (approximately 50 ml saline) was poured through the filter. Filtrate plus 50 ml of flask rinse was poured directly into the plankton centrifuge running at high speed. Centrifugation was considered complete with 3–5 ml of suspension remaining in the centrifuge cup. Material was removed from the sides of the cup by rubber spatula and added to the suspension. Myxozoans were diagnosed by microscopic examination of two drops of the centrifuge suspension. Total time con-

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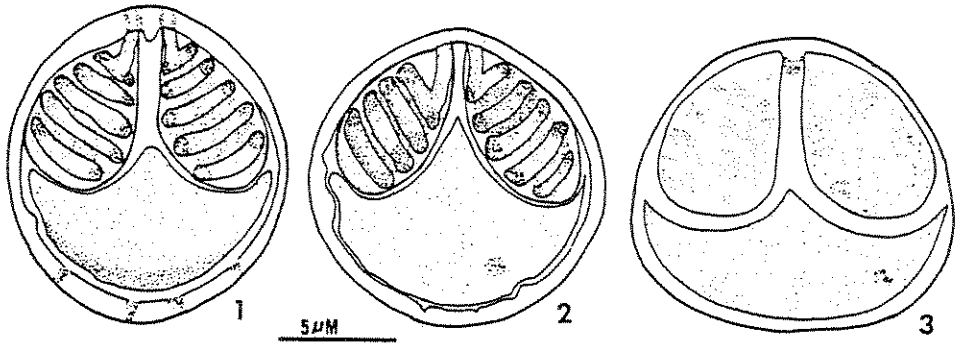

Figures 1–3. Free-hand drawings of spores of Myxobolidae from fathead minnows. *Myxobolus* sp. from brain and meninx primitiva.

spores are distinctly different (Guilford 1963; Bond 1938). Taxonomy, histopathology and ultrastructure of this unique myxobolid will be described in a future report.

Myxobolus sp. 2 occurred as minute cysts within and between skeletal muscle fibres. Cysts contained spores of three distinct shapes. Most numerous spores were ovoid in front view with maximum breadth anterior to the transverse midline (Fig. 4); ovoid spores similar in size and shape to those of *Myxobolus* sp. 1 (Fig. 1) were less common but regularly seen in muscle infections. Several ovoid spores showed a short (2–3 μm) caudal extension of the valves. The sporoplasm stained variously with Lugol's iodine; most spores showed a diffuse positive reaction; a few showed a distinct iodinophilous vacuole in the posterior third of the sporoplasm. Spore morphology and variability of *Myxobolus* sp. 2 closely resembled that of *M. muelleri* Bütschlii common in various tissues in many cyprinid hosts in Eurasia and North America (Mitchell 1970).

A third *Myxobolus* species produced small (up to 0·8 mm) white spherical cysts on gill filaments and large (up to 2 mm) white globose cysts in pharyngeal and buccal epithelium. Spores (Fig. 5) were relatively large (Table 1) and pyriform with elongate cnidocysts. A distinct iodinophilous vacuole was visible in fresh and Lugol's stained spores. Many gill-inhabiting *Myxobolus* species produce spores virtually identical to this pyriform type. *Myxobolus* sp. 3 may be conspecific with *M. angustus* Kudo infecting gills of the bullhead minnow, *Pimephales vigilax* (Baird & Girard), in the midwestern United States (Kudo 1934).

Spores of a fourth myxobolid, *Unicauda* sp, were seen only in plankton centrifuge harvests of minnow heads and whole minnows. Caudal processes of many spores were partially broken after plankton centrifugation. Spores (Fig. 6, Table 1) closely resembled those of *U. crassicauda* (Kudo) described from fins and integument of the stoneroller, *Campostoma anomalum* (Rafinesque), from Illinois, U.S.A. (Kudo 1934). Minchew (1981) described a similar species, *U. magna*, from fin tissues of two fathead minnows in Pennsylvania.

After spore types were determined, prevalence estimates of the three *Myxobolus* forms in fathead minnows were made by microscopic survey and by the PCT. In this

by spheroid and ellipsoid spores, Figs 2 & 3) appeared in harvests of all 10 minnows. Spores of *Unicauda* sp. and *Myxobolus* sp. 3 were found in five and nine of these fish respectively. The higher prevalences indicated for the brain and gill *Myxobolus* after whole-fish processing may reflect occurrence of these myxobolids in other tissues. In the microscopic survey *Myxobolus* sp. 3 was found in pharyngeal and buccal epithelium as well as in gills. Bond (1938) found *M. subtecalis* in connective tissue throughout the viscera and in the fins in *F. heteroclitus*, although its main site of infection is the brain. The similarity among some spores of the brain and muscle myxobolids in the fathead minnow made diagnosis of these two forms difficult in whole-minnow harvests. Without detailed microscopic study of spore shape and variability these two myxobolids could not have been separately diagnosed in the whole-fish samples. The marked similarity in some spores seen in the two sites provides doubt that they are separate species.

This study suggests the PCT is applicable to studies of population dynamics of histozoic myxobolid infections in feral fish. The technique may be used initially to determine general infection levels and variety of spore types in a host population. The PCT seems more sensitive and less time-consuming than the routine microscopic survey. Whole organs or entire fish may be processed and examined rather than small samples. After infections are found by the PCT, spore and cyst characteristics and sites of infection may be ascertained by systematic microscopic survey. Prevalence may then be monitored by the PCT. Although we have made no attempt to monitor intensity of these myxobolid infections, from studies with *M. cerebralis* (O'Grodnick 1975, 1979) it seems the PCT could also provide valid estimates of this population parameter.

Acknowledgments

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carbohydrates do not play a large part in their metabolism. The glycogen is almost absent from the cytoplasm of their plasmodia. It is revealed only in the iodinophilous vacuoles of mature spores which lie in the central part of the plasmodium (Fig. 17).

As to the coelozoic Myxosporidia, the picture is less clear. *Myxidium lieberkühni*, a parasite of the urinary bladder of pike, contains rather a large amount of glycogen in its cytoplasm, but at the same time displays a rather high succinate dehydrogenase activity (Fig. 18). Two explanations of this apparent contradiction are conceivable. It is possible that peculiarities of water-salt metabolism of freshwater fish create suitable conditions for a truly aerobic metabolism of the parasites of its urinary bladder. In fact, freshwater fish are hypertonic with respect to the external environment. To resist the effect of outside water entering through the gills, they have to eject constantly an enormous amount of water by means of cellular activity of the kidneys and urinary bladder (GINETSINSKY 1964; STRAUB 1965). On the other hand, the succinate dehydrogenase of the parasite may have another significance. A plentiful secretion of hypotonic urine is characteristic of their hosts. In pike, the urine is highly hypotonic and is almost lacking sodium. It is well known that cells of the organs connected with the active transport of sodium ions possess high succinate dehydrogenase activity. In freshwater fish, this function is performed by the excretory cells of the gills and by the cells of kidney distal channels, which accordingly show a high activity of this enzyme (GINETSINSKY 1964). It is possible that in *Myxidium lieberkühni*, whose plasmodia are surrounded by hypotonic urine, the high succinate dehydrogenase activity is also connected with the transport of sodium ions.

The inhabitants of the gall bladder (*Myxidium gasterostei* from stickleback and *Sphaeromyxa* species from marine fish) contain a significant amount of glycogen and small discrete drops of neutral fat. As to the succinate dehydrogenase activity, monoformazan was produced during the reaction in *Myxidium gasterostei* (USPENSKAYA 1969), while in *M. incurvatum* no activity was observed.

Thus, according to these incomplete data, Myxosporidia are neither obligate aerobes nor obligate anaerobes. Moreover, there are some data showing that one and the same myxosporidian species can live in aerobic as well as in anaerobic conditions. And also different life cycle stages of Myxosporidia — plasmodia in fish organs and spores in water — can have different types of metabolism.

The cytoplasm of coelozoic Myxosporidia as well as that of histozoic ones is rich in RNA (being stained with methyl green-pyronin, or by gallocyanin-chromalum, with ribonuclease control). The reactions for RNA are especially intensive at the beginning of sporogenesis, which is apparently connected with active protein synthesis.

The cytoplasm of generative cells and pansporoblasts is stained more intensively with mercuric bromphenol blue for the total protein than the rest of the plasmodium. The spore valves, polar capsules and polar filaments are also of protein nature. The cytoplasm of the sporoplasm cell (Fig. 19) contains glycogen either in granular form or in the so-called iodinophilous vacuole; in some species the cytoplasm is pyroninophilic due to RNA and also contains proteins and drops of fat.

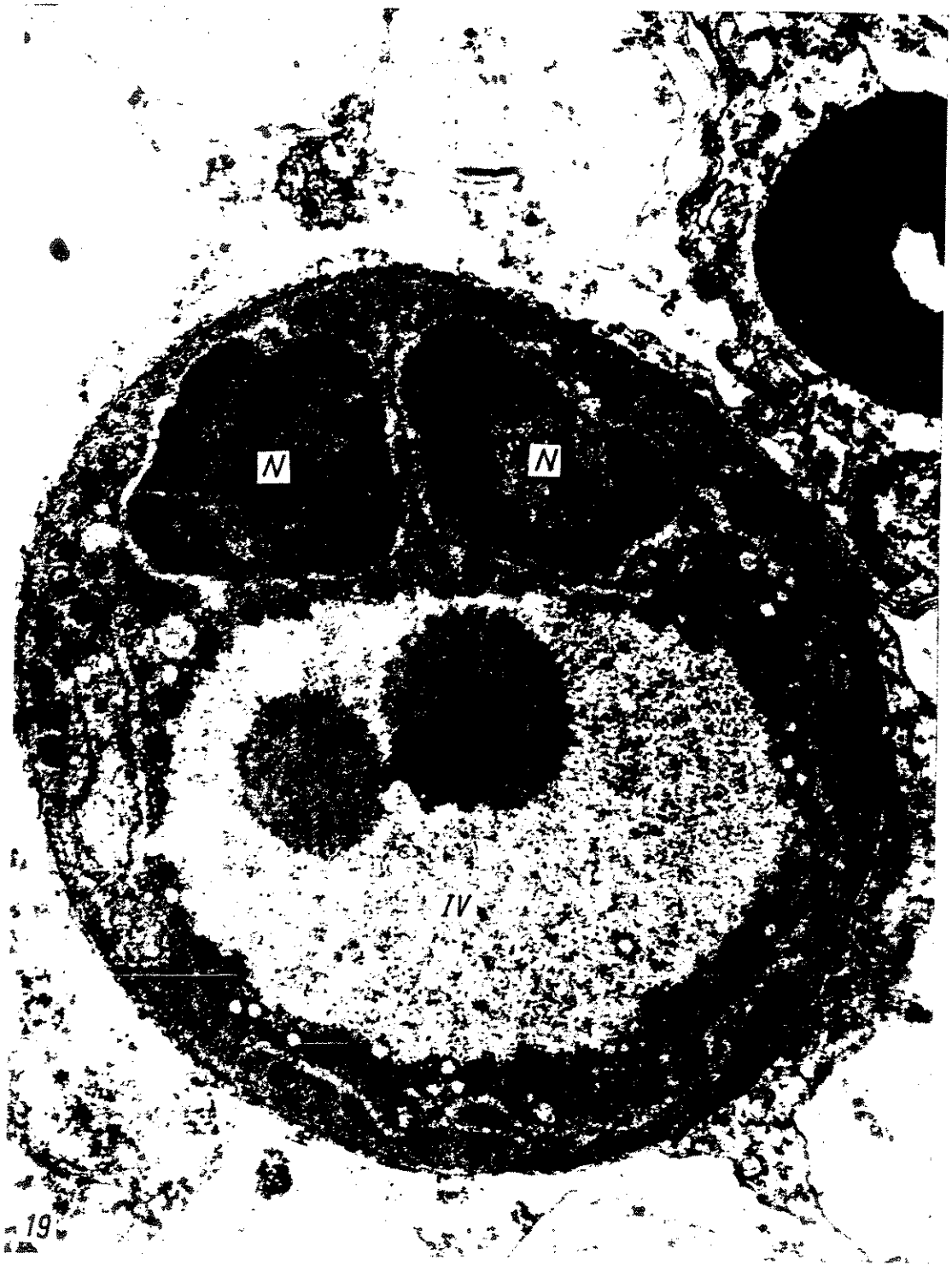

Fig. 19. Ultrastructure of *Myxobolus disparoides* sporoplasm, showing iodinophilous vacuole devoid of membrane. Two nuclei at top. ×22,590.

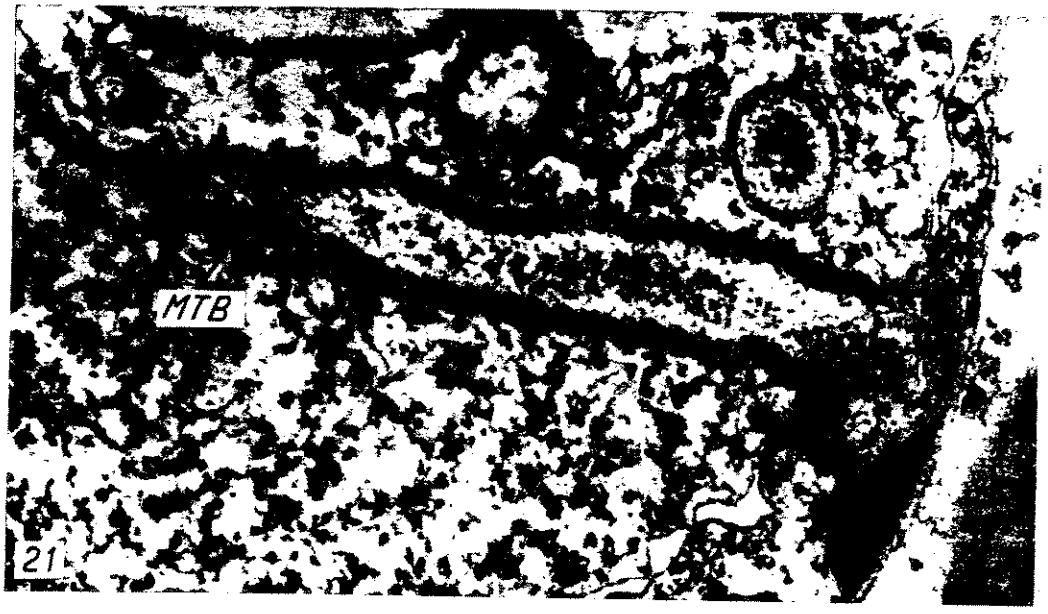

Fig. 20. Fine structure of polar filament wall in *Myxobolus disparoides*. ×90,000.

Fig. 21. Fine structure of external tube wall in *Myxobolus disparoides*. ×27,000.

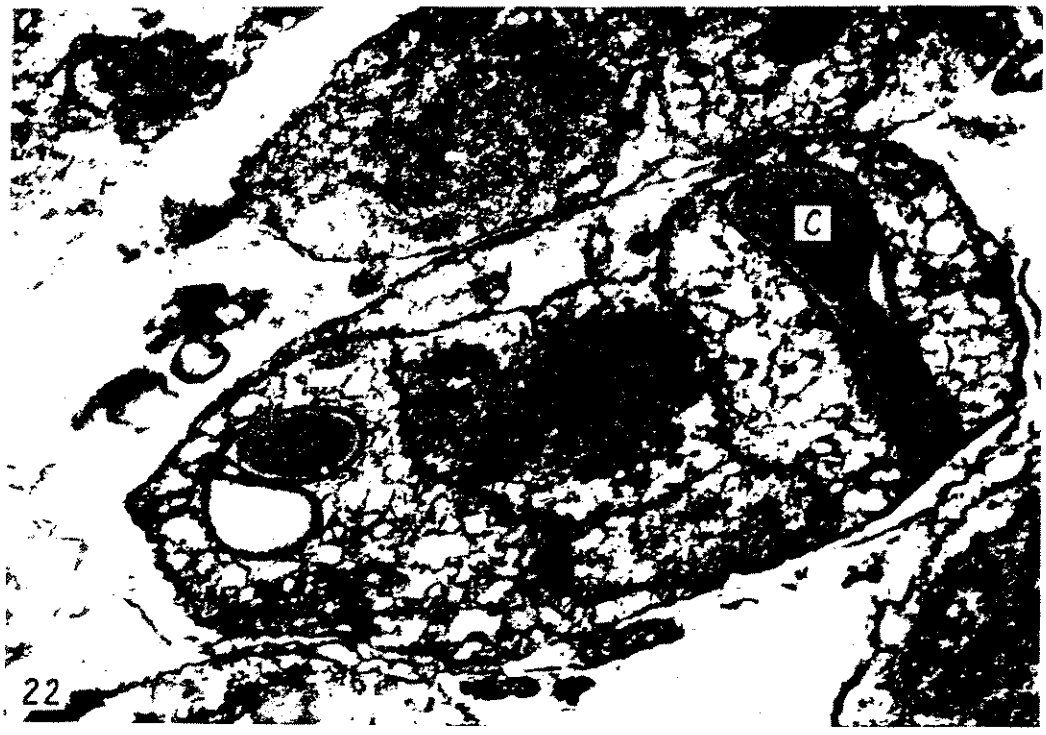

Fig. 22. Central core formed inside the external tube of *Myxidium gasterostei* polar apparatus. ×16,500.

Fig. 23. Apparent expulsion of polar body from a dividing sporoblast cell in *Myxidium gasterostei*. ×18,000.

Previously we found that spores of *Myxosoma cerebralis* had to be aging in water rather long (about 4 months) to become invasive (USPENSKAYA 1955, 1978b). Later these data were confirmed by HOFFMAN and PUTZ (1969) and HALLIDAY (1976). We investigated cytochemically the spores of *Myxosoma cerebralis* aging in water for 4 months and revealed no degenerative changes either in the cytoplasm or in the nuclear apparatus of the sporoplasm. During this period, some processes apparently take place in the polar capsule apparatus, which makes it ready for the discharge to occur.

5. Mechanism of polar capsule discharge

The investigation of polar capsule discharge mechanism using glycerinated spores shows that it is an active process connected with contractile systems.

The relaxation (discharge) of the polar filament takes place when calcium ions are withdrawn from the glycerinated spore model. Thus, 100% discharge occurs in $2 \cdot 10^{-3}$ M EGTA solution. The presence of ATP does not influence this process. ATP activity was observed inside the polar capsule. The energization of the system seems to take place during the development of polar capsule apparatus. The active component of the system is apparently composed of microtubules (Figs. 20, 21) which were found by many authors in the external tube and filament wall. Long microtubules are spiralling around the external tube (Fig. 21). The walls themselves may be contractile also. Some part in the inversion of the tube and discharge of the filament may be played by the central core (Fig. 22) of the external tube which may be contractile and similar to the spasmonema of the stalk in Vorticella. During the process of external tube inversion the pressure may increase inside the capsule and tension may arise in the filament as in a clock spring (USPENSKAYA 1976a, 1977; CARRÉ and CARRÉ 1980), but the destruction of the cork alone does not lead to the discharge, which depends on Ca^{++} withdrawal and relaxation of the contractile system as a result (USPENSKAYA 1976a, 1977).

Discussion

Many papers based on light microscopical studies are devoted to the nuclear cycle of Myxosporidia. However they are all based on morphological observations only. SCHULMAN (1966) gives a thorough review of these papers in his monograph. Up to now only we have carried out cytophotometric investigations of the nuclear ploidy at different stages of the myxosporidian life cycle. Our data permitted us to set forward a new concept of the nuclear and life cycles of Myxosporidia (USPENSKAYA 1975, 1976b, 1978a, 1979a, 1979b, 1981) and to discuss some recent works concerning the sporogenesis which were carried out at both light and electron microscopical levels. These works contain only few data on myxosporidian ploidy. SIAU (1979) who observed synaptinemal complexes in uninucleate cells of *Myxobolus exiguus* thinks that the sporoplasm and the schizozoite of Myxosporidia are diploid while all other stages are haploid. However, this point of view contradicts our data, according to which the

parasitic phase of Myxosporidia is diploid and the phase of dispersion (i.e., the spore with all its cells) is haploid. But as SIAU himself is not sure in the exact identificaiton of the stage with synaptinemal complexes, and as his paper is lacking the description of the material from which this stage was obtained, it is now difficult to evaluate his data and to compare them with ours.

The new evidence that the life cycle of Myxosporidia is heterophasic, i.e. includes alternation of haplophase and diplophase (USPENSKAYA 1975, 1976b, 1979a, 1981), gives possibility to speculate on phylogenesis and relationships of the Myxosporidia with other animal groups. Using our concept of the life cycle of Myxosporidia, SCHULMAN and PODLIPAEV (1980) tried to compare it with that of Actinomyxidia.

According to our data, meiosis in Myxosporidia holds an intermediate position in the life cycle, being neither zygotic nor gametic. This is characteristic of the cycles with alternation of haplo- and diplophase. Data on the morphology of myxosporidian meiosis, previously obtained with the light microscope, are contraversial and inexact. NOBLE (1944) reported that during reductional division before sporoplasm nuclei formation the chromosomes were distributed into two groups without fission, and so each nucleus got one half of the chromosomes. According to GEORGEVITSCH (1923, 1937), during meiosis each nucleus gets a diploid number of chromosomes (as during mitosis), but then only half of them is enveloped by the nuclear membrane, and the other half is resorbed in the cytoplasm. In more recent publications (SCHULMAN 1972; SCHULMAN and SEMENOVITCH 1973) the myxosporidian meiosis was claimed to be one-step and that was used as a proof for the primitivity of Myxosporidia.

Our cytophotometric data do not give us the possibility to decide whether meiosis includes one or two nuclear divisions. The nuclear DNA distribution curves of three successive generations of pansporoblast nuclei of *Sphaeromyxa elegini* (2, 4, and 8 nuclei inside the pansporoblast) are identical and each of them has two peaks (Fig. 24). We suppose that this bimodality is due to the existence of both pre- and postsynthetic nuclei in each of these groups. Thus the amount of DNA at these stages would correspond to 2c and 4c levels. At the next stage, only the 1c level is present, and it is not clear enough how a single nuclear division could result in the decrease of the DNA content not only from 2c to 1c, but also from 4c to 1c (Fig. 24). Such a decrease could be explained, for instance, by the eliminatinon of some chromosomes, as reported by GEORGEVITSCH (1923, 1937), or by the expulsion of a polar body, as described by JANISZEWSKA (1957) in Actinomyxidia. According to JANISZEWSKA, the polar body is pushed out of the cell at the end of a spindle. Once we had a chance to observe under the electron microscope a pattern closely resembling the expulsion of a polar body at the end of a telophase spindle (Fig. 23). In our previous paper (USPENSKAYA 1981) we interpret this electron micrograph as a way of formation of residual pansporoblast nucleus, but it can be considered as a polar body expulsion as well. This could mean that there is one more nuclear division in the pansporoblast which is heteropolar (with one nucleus degenerating) and which was heretofore overlooked. Together with such a cryptic division, the meiosis in Myxosporidia can well be a two-step process.

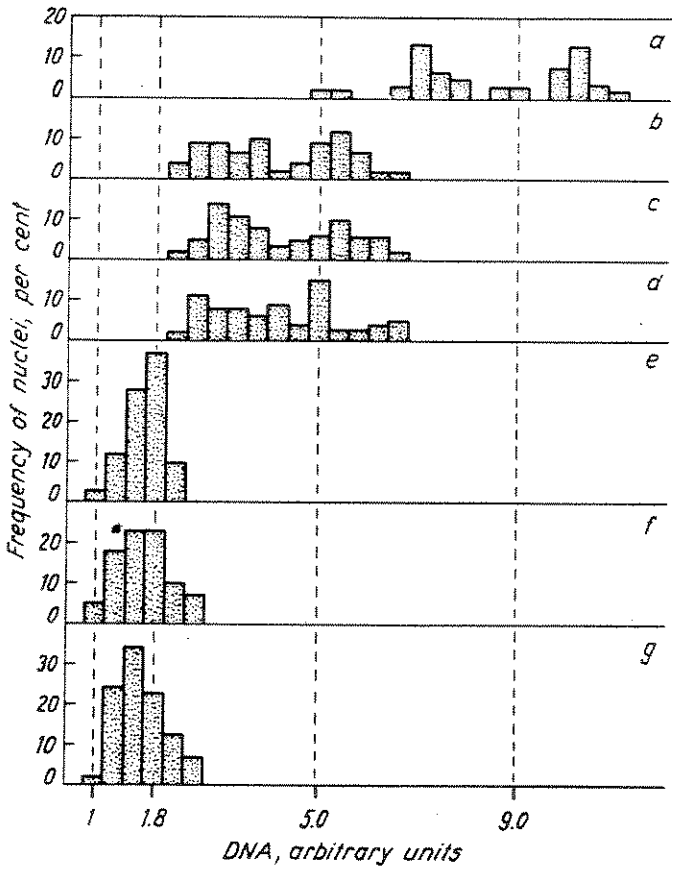

Fig. 24. Frequency distribution of DNA values of nuclei of different stages of *Sphaeromyxa elegini*: a — vegetative nuclei in trophozoite cytoplasm, b—d — sporoblast nuclei (b — two, c — four, d — eight nuclei inside the pansporoblast), e — sporoplasm nuclei, f — capsulogenic nuclei, g — valvogenic nuclei.

No other micrographs of meiosis have so far been obtained with the electron microscope. Thorough investigations of this part of the life cycle are necessary. At present we have no sufficient data to either deny or confirm the existence of one-step meiosis in Myxosporidia, which was supposed to exist in the works based on light microscopic observations and chromosome counts (Georgevitsch 1937; Schulman 1972; Schulman and Semenovitch 1973).

The variability of ultrastructure on the myxosporidian surface which we have observed adds to the data already available. Many authors pointed out the difference in surface ultrastructure between coelozoic and histozoic species. It is usually considered that the former typically have microvilli while the latter display pinocytotic activities (Lom 1969; Current et al. 1979). However, Current et al. (1979) have shown that different surface structures can exist in specimens of one and the same species inhabiting different parts of gill filaments. They considered this difference to be connected with the degree of pathogenicity of the respective forms.

The vegetative stages of histozoic Myxosporidia were formerly taken for "cysts" until it was established, at the light microscopic level, that most of them were large plasmodia. The capsule, if it exists around the plasmodium, is a derivative of the host tissue and has nothing in common with a cyst. There was an opinion that all histozoic Myxosporidia have one unit membrane at the surface (Schulman 1966; Ginetsinskaya and Dobrovolsky 1978). Controversial data on this point have been obtained by electron microscopy: in some cases, only one unit membrane was observed (Lom and Pytorac 1965a, 1965b; Schubert 1968; Current 1979), in other cases, two membrane-like layers were described (Desser and Paterson 1978). Further variants are one membrane covered by a granular coat (Current and Janovy 1978; Current et al. 1979), two membranes in some cases covered by a granular layer (Lom and Puytorac 1965a; Current and Janovy 1976), and even a syncytial wall bordered by two membranes (Desser and Paterson 1978). Our data on the surface ultrastructure of vegetative stages of *Kudoa quadratum*, *Henneguya oviperda* and *Myxobolus disparoides* clearly show that they are not cysts but large plasmodia bordered by a single membrane which either lacks additional layers *(Kudoa quadratum, Henneguya oviperda)* or has a complex outer supramembrane layer *(Myxobolus disparoides)*.

It is clear from our data concerning *Kudoa quadratum* that the existence of small plasmodia is not a characteristic feature of this genus, and that the existence of small trophozoites with less than 8 sporoblasts is not a characteristic feature of the order Multivalvulea, though considered as such by Schulman (1978); Kovaleva and Schulman (1978).

There is not much data concerning cytochemistry of Myxosporidia. Glycogen and fat were observed in the plasmodium cytoplasm as early as in 1932 (Petruschewsky 1932). Podlipaev (1972) described fat in myxosporidian cysts. Chaudhuri and Chakravarty (1970) pointed out the presence of RNA in the sporoplasm cytoplasm of *Myxobolus*. Many investigators have described a polysaccharide related to glycogen in the iodinophilous vacuole of the sporoplasm. This was revealed by the PAS reaction, by Best's carmine, and by the Lugol mixture (Kudo 1918; Petruschewsky 1932; Bond 1940; Walliker 1968; Galinsky and Meglitsch 1970; Podlipaev and Schulman 1978). With the electron microscope, the polysaccharide of the iodinophilous vacuole was identified as β-glycogen (Desser and Paterson 1978). Schubert (1968); Desser and Paterson (1978) as well as we did not find any membrane around this glycogen cluster.

Up to now there has not been any data concerning the type of metabolism in Myxosporidia. Only a few papers (Galinsky and Meglitsch 1970; Podlipaev 1974; Podlipaev and Schulman 1978) mentioned a decrease of the polysaccharide content of the iodinophilous vacuole when spores were outside the host. This vacuole is generally regarded as a nutrient stock, and the decrease of the glycogen amount inside it, as a result of the metabolism of sporoplasm.

Various types of nutrition in Myxosporidia were mentioned in the papers describing phagocytosis (Guyenot and Naville 1922; Greven 1956), outside enzymatic diges-

tion (HOFER 1903; PLEHN 1924; USPENSKAYA 1957), and membrane digestion (USPENSKAYA 1966). SCHULMAN (1966) summarizes these data in his monograph.

GRASSÉ (1960) supposed that microvilli on the surface of myxosporidian trophozoites play a part in osmotrophic nutrition. After the theory of contact digestion was formulated (UGOLEV 1962). SCHULMAN (1964) suggested this type of nutrition for Myxosporidia, basing on microvilli existence. But the presence of microvilli cannot be considered sufficient to prove the contact digestion. To support the existence of membrane digestion in Myxosporidia, we have cytochemically demonstrated phosphatase activity on the outer surface of microvilli membrane (USPENSKAYA 1966, 1979b). SCHULMAN (1964, 1966) considers membrane digestion to be the main type of nutrition in Myxosporidia and thinks that it played a significant role in the evolution of myxosporidian vegetative stages.

Our data on Myxosporidia from different habitats in the fish organism show that their vegetative stages may possess all possible types of nutrition which occur in various combinations in different species. The mode of nutrition is closely connected with the surface ultrastructure of the trophozoites and with the shape and dimensions of their body.

The data on the mechanism of polar filament discharge are not abundant. They have been summed up elsewhere (USPENSKAYA 1977). There is a lot of data on the influence of different chemicals on the discharge. LOM (1964) compared his observations on the discharge mechanism in Myxosporidia with those of YANAGITA (1973) concerning the discharge of the nematocysts in Coelenterata. He supposes that there is some stopper-mechanism in the nematocysts which prevents the release of pressure or tension existing inside the nematocyst capsule. LOM identified the "cork" of the myxosporidian polar capsule with the stopper mechanism which is composed, according to his view, of globular protein. The destruction of the "cork" leads to some changes inside the capsule and the discharge occurs as a result. He rejects the role of osmosis in the discharge process in Myxosporidia, contrary to Microsporidia of which the osmotic mechanism is characteristic. Our data speak in favour of a leading role of contractile proteins in the discharge mechanism of Myxosporidia and provide a new approach to the future investigations of the event.

Morpho-physiological characteristic of the Myxosporidia

Summing up, we would like to give a brief morpho-physiological characteristic of the Myxosporidia based on previous ones (GRASSÉ 1960; SCHULMAN 1966; RAIKOV 1967, 1978; LOM 1973; GRASSÉ et LAVETTE 1978) but taking into account the new cytophotometric, cytochemical and electron-microscopic data.

Myxosporidia is a group of parasitic animals. They are chiefly parasites of Teleostei, sometimes of other fish and poikilothermal water vertebrates. They may be coelozoic, histozoic or intracellular parasites. Many of them are agents of serious fish diseases.

The Myxosporidia possess both features relating them to Protozoa and features placing them at the supercellular and even multicellulsr level. They have a complex life cycle with the alternation of diplo- and haplophase, and with the intermediate

meiosis. The exact type of meiosis characteristic of Myxosporidia is unknown. According to light microscopy, mitosis is intranuclear and acentric.

The myxosporidian diplophase is represented by the parasitic trophozoite in form of multinucleate polyenergidic plasmodia which can vary in shape and size. The cytoplasm of plasmodia (and that of cells of other stages of development) contains typical cell organelles: microtubuli, microfilaments, smooth and rough endoplasmic reticulum, mitochondria, Golgi bodies (dictyosomes), vacuoles, free ribosomes, as well as various inclusions such as granules or clusters of glycogen, drops of fat and so on. The plasmodia have one unit membrane with or without additional supramembrane layers of varying structure. The membrane can form microvilli to increase the surface area and/or to form pinocytotic channels or vesicles. Sometimes the plasmodium becomes surrounded by a capsule which is a derivative of the host tissue. Plasmodia themselves do not form cysts in the exact meaning of the term.

The Myxosporidia are neither obligate aerobes nor obligate anaerobes. All known types of nutrition occur in Myxosporidia (phagocytosis followed by intracellular digestion inside food vacuoles; extracellular digestion by means of secreted enzymes or membrane digestion followed by pinocytosis or active transport of nutrients). These types can be combined in different ways depending on the localization of the given species in fish organism. The type of nutrition is closely connected with the surface ultrastructure, shape, and size of the plasmodium.

Polyenergidic plasmodia multiply asexually (by budding or plasmotomy). They represent the reproduction or agglomeration phase. The plasmodia display nuclear differentiation. The vegetative nuclei are in common cytoplasm (as in other heterokaryotic Protozoa), but the generative nuclei are separated into peculiar generative cells which are analogous to the sexual cells of multicellular organisms.

The vegetative nuclei of large plasmodia are polyploid. Both polyploidisation and multiplication of these nuclei take place inside the plasmodia. During sporogenesis, not only generative nuclei but also the respective cells divide. At the beginning of sporogenesis, the generative nuclei divide mitotically and later meiosis takes place and haploid multicellular spores arise. At the end of sporogenesis, the differentiation of the somatic spore cells into capsulogenic and valvogenic ones occurs, which is similar to the somatic differentiation in Metazoa. This is followed by morphogenesis in both types of cells (formation of a complex polar apparatus and of valves, respectively).

The haplophase in Myxosporidia is represented by the multicellular spore composed of both somatic elements (cells producing valves and polar capsules) and sexual elements (sporoplasm cells). There may be one or two sporoplasm cells (in the former case it is binucleate, with two haploid nuclei), from 2 to 6 valves and from 1 to 6 polar capsules in each spore.

The spores serve for the distribution of the parasite; this is the resting phase of the life cycle and also the phase of dispersion. Typical of the spore is the existence of a polar apparatus, consisting of polar capsules, with a polar filament coiled inside each capsule. The polar capsule is derived from the Golgi apparatus. The development

of polar capsules and polar filaments is very similar to that of coelenterate nematocysts, but unlike the nematoblast the capsulogenic cell degenerates at the end of the capsule formation. There is a contractile system at the base of the discharge mechanism of polar capsules; this system is operated through the removal of Ca^{++} from the system.

A primitive sexual process (autogamy or pedogamy) takes place at the moment when the sporoplasm is leaving the spore engulfed by a fish. This consists in fusion of two haploid sporoplasm nuclei, thus restoring the diploidy.

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Myxosoma cerebralis: *In Vitro* Sporulation of the Myxosporidan of Salmonid Whirling Disease

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SYNOPSIS. Trophozoites and other pre-spore stages of the myxosporidan *Myxosoma cerebralis* were taken from infected rainbow trout (*Salmo gairdneri*) and cultured *in vitro*. Cultures eventually yielded mature spores capable of discharging their polar capsules. This is the first report of culture of a myxosporidan.

Index Key Words: *Myxosoma cerebralis*; salmonid whirling disease; *in vitro* culture.

MYXOSOMA cerebralis (Hofer) is a histozoic myxosporidan found in cartilage of salmonid fishes. Light infections are often subclinical, while moderate involvement results in extensive lysis of cranial or vertebral cartilage, and leads to neurologic impairment producing the typical "black tail" symptom (Fig. 1). If severe damage occurs, it is followed by abnormal ossification, which in turn disfigures and cripples the fish and produces clinical "whirling disease." Severe infections kill the host.

Whirling disease is recognized internationally as one of 6 major communicable diseases in salmonid fish husbandry (1). There are reviews on the disease and known portions of the life cycle of the parasite (1, 2, 4), attempted control measures (2), biologic properties of spores (3), and biophysical procedures for releasing and concentrating spores (5, 6).

Our purpose was to develop a method for *in vitro* maturation of *M. cerebralis* spores from pre-spore stages. Such a method will be useful in testing drugs and for investigating the physiology, metabolism, transmission, and life cycle of the parasite.

MATERIALS AND METHODS

Trophozoites were obtained from cartilage of naturally or experimentally infected rainbow trout (*Salmo gairdneri* Richardson). Experimental infections were produced at 12.5 C by the method of Hoffman & Putz (3). When naturally infected tissue was used, the absence of spores in the test tissues was ascertained both by histologic examination and by the detection procedures of Markiw & Wolf (5). Experimentally infected fish were kept at 12.5 C and used at 70 to 87 days postexposure. At that temperature spore formation usually requires ~ 100 days.

To minimize tissue contamination by feces and regurgitated food, we withheld food from donor fish for 24 hr before use. Fish were anesthetized and gill arches were excised, rinsed twice in sterile water, and placed in sterile Hanks' balanced salt solution (BSS). Gill filaments were then removed, and cartilage was minced. Cartilage fragments were transferred to phosphate-buffered saline containing 500 IU polymyxin B, 40 IU

TABLE 1. *Summary of attempts to induce in vitro sporulation of trophozoites and other pre-spore stages of* Myxosoma cerebralis.

Trial No.	Duration of infection in host (days)	Type of culture	Incubation temperature (°C)	Number of cultures	Duration of incubation (months)	Spores
1	Unknown (natural infection)	Explants of cartilage in MEM-10*	15	4‡	18	Present at 8 months (when first examined) and still present at 18 months
2	39	"	15	18 18§	3.5	Failed to develop
3	68	"	15	20	6.5	Present at 81 days
4	70	"	15 20	11 11	6.5	Present at both temperatures at 79 days; one pansporoblast at 15 C
5	86	Tissue-free suspension in MEM-10 + FCE†	15 20	2 2	6	Present at 21 days at both temperatures in all cultures
			15 20	4 4	6	
6	87	MEM-10 + FCE	15	8	6	Present at 32 days in all cultures, one pansporoblast
"	70	Tissue-free suspension in plasma clot + FCE	15	9	3	Present at 39 days

* Eagle's minimal essential medium.
† Fish cartilage extract, 10%.
‡ Except as indicated, the antibiotics were polymyxin B, 500 IU/ml; zinc bacitracin, 40 IU/ml; and neomycin 500 μg/ml.
§ Gentamicin, 500 μg/ml.

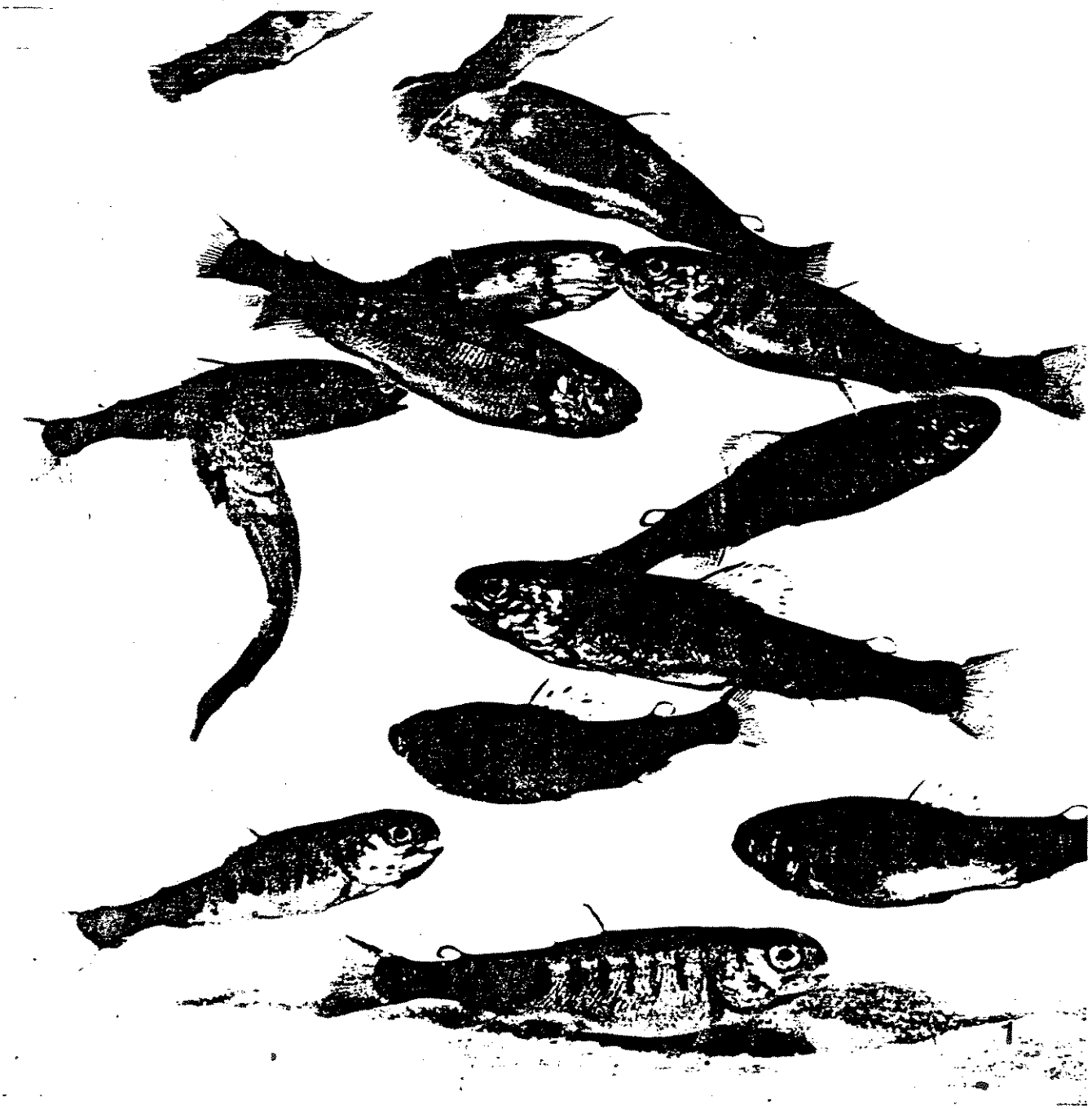
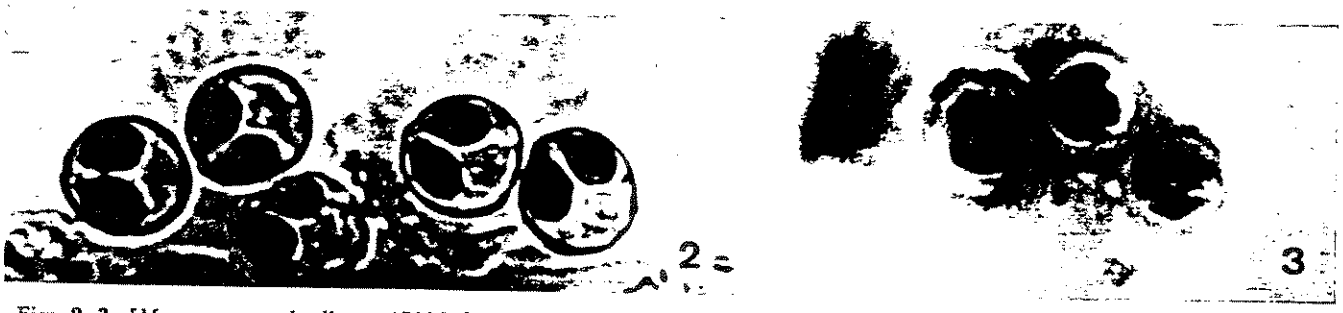


Fig. 1. Rainbow trout fingerlings infected with *M. cerebralis*. Note the "black tail" symptom due to impairment of melanophore control in infected fish. × 1.5.

zinc bacitracin, and 500 μg neomycin/ml; held at 4 C for 2-3 hr, and then washed 3 times with cold sterile BSS.

Samples of cartilage were placed in chicken plasma clots on coverglasses and immersed in Eagle's minimal essential culture medium (MEM), Earle's BSS containing 10% fetal bovine serum (MEM-10). Other samples were finely minced, suspended in BSS, and passed through glass wool to remove the larger particles. The resulting filtrate was centrifuged at 1200 *g* for 10 min at 4 C, and the pellets of trophozoites and pre-spore stages were gently resuspended in distilled water for 1-2 min to lyse erythrocytes. Hanks' BSS was added, the suspension was re-centrifuged, and parasite cells were placed directly in tube cultures with MEM for incubation at 15 and 20 C for 1.5 to 6 months.

We prepared cartilage extract from healthy rainbow trout, after heating the fish in water at 45 to 50 C for 5 min and then defleshing the axial skeleton. Skeletal tissue was minced then added to 2 volumes of MEM-10. The mixture was sonicated



Figs. 2, 3. [*Myxosoma cerebralis*. × 1700.] 2. Spore, 8-10 μm in diameter. Silver nitrate stain. 3. Sporulation achieved *in vitro*. May-Grünwald-Giemsa stain.

with a Heat Systems Sonifer (Ultrasonics, Inc., Model W185D) using a microtip and 65 W of energy at 0 C for 30 min. Decontamination was effected by 1-hr centrifugation at an average 54,500 *g*. Cartilage extract was added to culture medium to a final concentration of 10%.

RESULTS AND DISCUSSION

In 6 of 7 trials, *in vitro* development of *M. cerebralis* proceeded from trophozoites to spores (Figs. 1-3, Table 1). Spores failed to develop in a tissue that had been infected for only 39 days. Although sporulation occurred at both 15 and 20 C, twin-spored pansporoblasts were found only twice, both times in cultures at 15 C. Sporulation was not inhibited by the bactericidal antibiotics used for decontamination and inhibition of microbial growth.

We do not yet know if spores that mature *in vitro* can give rise to clinical whirling disease. Judged by their ability to discharge their polar capsules, however, the cultured spores are viable. Comparisons of *in vivo* and *in vitro* sporulation rates were not made. Fish cartilage extract was not needed for sporulation, possibly because there were adequate nutrients in the culture medium, or residual cartilage, or both.

In this report we describe the first known instance that any stage of a myxosporidan has been cultured *in vitro*.

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Myxosoma cerebralis: a Method for Staining Spores and Other Stages with Silver Nitrate

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WOLF, K., AND M. E. MARKIW. 1979. *Myxosoma cerebralis*: a method for staining spores and other stages with silver nitrate. J. Fish. Res. Board Can. 36: 88–89.

Eleven dye substances were tested for their ability to stain *Myxosoma cerebralis* and to be retained through either the pepsin-trypsin-dextrose or the plankton centrifuge method of releasing and concentrating spores. Silver nitrate proved to be the best; it produced a distinctive brown color that has been retained by spores through 2.5 yr of storage. The method has applications in histologic studies of whirling disease, in quantifying the efficiency of spore detection procedures, and in teaching.

Key words: myxosporidans, *Myxosoma cerebralis*, whirling disease, spore staining

WOLF, K., AND M. E. MARKIW. 1979. *Myxosoma cerebralis*: a method for staining spores and other stages with silver nitrate. J. Fish. Res. Board Can. 36: 88–89.

Nous avons essayé onze colorants en vue de déterminer leur aptitude à colorer *Myxosoma cerebralis* et leur rétention lorsque soumis soit à la méthode pepsine-trypsine-dextrose, soit de centrifugation du plancton, pour la mise en liberté et la concentration des spores. Le nitrate d'argent s'avère le meilleur; il produit une coloration brune distinctive retenue par les spores pendant un entreposage de 2,5 mois. La méthode peut être utilisée dans des études histologiques de la maladie giratoire, la quantification de l'efficacité des méthodes de détection des spores et enfin l'enseignement.

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SEVERAL procedures have been developed for releasing and concentrating spores of *Myxosoma cerebralis* and thereby facilitating whirling disease diagnostic work, detection, and research applications (Landolt 1973; Markiw and Wolf 1974; O'Grodnick 1975). Considering such factors as purity of final product, the time required for processing, simplicity, and the sensitivity of detection, each method has certain advantages. However, the Fish Health Section of the American Fisheries Society (Anon. 1975) has suggested only two methods for detecting asymptomatic carriers of *M. cerebralis* — the pepsin-trypsin-dextrose (PTD method) of Markiw and Wolf (1974) or the plankton centrifuge method reported by O'Grodnick (1975).

When the number of spores in a pooled sample of fish is low (as in light infections or low incidences) there is some risk of failing to detect the organism — especially if personnel are not well versed in the procedures. Accordingly, we sought a dye substance that would stain spores and persist through either the PTD or plankton centrifuge method of processing, and thereby provide a means of measuring the percentage recovery and hence efficiency.

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Methods — Heads of infected rainbow trout (*Salmo gairdneri*) were immersed for several minutes in water at 50°C, the soft tissues were removed, and the skull and gill arches were minced and then crushed in a mortar and pestle. The resulting material was suspended in water, and the portion that passed through a 125-μm screen was centrifuged at 1200 × *g* for 10 min. The pelleted tissues fragments and spores were subjected to digestion by pepsin for 60 min and trypsin for 30 min, and then further purified by centrifugation through 55% dextrose. Spores were washed in distilled water and fixed in 5% neutral buffered formalin for 3 or 18 h.

Subsamples of preserved spores were stained with 1 of 11 different biological dye substances (Table 1), subjected to the standard PTD procedure, and examined for color retention and structural integrity.

The efficiency of recovery of stained spores was quantified by seeding known numbers in duplicate portions of finely minced cranial elements and gill arches of healthy rainbow trout fingerlings — each portion equivalent to a pooled sample of 60 fish. The samples were processed by the PTD method, the resulting harvest was brought to the original volume seeded, and the spores were counted in a hemacytometer.

Results and discussion — Silver nitrate proved to be the stain that was retained through PTD processing with little loss of its distinctive yellow to brown colora-

TABLE 1. Biological stains applied to spores of *Myxosoma cerebralis* and tested for persistence through PTD processing[a].

Stain	Concentration (%)	Staining time
Aceto-orcein	1.0	10 min
Crystal violet	1.0	10 min
Crystal violet + Lugol's iodine	1.0	5 min
Hematoxylin-eosin Y (Delafield's)	0.7	4 h
Hematoxylin, iron (Weigert's)	0.5	4 h
Malachite green	1.0	16 h
Methylene blue	1.0	30 min
Osmium tetroxide	1.0	1 h
Pinacyanol	0.25	15 min
Potassium permanganate	1.0	10 min
Silver nitrate	10.0	3 h[b]

[a]Pepsin-trypsin-dextrose centrifugation method of Markiw and Wolf (1974).

[b]After spores were treated with 10% aqueous silver nitrate (pH 4.7) they were exposed to midday sunlight for 2 to 3 h to photo-oxidize the compound.

tion. Aceto-orcein and potassium permanganate were unsatisfactory because they damaged spores. Although they stained spores well, crystal violet, malachite green, and methylene blue pigments were lost during PTD processing. Osmium tetroxide did not stain adequately. The hematoxylin stains and pinacyanol imparted good pigmentation, but their retention was less than that of silver nitrate.

Spores stained with silver nitrate were preserved at 4°C in a 12.5% solution of polyvinylpyrrolidone (M.W. 40,000), 5% formalin, or water. The least loss of pigment occurred in water; polyvinylpyrrolidone was wholly unsatisfactory (Fig. 1). In spite of the small size of the spores, a 3-h fixation time in formalin before staining with silver nitrate was inadequate. Periodic counts of stained spores held in water after a 16-h fixation in formalin showed that spore numbers were stable for at least 2.5 yr.

As a result of impregnation with silver nitrate and later exposure to sunlight at midday, spore valves were stained a yellowish brown and the polar capsules or the extruded filaments an intense brown. Pre-spore stages were stained like the valves, and presumptively identified trophozoites were amorphous masses showing multiple brown pigment spots. Because of differential avidity for the silver compound, histologic sections of fish tissue with *M. cerebralis* showed the parasite in marked contrast to surrounding host cells.

Minced normal trout bone and cartilage was seeded with silver stained spores at rates of from 1000 to 2000 per head. In eight trials, the average recovery was 91% of the number seeded (range, 67–131%).

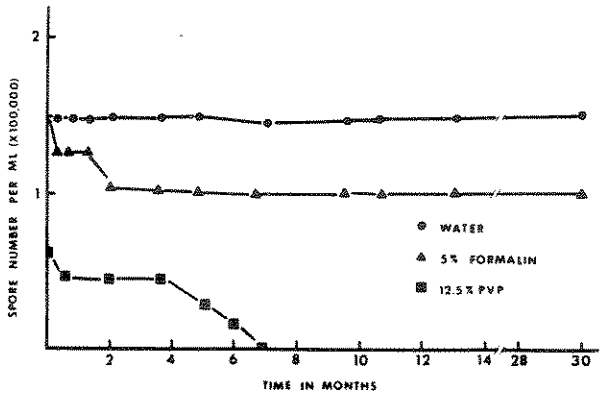

FIG. 1. Comparison of intact survival of *M. cerebralis* spores stained with silver nitrate and held in water, 5% formalin, or 12.5% solution of polyvinylpyrrolidone (PVP).

We suggest that silver stained spores be used to measure the efficiency of recovery when critical detection is required, or when persons first use either the PTD or the plankton centrifuge method. However, the stained spores should be used in a separate but parallel mince of normal tissue and not with the unknown, for about 7% of the spores blanched to a pale yellow color. The inexperienced investigator might confuse the lightly stained spores with untreated spores and report a false positive.

Judging by our work with stained spores, a few spores can and often do adhere to plankton centrifuge walls or rotor, or to blenders, food choppers, and laboratory ware. Presumably, unstained spores can similarly adhere to walls of utensils and conceivably could give rise to a false positive in later work. Soaking such equipment for 30 min in household bleach diluted with an equal volume of water is sufficient to dissolve or otherwise decontaminate the utensils.

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Myxosoma cerebralis: Inactivation of Spores by Hot Smoking of Infected Trout

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WOLF, K., AND M. E. MARKIW. 1982. *Myxosoma cerebralis*: inactivation of spores by hot smoking of infected trout. Can. J. Fish. Aquat. Sci. 39: 926–928.

We processed 2-yr-old rainbow trout (*Salmo gairdneri*) and brook trout (*Salvelinus fontinalis*) with clinical whirling disease, which is caused by *Myxosoma cerebralis*, in one of the following ways: iced, iced and brined, or iced, brined, and hot smoked at 66°C for 40 min. Skeletal elements of each group were added to tanks containing soil samples from an aquatic environment free of *M. cerebralis* and aged for 4 mo at 12.5°C. Following this we assayed for infectivity by holding susceptible rainbow trout fry for 3 mo in the tanks of aquatic soil and skeletal elements. The fry were then examined for *M. cerebralis* spores. Spores were found in the fry from tanks that had received iced and iced and brined samples, but not in fry from the tanks containing hot smoked tissue, healthy tissue, or no tissue — the last two being negative controls.

Key words: whirling disease, *Myxosoma cerebralis*, heat inactivation, hot smoking

WOLF, K., AND M. E. MARKIW. 1982. *Myxosoma cerebralis*: inactivation of spores by hot smoking of infected trout. Can. J. Fish. Aquat. Sci. 39: 926–928.

Des truites arc-en-ciel (*Salmo gairdneri*) et des ombles de fontaine (*Salvelinus fontinalis*) de 2 ans, atteints de la maladie clinique gyratoire causée par *Myxosoma cerebralis*, ont été soumis à l'un des traitements suivants : glaçage; glaçage et saumurage; ou glaçage, saumurage et fumage à chaud à 66°C pendant 40 min. Des éléments squelettiques de chaque groupe furent ajoutés à des bassins contenant des échantillons de sol provenant d'un environnement aquatique libre de *M. cerebralis* et laissés pendant 4 mois à 12.5°C. On a ensuite procédé à des essais de contamination en maintenant des alevins de truite arc-en-ciel pendant 3 mois dans des bassins contenant des échantillons de sol et des éléments squelettiques. Les alevins ont ensuite été examinés en vue d'y détecter la présence de spores de *M. cerebralis*. On a

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trouvé des spores chez les alevins maintenus dans les bassins contenant les échantillons glacés, et glacés saumurés, mais non chez ceux contenant des tissus fumés à chaud, des tissus sains, ou absence de tissus — ces deux derniers étant des témoins négatifs.

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Myxosoma cerebralis, the myxosporidan protozoan that is the etiologic agent of salmonid whirling disease, produces infections ranging from subclinical to lethal. In addition, fish that survive light to intermediate infection are lifelong carriers. If such infected fish are transported to, or stocked in, waters where the disease has not occurred, they have the potential of introducing the pathogen. Accordingly, in some places fish health authorities have effected measures directed toward eradicating the parasite.

In the United States whirling disease is known from limited areas of the Northeast and West (Wolf 1974). The condition has been identified at two federal and several state hatcheries and also at commercial facilities. Where eradication was attempted, clean-up operations resulted in destruction and burial of several million fry and fingerlings and tens of thousands of kilos of fish of edible size.

In Europe, whirling disease is controlled by sanitary measures and rearing susceptible fish to the advanced fingerling stage in uncontaminated spring or well water. The older and more resistant fish may then be held in contaminated waters without undue loss or disfigurement, even though they become subclinically infected and asymptomatic carriers. Such infected fish appear normal, but fish health regulations in several countries prevent their importation.

Myxosoma cerebralis poses no threat to human health; in fact, it is likely that many of the trout produced for human food in Europe have subclinical infections with whirling disease. It has been assumed that cooking kills the spores of *M. cerebralis*, just as it does the trichina worm in pork, and other parasites in meat and meat products.

It is unknown if smoking of salmonids inactivates the spores of *M. cerebralis*. If smoking were effective, infected fish destined for destruction in eradication procedures could be used as human food. Also, the nations now barring fresh or live fish with *M. cerebralis* infection might well reduce the strictness of their regulations and allow importation of an appropriately smoked product.

Materials and methods — Fourteen 2-yr-old rainbow trout (*Salmo gairdneri*) having a mean total length of 31 cm and a mean weight of 327 g, plus 19 brook trout (*Salvelinus fontinalis*) averaging 25 cm and 171 g, each with clinical whirling disease were selected from an affected population. Presence of the disease was confirmed by the most simple laboratory examination; in fact, every fish in a subsample had abundant spores. Yearling rainbow trout from Leetown National Fish Hatchery (West Virginia) stock were used as a source of uninfected skeletal elements.

About 100 L of soil from a fish hatchery pond and from an effluent basin containing vegetation and entrapped invertebrate fauna were obtained at West Virginia's Department of Natural Resources facility at Ridge, a hatchery with no known history of whirling disease. The materials were distributed at the rate of 20 L per tank (52 cm in diameter), which was then filled with spring water, free of *M. cerebralis*, to a depth of 36 cm. Water (see Warren 1963 for chemistry), was supplied at a rate of 600 mL per minute. Because of the presence of plant material, the tanks were kept under continuous fluorescent illumination to prevent oxygen depletion.

All whirling disease fish, a total of 33, were killed on the same day, eviscerated, bagged in plastic, and iced. The following morning, two-thirds of the fish were flown to the testing facility, placed in 8% brine solution (w/w) at the rate of 2 kg of brine per kilogram of fish, and held at 3.0 ± 0.8°C for 16 h. Fish were then removed from the brine, rinsed for 15 s in 0°C water, and blotted. Half (11 fish) were bagged and iced; the remaining 11 fish were further dried in a forced-air draft at 1°C for 1 h to help firm tissues before the smoking was begun. The process of smoking with hickory chips and sawdust began with a 100-min period of drying and further firming of flesh during which the temperature was gradually raised from 10 to 37.8°C. During the next 40 min the temperature was raised to 66°C and held for 40 min. Heat and smoke were then withdrawn. After being smoked, the 11 fish were cooled, bagged, and iced, and with the 11 brined fish flown back to the laboratory. Skeletal elements, principally those of the head, were removed, minced, and added to test aquatic environments the following day. One environmental unit was seeded with smoked fish material, another with brined fish, and a third with iced fish (to serve as a positive control). A fourth unit was seeded with tissues from healthy fish (the first negative control), and a fifth was kept as an environmental or second negative control to demonstrate that the aquatic soil itself harbored no *M. cerebralis*. Materials were allowed to age for 4 mo, before 60 susceptible rainbow trout fry were added. Eighty days later, after sufficient time for spore development, 55 fry from each unit were processed by the pepsin–trypsin–dextrose centrifugation method for spore release and concentration (Markiw and Wolf 1974).

The smoking operation was patterned after that described by Seagran et al. (1970) for compliance with the U.S. Food and Drug Administration's regulations governing the smoking of fish. The brined and air-dried trout were hung in the smoke chamber of an Afos brand forced draft mini-kiln (manufactured under work order 10415/8, Patent 1184363, Afos. Ltd., Anlaby, Hull, England[1]). Thermocouples from a Honeywell Elektronic 15 thermograph were placed in the tissues of five fish, in ambient air, in the atmosphere of the chamber, in a receptacle of water in the chamber, and in the stack. External charting permitted recording of temperature at critical points throughout the processing.

Results — Susceptible fry were held in the test environments for 80 d. At the end of that time the pepsin–trypsin–dextrose centrifugation method of spore release and concen-

[1]Mention of product name does not indicate Government endorsement of commercial product.

tration showed that viable spores were present only in the brined fish and in those of the positive control group, the group held continuously on ice. The test soil itself and the tissues from fish known to be free of *M. cerebralis* showed no evidence of infectivity.

Discussion — *Myxosoma cerebralis* spores were inactivated during the smoking process, but it is most likely that the heat and, more specifically, the peak temperature held for 40 min inactivated the spores. *Myxosoma cerebralis* survived brining that resulted in a mean NaCl content of tissue of 1.21% (range 0.95−1.57%), a value more than twice physiological levels. It seems logical, therefore, to attribute spore inactivation to temperature alone, i.e. the 40 min at 66°C. Hoffman and Markiw (1977) have shown that, for spores heated at 70°C for 40 min, an average of 82% are penetrated by methylene blue dye; moreover, 100% of the spores heated at 90°C for 10 min are dyed and presumed killed. Our results show that the lower temperature of 66°C for 40 min is lethal.

We conclude that hot smoking of trout infected with whirling disease offers a biologically safe way of preparing fish for human food that would otherwise be destroyed where eradication procedures are used as a control measure for whirling disease.

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Biology Contravenes Taxonomy in the Myxozoa: New Discoveries Show Alternation of Invertebrate and Vertebrate Hosts

Ken Wolf and Maria E. Markiw

Myxozoans are small, multicellular, spore-forming parasites that are currently accorded the rank of phylum in the subkingdom Protozoa (*1*). Two classes of myxozoans are recognized: Myxosporea, which occur almost exclusively in cold-blooded vertebrates (predominantly fish), and the morphologically different Actinosporea, which occur in invertebrates (notably annelid worms). Actinosporea and Myxosporea have both been known for 80 years; however, even though developmental stages have been described in their respective hosts, complete life cycles—particularly the infectious stages—have eluded identification.

Abstract. *For 80 years the infectivity of salmonid whirling disease has eluded discovery. New findings now show that this myxosporean disease of fish is initiated by what is regarded as an actinosporean produced in a tubificid oligochaete. Experimental results provide evidence that, instead of being considered as representatives of separate classes in the phylum Myxozoa, the myxosporean and actinosporean are alternating life forms of a single organism.*

In this article we report that, in at least one situation, an actinosporean and a myxosporean are not separate entities, but are alternating life stages of a single organism. We tracked the complete life cycle of the myxosporean that causes whirling disease in salmonid fish. We further demonstrated, under controlled laboratory conditions, a previously unrecognized identity that is shared by a myxosporean and an actinosporean. In essence, whirling disease in the fish results in the formation of myxosporean spores that are incapable of infecting other salmonids but which, when released into the environment, initiate an infection in an oligochaete of the family Tubificidae. Infection in the tubificid results in the production of an actinosporean that is incapable of infecting other worms, but which is the long-sought infective agent of salmonid whirling disease.

Our explanation of the myxozoan life cycle should not be considered radical, for the use of different hosts and the occurrence of comparable changes in morphology are well established in parasitology. Such alternations or transformations are common among the helminths and universal in malarial organisms.

These findings suggest that a reappraisal of myxozoan taxonomy should be considered. They also provide a model for investigating life cycles of other myxosporeans and actinosporeans and open new avenues for controlling myxosporean diseases of fish.

Background Biology of Myxosporea

Whether histozoic or coelozoic, most myxosporean infections are well tolerated by the host fish—as, for example, *Myxosoma cerebralis* is in its original host, the European brown trout (*Salmo trutta*). In contrast, in a new host, the rainbow trout (*Salmo gairdneri*), the parasite produces a virulent infection termed "whirling disease" (*2*, *3*).

Whirling disease derives its name from the tail-chasing behavior of afflicted salmonids. Histozoic *M. cerebralis* attacks cartilage, and, if the organs of equilibrium are involved, motor control is deranged. Spores of *M. cerebralis* are universally present in salmonids with whirling disease. However, fresh spores of *M. cerebralis* are not infectious in fish (*3–5*). Instead, spores must first be "aged" in an aquatic environment (3 to 4 months) before infectivity is produced (*4*). However, it has not been shown that the spores per se are infectious after aging. And, although the nature of the infectivity has been diligently sought, it has eluded identification.

We have found that whirling disease infectivity for fish does not develop endogenously in the spores of *M. cerebralis*. To arrive at this conclusion, we aged spores in aquatic environments consisting of springwater over inert substrates such as sand, glass wool, or pasteurized or sterilized aquatic soil and then added rainbow trout. In all trials the spores failed to yield infectivity. In contrast, rainbow trout that were held in containers of pond soil from fish hatcheries where whirling disease was enzootic readily acquired the infection. Because these trout developed whirling disease after being fed worms, tubificid oligochaetes were, by virtue of their abundance, implicated as an intermediate host.

Conclusive support of this premise was obtained by populating each of a number of containers of pasteurized trout pond soil with a different species of tubificid oligochaete and then adding fresh spores of *M. cerebralis* (*5*). After 4 months we added susceptible rainbow trout fry. Whirling disease occurred only among fry in containers that had received tubificids from a fish hatchery where the disease was epizootic or in containers that had received normal tubificids plus spores of *M. cerebralis* (*5*).

Background Biology of Actinosporea

Actinosporea have been known since 1899, when A. Stolc described a representative organism from a tubificid oligochaete (*6*). The postulated life histories of several actinosporeans of the genus *Triactinomyxon* have been described and illustrated in detail (*7–9*). However, this research was based on tubificids taken from nature: the elapsed time of development was not known and the authors were not able to identify the initial stage of the developmental cycle. Various investigators have assumed that the Actinosporea are infectious for the tubificids in which they are found; however, such infectivity has never been demonstrated.

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Having implicated tubificid oligochaetes in the so-called aging process of *M. cerebralis* (*5*), we next sought to identify the nature of the infectivity of whirling disease. This phase of the investigation led to the discovery that, when spores of *M. cerebralis* were kept in the presence of tubificids for several months, a previously unrecognized stage in the myxosporean life cycle appeared, an actinosporean of the genus *Triactinomyxon* (Fig. 1).

Known numbers of spores of *M. cerebralis* were added to quantified populations of tubificids and the two were kept together for periods as long as several months (*10*). A stock of about 180,000 worms was placed in a container holding axenic aquatic soil; spores were then added in the amount of 100 per worm. To assess the effect of contact time, we removed half the worms after 10 days, thoroughly washed them, and transferred them to a second container of soil. After 20 days, one-sixth or about 30,000 worms were similarly transferred to a third container. Two containers, each with 30,000 worms but no spores, served as negative controls. All containers received a slow flow of springwater at 12.5°C. During the ensuing several months, samples of worms were crushed for microscopic examination and others were fixed for later scrutiny and histological examination.

On day 104, but not earlier, crushed tubificids from the first container showed a previously unrecognized organism that we identified as an actinosporean of the genus *Triactinomyxon* (Fig. 1). The organism was present in all later samples from the first container and from containers that held tubificids that had been in contact with *M. cerebralis* spores for only 10 or 20 days. The *Triactinomyxon* could not be found in the tubificids that had had no contact with the myxosporean.

Features of the *Triactinomyxon*

Morphology. The *Triactinomyxon* is anchor-shaped and is topped by three polar capsules. The cavity of the anterior end or enispore is about 36 μm long and contains 32 to 50 spherical sporozoites (Fig. 2). The sporozoites reacted to bisbenzamide stain and were positive for DNA (*11*). Beneath the short column of sporozoites, the style extends about 90 μm, and the tapering arms project about 170 μm further.

Relatedness to M. cerebralis. The enispore reacted strongly with fluorescein isothiocyanate–conjugated rabbit antiserum to *M. cerebralis* (Fig. 3) (*12, 13*). The gossamer structural material of the style and arms did not react with conjugated antiserum.

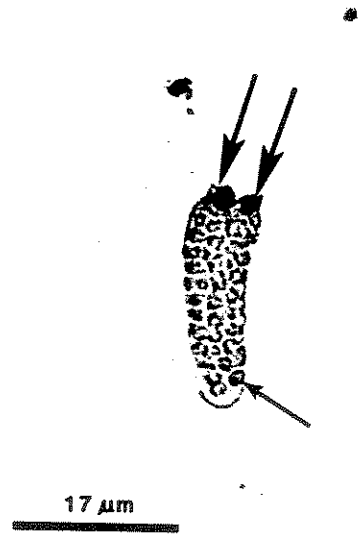
Production dynamics. The *Triactinomyxon* proved to be waterborne as well as present within tubificids. Three weeks after we found the organisms in the tubificids, effluent from the first container (containing about 60,000 tubificids) yielded *Triactinomyxon* at rates of 2,400 to 3,100 per minute at a flow of 120 ml/min. Production declined during the following month but continued at a detectable level for 7 months. *Triactinomyxon* never appeared in the effluent from the negative control containers.

The *Triactinomyxon* readily passed screen mesh of 200 μm but was retained by 50-μm mesh, in agreement with earlier results showing that the major peak of the then-unknown infectivity of whirling disease was trapped on 50-μm screen (*5*). The finding that *Triactinomyxon* is waterborne agrees with our earlier observation that whirling disease occurs among trout fry held solely in the water column as well as among fry having access to the soil (*5*).

Triactinomyxon and Whirling Disease

Data from three sets of experiments conducted separately provide evidence that the *Triactinomyxon* initiates the clinical condition known as salmonid whirling disease and that within the vertebrate host a morphological change results in the development of a myxosporean of the genus *Myxosoma* (Fig. 4).

In the first trial, 20 rainbow trout fry were simply exposed to *Triactinomyxon* (*14*). The test fry developed whirling disease, whereas the nonexposed source stock of fish remained healthy. At 19



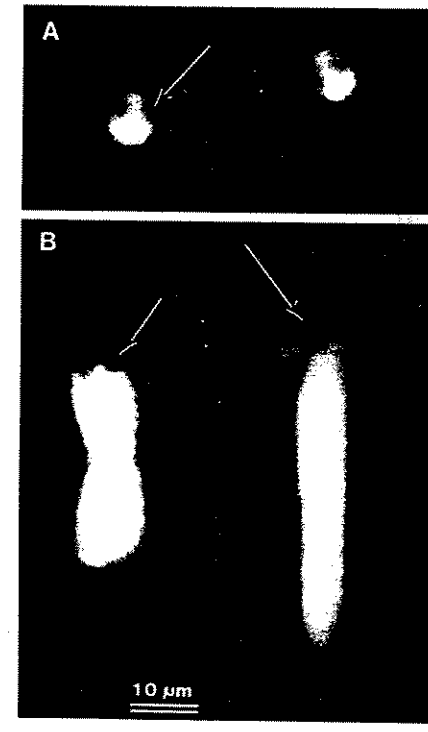


Fig. 1 (left). Mature waterborne *Triactinomyxon* produced in tubificid oligochaetes after exposure to *M. cerebralis*. Fig. 2 (center). Enispore of *Triactinomyxon* showing two of the three pyriform polar capsules (large arrows) and the numerous internal sporozoites (small arrow). Fig. 3 (right). Comparative response of enispore of a mature *Triactinomyxon* and of *M. cerebralis* to rabbit antiserum prepared against *M. cerebralis* and conjugated with fluorescein isothiocyanate. (A) Positive reaction of mature spores of *M. cerebralis* to the homologous antiserum. (B) Comparable reactivity of triactinomyxons to antiserum against *M. cerebralis*. Arrows point to nonreactive polar capsules.

weeks after exposure, a group of 12 fry was processed by the pepsin-trypsin-dextrose (PTD) centrifugation method of myxosporean spore release and concentration (*15*). The affected fry harbored 680,000 myxosporean spores each.

In the second experiment signs of whirling disease appeared in a group of 20 fry held for 6 days (*16*) on 50-μm mesh screen that was collecting experimentally produced waterborne *Triactinomyxon*. Whirling disease also developed in another 20 fry placed for 6 days directly in a container of tubificids that were producing the actinosporean. Both groups of fish also produced spores of *M. cerebralis* (Table 1). Measured numbers of *Triactinomyxon* administered to other groups of 20 fry by mouth or by intraperitoneal injection (*17*) resulted in the production of spores of *M. cerebralis* but not in signs of whirling disease. Intramuscular injection of *Triactinomyxon* produced neither the disease nor myxosporean spores. Negative controls remained free of *M. cerebralis* spores (Table 1).

In the third trial, duplicate negative controls, each consisting only of tubificids (100 g, or about 14,700), failed to produce *Triactinomyxon*, and showed no infectivity for rainbow trout fry. Positive controls also consisted of 100 g of tubificids, but *M. cerebralis* spores were added at the rate of 40 per worm in one case and 400 per worm in the other. Both containers of worms and myxosporean spores produced *Triactinomyxon*, but the differential was twofold instead of tenfold. *Triactinomyxon* from the more productive container was tested in duplicate; both of two groups of 20 trout fry that were exposed for 3 days developed whirling disease.

Table 1. Occurrence of whirling disease and spores of *M. cerebralis* in groups of 20 rainbow trout fry variously exposed to spores of an actinosporean of the genus *Triactinomyxon*. Numbers in parentheses indicate sample size.

Treatment	Signs of whirling disease	Average number of *M. cerebralis* spores per fish at	
		4 months	4.5 months
Exposure (6 days) to tubificids producing *Triactinomyxon*	Yes	245,400 (3)	(0)*
Exposure (6 days) to waterborne *Triactinomyxon*	Yes	94,200 (3)	400,000 (4)
Intraperitoneal injection of 14,200 *Triactinomyxon*	No	1,000 (3)	11,000 (11)
Intubation of 7,100 *Triactinomyxon*	No	300 (3)	26,400 (9)
Intramuscular injection of 7,100 *Triactinomyxon*	No	0 (3)	0 (3)
None (contact with *Triactinomyxon* prevented)	No	0 (3)	0 (1)†

*Accidental loss. †Sole survivor of accidental loss.

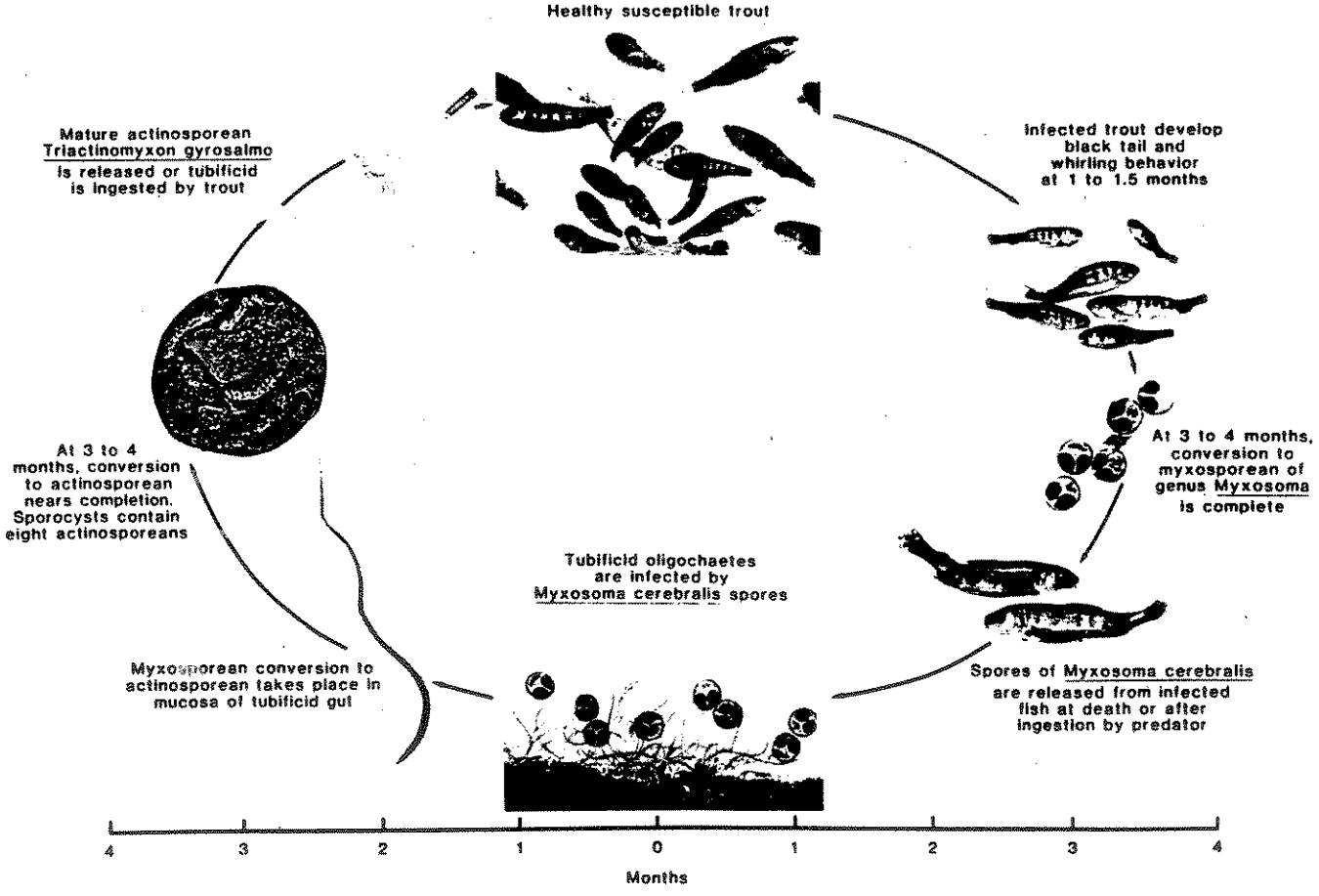


Fig. 4. Biphasic life cycle of a myxozoan. Clockwise from bottom center: Spores of *M. cerebralis* infect tubificid oligochaetes and initiate the actinosporean phase. At 12°C and 3 to 4 months later conversion to the actinosporean is nearly complete as multiple parasite cysts mature in the worm gut. Young salmonid fish ingest worms or waterborne *Triactinomyxon* infects fish via the gut or a branchial route and the myxosporean phase begins (top center). At 1 to 1.5 months the fish show signs of whirling disease; at 3 to 4 months the myxosporean phase is completed with maturation in the cartilage of spores of *M. cerebralis*. The myxosporean phase is not capable of infecting other fish, nor can the actinosporean phase infect oligochaetes.

Tubificid Resistance to *Triactinomyxon*

We challenged tubificids with *Triactinomyxon* but were unable to show autoinfection. A population of about 14,700 worms in axenic soil was exposed on eight occasions during a 3-week period. The *Triactinomyxon* were not quantified; the challenge consisted of organisms collected during 2- to 5-day periods and simply added to the container of worms. Four months after this challenge, 140 groups of five worms each were crushed and examined microscopically. No *Triactinomyxon* were found. In addition, three groups of about 800 worms each were crushed in a mortar and the coarse material was allowed to settle. The supernatant material was centrifuged for 10 minutes at 600*g*; it too was devoid of *Triactinomyxon*. The tubificids did not produce whirling disease in trout fry, and when the fish were digested by the PTD method no spores of *M. cerebralis* were found.

Form and Function of *Triactinomyxon*

Janiszewska (*8*) postulated that the great tapering processes of *Triactinomyxon* provide buoyancy. We agree, but suggest that a more important function of the elongate valves could be to lodge the organism between gill lamellae of host fish. The anchor-like processes could provide a temporary hold whereby the long-unknown function of polar capsules would come into play for a more secure hold, so that sporozoites could be transferred to the vascular system.

To evaluate these possibilities, we sprayed a suspension of *Triactinomyxon* directly into the gill chambers of ten trout fry, held them individually for 20 seconds, and then rinsed the fry and transferred them to springwater for 4 months. Signs of whirling disease did not appear, and eight of the fry survived for 4 months, at which time they were individually processed for *M. cerebralis* spores by the PTD method. Five of the eight fry harbored spores of *M. cerebralis*, but the numbers were low—only 600 to 3100 per fish.

Thus far, all evidence indicates that the whirling disease infectivity is, initially at least, inside the tubificid. Further evidence was obtained by feeding infection-bearing tubificids to rainbow trout fry. In duplicate, ten fry were fed thoroughly washed tubificids in four consecutive feedings. Duplicate groups of ten other fry were fed tubificids that had been thoroughly washed and externally decontaminated in 40 parts per million sodium hypochlorite for 2 minutes; the chlorine was then neutralized with sodium thiosulfate. Decontamination was terminated before the worms were dead and discolored. Palatability was not affected, for the fry avidly ate them.

After 4 months at 12.5°C, the four lots of fry were killed and processed by the PTD method. All yielded *M. cerebralis* spores.

Conclusion

Fresh spores of *M. cerebralis* have never been shown to infect fish; neither have *Triactinomyxons* been shown to infect their annelid hosts. The explanation is that *M. cerebralis* infects the tubificid and does so in a matter of 10 days or less. A new development cycle occurs and results in a *Triactinomyxon* that infects the trout.

In this particular case, the life forms that have previously been known as an actinosporean and a myxosporean have been shown to be simply alternate life stages of a single organism. It seems likely that comparable alternations of form and host will be found among other Myxozoa. Indeed, Janiszewska (*8*) noted similarities of spore formation in what were then termed Actinomyxidia and Myxosporidia. We propose the name *Triactinomyxon gyrosalmo* for identifying the specific cause of salmonid whirling disease. We leave to the systematists the decision as to which name—*Myxosoma cerebralis* or *Triactinomyxon gyrosalmo*—will be used to identify the organism.

We have not yet determined which species of tubificid is involved in whirling disease (*5*). Considering the biology of aquatic oligochaetes, prevention of whirling disease could be achieved if the host worms could be eradicated. Chemicals selectively lethal for annelids could achieve eradication, but such compounds are not yet known.

Two investigators have independently claimed to have infected trout with whirling disease by using *M. cerebralis* spores simply aged in water (*18*, *19*). In neither study were details of the work sufficient to judge whether tubificids were involved.

The tubificids we used do not permit us to equate our system with pure culture techniques that are basic to microbiology and virology. Accordingly, we cannot claim to have fulfilled Koch's postulates for whirling disease. We have, however, offered the only explanation we know of that is supported by experimental data.

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13. When reacted in direct tests with 11 different fish myxosporeans, the conjugated rabbit antiserum to *M. cerebralis* showed heterologous activity only against another member of the genus *Myxosoma*.
14. Fry were kept in a 3-liter container of aerated springwater to which was added a 48-hour-long (but otherwise unquantified) harvest of *Triactinomyxon* from the initial container of 60,000 tubificids. At 2-day intervals, the procedure was repeated twice with fresh water and *Triactinomyxon*.
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SHORT COMMUNICATION

Salmonid whirling disease: *Tubifex tubifex* (Müller) identified as the essential oligochaete in the protozoan life cycle

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The life cycle of the protozoan that causes salmonid whirling disease has been subjected to investigation and speculation for decades, but only recently have the facts been elucidated. First, Markiw & Wolf (1983) showed that spores of the myxosporean *Myxosoma cerebralis* could not of themselves generate infectivity for fish, and that a tubificid oligochaete played an essential but undetermined role in the development of the unknown infectious stage. Second, Wolf & Markiw (1984) identified the infectious stage for fish to be an actinosporean of the genus *Triactinomyxon*, which was produced in the gut of tubificids that had been exposed to spores of *M. cerebralis*. Wolf & Markiw (1984) suggested that the descriptive name *Triactinomyxon gyrosalmo* be used to designate the initiator of salmonid whirling disease. Such usage distinguishes the organism from other triactinomyxons that have been given species names but whose biological role is thus far known only as a parasite of an annelid.

Just as myxosporeans have long been recognized as parasites of fishes, actinosporean protozoans have long been recognized as parasites of annelid worms (Mackinnon & Adam 1924; Janiszewska 1955). In addition to identifying the infective stage of whirling disease, Wolf & Markiw (1984) strongly suggested that myxosporeans and actinosporeans were not separate classes in the phylum Myxozoa (Levine *et al.* 1980) but were actually alternating life-stages of a single organism. Accordingly, the biology of whirling disease was described as comparable to that of some helminths, and more specifically to that of the sporozoans that cause malaria.

The vertebrate phase of the whirling disease life cycle takes place in fish of the family Salmonidae, all members of which are considered to be susceptible to infection. The invertebrate phase requires an oligochaete of the family Tubificidae, but identification of specific susceptible members is thus far lacking. Markiw & Wolf (1983) noted that the tubificids they used were taken from natural populations that consisted of four species: *Ilyodrilus templetoni, Limnodrilus hoffmeisteri, Quistadrilus multisetosus*, and *Tubifex tubifex*. The purpose of the present study, therefore, was to determine which of these species was involved in the life cycle of the whirling disease protozoan.

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Tubificids free of actinosporeans were stocked in containers of axenic soil and exposed to fresh spores of *M. cerebralis*. After 16–18 weeks, the time required for development and maturation of *Triactinomyxon gyrosalmo*, representative worms were removed, killed and fixed for later examination. Specimens that differed from normal coloration and morphology were selected and a small section of the posterior body was crushed on a microscope slide and searched for triactinomyxons. In time, a pattern of infection became apparent; the organisms were abundant in worms that were pale, that had generalized anterior swelling, and displayed an opaque outer layer. In two groups of worms that were examined, an overall 9% prevalence rate of experimental infection was found. In contrast, the prevalence rate among naturally infected specimens was only 0·6%. Diagnostic accuracy of the abnormal signs was about 30% for the first group, but our greater experience increased this percentage to about 70 in the second group. Eighteen of the worms that harboured cysts of *Triactinomyxon* were examined by one of us (J.K.H.) to determine species; of that number, one was reproductively mature and proved to be *Tubifex tubifex*, and the remainder were immature and therefore could not be conclusively identified to species. These individuals were, nevertheless, judged to be the young of *T. tubifex* because of their close resemblance to that species.

The determination that 17 of the infected worms were immature *T. tubifex* agrees with an earlier observation that the species was the most numerous among the several present in samples from two widely separated hatcheries—one where whirling disease was epizootic and the second, a trout hatchery that was free of the infection (Markiw & Wolf 1983).

Anatomically, cysts of *T. gyrosalmo* were in segment 17 of the annelids and posteriad, but not in the more anterior segments.

Whether other resident tubificids are susceptible to spores of *M. cerebralis* remains to be determined. Low representation of other species in the mixed populations as well as the surprisingly low prevalence of infection of *T. tubifex* suggest that although a possibility exists the probability is small. Definitive answers could be obtained by exposing monospecies populations of various tubificids to *M. cerebralis* spores and examining the worms for infection and confirming their identity. Assay for water-borne infectivity should not be considered definitive, because contaminating susceptible tubificids could be present and could account for the infection.

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Occurrence of Whirling Disease of Trout in Western United States

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YASUTAKE, W. T., AND H. WOLF. 1970. Occurrence of whirling disease of trout in western United States. J. Fish. Res. Bd. Canada 27: 955–956.

Whirling disease of trout, caused by *Myxosoma cerebralis*, was diagnosed in 1966 for the first time in rainbow trout (*Salmo gairdneri*) from California and Nevada hatcheries. In 1969 the first incidence of this disease in cutthroat trout (*S. clarki*) was reported in Nevada. To date these have been the only confirmed cases of whirling disease in western United States.

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THE HIGHLY INFECTIOUS WHIRLING DISEASE of trout caused by *Myxosoma cerebralis* has been prevalent in hatcheries in the eastern United States since 1956 (Hoffman et al., 1962). This disease, however, was not diagnosed in any of the western hatcheries until 1966 when specimens sent to the Western Fish Disease Laboratory were confirmed histologically as having *M. cerebralis* infection. This report documents the first occurrence of the disease in the western United States.

In January of 1966 we received moribund, 9-month-old rainbow trout (*Salmo gairdneri*) from a private trout hatchery located on Garapata Creek just south of Carmel, California. In an accompanying letter the hatchery owner described the behavior as "an extremely nervous twisting action as though the fish were hooked in the mouth and then got off the hook. They spiral around and around as though they were tied by the nose. This becomes so violent at times that some of them come out onto the bank. This action seems to take place mainly when they are startled or when you approach the pond to feed." These signs were first noticed in a few fish when they were approximately 6 months old. At that time the hatchery owner thought that the problem was caused by either overcrowding or rising water temperature. However, the whirling behavior persisted and increased through the summer and winter months.

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FIG. 1. *Myxosoma cerebralis* spores in the cranial area of rainbow trout. May-Grunwald-Giemsa stain; ×2060.

Histopathological examination of these fish revealed an extensive myxosporidian infection in the cranial cartilaginous tissue (Fig. 1), particularly in the auditory capsule and base of the gill areas. Fresh wet mount preparations of the spores (about 6–10 μ in diameter) exposed to Lugol's iodine solution indicated the absence of iodinophilous vacuoles; this plus the cartilaginous location verified the identification.

In June of 1966 a group of 4½-month-old rainbow trout suspected of having whirling disease was sent to Western Fish Disease Laboratory from a state hatchery on the Truckee River in Nevada. Signs described by the biologist were those of typical *M. cerebralis* infection: tail chasing, whirling or twisting, and black tail. Histopathological examination revealed many trophozoitelike cells along the cartilaginous tissues of the cranial area, but no spores were observed. Subsequent examination of 7-month-old fish from the same lot disclosed the spores.

In late November 1969, a third case of whirling disease was reported from a population of rainbow and cutthroat (*S. clarki*) trout at a National Fish Hatchery in Nevada (W. J. Walsdorf, personal communication). Water supply was from wells and the Carson River. This is believed to be the first incidence of *M. cerebralis* reported in cutthroat trout.

Diagnosis of the California and the Nevada materials was confirmed by Dr Glenn Hoffman, parasitologist at the Eastern Fish Disease Laboratory.

The 1966 Nevada fish were planted in several water sheds before a definitive diagnosis was made. When *M. cerebralis* was definitely identified, the concrete troughs, tanks, and ponds were treated with live steam and thoroughly dried. No evidence of a recurrence has been found in the 3 years since the facilities were treated. The source of infection at this Nevada hatchery is unknown; however, it has a history of receiving wild and domestic strains of trout regularly from many sources in the western United States.

The approximately 100,000 rainbow trout from the Garapata Creek Hatchery in California were all destroyed by burial in compliance with California law. Again the source of infection is unknown. The hatchery, however, had a long history of receiving only trout eggs that are not believed to carry the disease (Hoffman et al., 1962). The water supply, however, contains a population of steelhead (*S. gairdneri*), and although infected specimens were not found at the time of the outbreak (12 fish were examined) they still remain as a suspected source of the infection.

To date the three cases noted above have been the only confirmed cases of *M. cerebralis* epizootics reported in the western United States.

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(FIG. 1 *opposite*)

THE SPREAD OF *Myxosoma cerebralis* INTO NATIVE TROUT POPULATIONS IN MICHIGAN

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WHIRLING DISEASE, caused by the Myxosporidian (Protozoa) *Myxosoma cerebralis*, has spread across the United States since it was first identified at a Pennsylvania trout hatchery in 1956 [2]. In August 1968, this disease was discovered in Michigan in three commercial trout hatcheries. Subsequent investigations revealed that whirling disease had been spread to other trout ponds, both commercial and private, by fish transfers from the originally infected hatcheries (unpublished data). The fish infected were rainbow trout (*Salmo gairdneri*).

The spread of whirling disease from infective sources into natural watersheds has not been well documented. Outbreaks of the disease have been reported from hatcheries, but fish in natural watersheds below these infected sources were not monitored for the disease. However, there is evidence that whirling disease is carried to locations downstream. In 1957 and 1958, the disease was present in a private hatchery in Pennsylvania upstream in the watershed from the Lamar National Fish Hatchery [2]; by 1960 the disease was discovered in fish at the Lamar Hatchery.

The only reference reporting infected fish in a stream is from Russia [5]. There, in 1954, *M. cerebralis* was found in a trout hatchery where brood stock rainbow trout (*Salmo irideus*) had been introduced several years earlier. In 1956, wild brown trout and rainbow trout infected with this parasite were found in the watershed downstream from the hatchery.

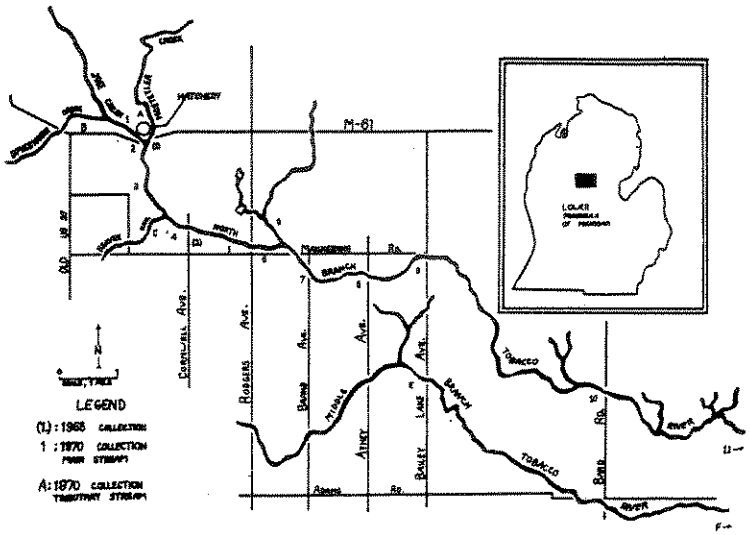
The *M. cerebralis* infection studied in this report occurred in the upper watershed of the North Branch of the Tobacco River, Clare County, Mich. (see map). The commercial hatchery which was initially contaminated is located on the uppermost tributary (Jose Creek); this hatchery reared only rainbow trout. The purpose of the present study was to assess the spread of *M. cerebralis* from the hatchery to wild trout, and to determine the extent of infection in the watershed. The watershed flows into Lake Huron. Since the Tobacco River produces only brook trout (*Salvelinus fontinalis*) and brown trout (*Salmo trutta*), the two rainbow trout which I examined were assumed to be escapees from the hatchery.

PROCEDURES

Trout samples were collected from the streams with electrofishing equipment, and they were kept fresh on ice in the field or preserved in 10 percent formalin. The fresh samples were brought to the Grayling Research Station, separated according to species, and frozen until used. The collection sites were selected on the basis of ease of access, distance from the commercial hatchery, and as tributaries to the main contaminated stream. The first collections were made in November 1968, approximately 3 months after the initial infection was found in the trout hatchery on Jose Creek. The 1970 collections were started some 16 months after the initial infection was discovered; 17 collections were made on the mainstream and tributaries. Eleven of the 17 collections came from Jose Creek and the North Branch of the Tobacco River.

The diagnosis of whirling disease was based on the presence or absence of *M. cerebralis*

NOTE.—This study was conducted in cooperation with the Department of the Interior, Bureau of Commercial Fisheries, under Public Law 89-304, Project AFC-9-1.



Myxosoma cerebralis infection in watershed of the North Branch Tobacco River, Clare County, Michigan.

spores. The trout from the collections were grouped into lots of about five trout each; occasionally single fish were examined. The heads were removed from the fish and placed in (40° to 45°C.) water for 2 to 3 minutes or until the skin and flesh readily fell away from the cranium [4]. The craniums were dissected, and portions of bone, cartilage, and the auditory capsule were placed in a mortar. The materials were mascerated and then ground to a pulp [3]. About 10 to 15 milliliters of distilled water was added to the pulp to create a slurry. Drops of this slurry were then examined microscopically at 450× for spores of *M. cerebralis*.

RESULTS

The 1968 stream collections (table 1) contained brook trout, brown trout, and one rainbow trout. The single rainbow trout (5.6 inches long) was infected with *M. cerebralis*. This rainbow trout was collected about 1 mile downstream from where the hatchery outlet enters Jose Creek (see map). It was considered to be an escapee from the infected hatchery. The brook trout (6 slurries) and brown trout (7 slurries) were not infected. The brook trout ranged in length from 2.5 to 5.8 inches, and the brown trout from 3.1 to 4.8 inches.

The 1970 collections (table 1 and fig. 1) consisted again of brook trout (2.3 to 10.7 inches), brown trout (1.5 to 15.1 inches), and 1 rainbow trout (9.6 inches). Brook trout were collected at eight of the 11 mainstream stations (Jose Creek and North Branch Tobacco River), and infected fish were found at each of the first six stations (fig. 1); for these six stations, 15 of 22 slurries (or 68 percent) were infected. Brown trout were collected at all 11 mainstream stations; infected brown trout were found only at stations 2 and 3; for stations 1 to 3, 4 of 30 slurries (13 percent) were infected. No infected trout were found in 47 slurries from 221 brown trout and 3 brook trout at stations 7 to 11. The single rainbow trout from station 2 was not infected.

In the six collections taken during 1970 from tributaries of Jose Creek and the North Branch Tobacco River (fig. 1), no infection was found among 112 brook trout and 152 brown trout (table 2).

During the 2 years, from 1968 to 1970, *M. cerebralis* had become established among native brook trout and brown trout, and had spread downstream about 6 miles below the hatchery where it started. It was more prevalent among brook trout than among brown trout, and I did not find it in tributaries.

Table 1.—Occurence of M. cerebralis *infection in trout collected from the mainstream of the North Branch Tobacco River*

Station [1]	Collection date	Trout species	Number of fish	Examination results [2]	Number of slurries [3]
	1968				
(1)	Nov. 21	Rainbow	1	+	1/1
		Brook	1	−	0/1
		Brown	12	−	0/5
(2)	Nov. 21	Brook	9	−	0/5
		Brown	6	−	0/2
	1970				
1	Apr. 13	Brook	20	+	1/4
		Brown	38	−	0/8
2	Jan. 28	Rainbow	1	−	0/1
		Brook	13	+	1/2
		Brown	51	+	3/19
3	Mar. 31	Brook	11	+	2/2
		Brown	15	+	1/3
4	Apr. 13	Brook	17	+	4/6
		Brown	33	−	0/5
5	Apr. 14	Brook	29	+	5/6
		Brown	21	−	0/4
6	May 11	Brook	10	+	2/2
		Brown	43	−	0/8
7	Apr. 14	Brown	49	−	0/10
8	Apr. 14	Brook	1	−	0/1
		Brown	49	−	0/10
9	Apr. 23	Brook	2	−	0/2
		Brown	48	−	0/9
10	Apr. 23	Brown	25	−	0/5
11	Apr. 18	Brown	50	−	0/10

[1] Numbered consecutively downstream.
[2] +=Evidence of infection. −=No evidence of infection.
[3] Number of infected slurries per total slurries.

Table 2.—Occurrence of M. cerebralis *infection in trout collected from tributaries of the North Branch Tobacco River*

Station [1]	Collection date	Trout species	Number of fish	Examination results [2]	Number of slurries
	1970				
A	Mar. 25	Brook	50	−	10
		Brown	1	−	1
B	Mar. 31	Brook	24	−	5
		Brown	39	−	8
C	Apr. 21	Brook	26	−	5
		Brown	28	−	6
D	Apr. 24	Brook	9	−	2
		Brown	10	−	2
E	Apr. 18	Brook	3	−	1
		Brown	47	−	10
F	Apr. 18	Brown	27	−	6

[1] Lettered consecutively downstream.
[2] No evidence of infection.

DISCUSSION

It is apparent that *M. cerebralis* spread down the first 6 miles of stream. The spread was favored by heavy disease incidence at the hatchery, abundance of susceptible trout, and trout movement. The disease at the hatchery was present in rainbow trout in six of seven dirt ponds (unpublished data). This infection of the hatchery was assumed to have begun in the late summer of 1967. Thus, *M. cerebralis* had at least a year to increase in the ponds and infect the stream before it was discovered in August of 1968. The total timespan, from introduction to discovery among native trout in 1970, was about 28 months.

Some infected trout moved upstream into Jose Creek (station 1) above the hatchery outlet. Trout were collected from this stretch of stream, beginning just above the outlet and extending 1,500 feet upstream. The incidence of *M. cerebralis* here was lower than below the outlet (station 2). Above the outlet, 1 of 4 slurries showed infection of brook trout; none in 8 for brown trout. Below the outlet brook trout were infected in 1 of 2 slurries, and brown trout in 3 of 19 slurries.

The pooling of trout into the examination slurries does not give an accurate percentage of infected fish, but brook trout were estimated to be 50 percent infected, and the brown trout 1 percent, in the 6 to 7 miles of stream immediately below the hatchery outlet. The rates of infection should be related to the abundance of the two trout species. In the upper 6 to 7 miles of the North Branch Tobacco River, the brook trout and brown trout are about equally abundant. Progressively downstream the brown trout is more abundant. The natural resistance of brown trout to whirling disease, along with a diminished spore concentration downstream, may explain why brown trout were not infected beyond the first mile or two below the hatchery. The occurrence of whirling disease in brook trout and brown trout in the Tobacco River corresponds to host reports from the United States [*2*].

Based on the results of this study, Jose Creek, the North Branch Tobacco River, and their tributaries were treated in 1970 to remove the infected fish population [*1*].

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A New Collection Method of Actinosporeans—A Probable Infective Stage of Myxosporeans to Fishes—from Tubificids and Experimental Infection of Goldfish with the Actinosporean, *Raabeia* sp.

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We tried several transmission experiments of naturally infected actinosporeans in the tubificid oligochaete, *Branchiura sowerbyi*, to myxosporean-free goldfish, *Carassius auratus*.

Almost all actinosporean spores collected by squashing tubificids or filtering effluent from a container with tubificids were physically damaged and/or unclean, and all infection experiments using such spores failed. However, coexistence of uninfected goldfish with a stock of *B. sowerbyi* shedding spores of several actinosporeans in the same container resulted in infections with three species of myxosporeans: *Zschokkella*, *Myxobolus*, and *Thelohanellus* spores in 15-20% of goldfish after 2.5-4 months. We improved the collection method of actinosporeans; tubificids were individually placed in the cell-well plates with 24 2 m*l*-wells and waterborne actinosporeans were detected by inverted phase-contrast microscopy. Actinosporeans harvested by this method were intact and purely concentrated. Eventually, in goldfish kept with tubificids harbouring the actinosporean *Raabeia* sp. in the same tank for 3 weeks, and in goldfish exposed to *Raabeia* suspension for 1 hour, spores of an unidentified *Myxobolus* were found 3-4 months after the exposure. Unexposed goldfish and ones exposed to actinosporean-free tubificids were both negative for the infection.

We developed the collection method of actinosporeans, and confirmed that the actinosporean *Raabeia* infecting the oligochaete *B. sowerbyi* was transformed into *Myxobolus* in goldfish.

Over 1000 species of myxosporeans have been recorded, and some can cause serious losses at fish culture farms (Lom, 1987). However, the life cycle, especially the cycle outside the fish hosts, and the mode of transmission have remained unknown. Until now, all infection trials with fresh spores, coexistence with infected fishes, and feeding the infected tissue have failed (Fryer and Sanders, 1970; Walliker, 1968). But in the studies on *Myxobolus*(= *Myxosoma*) *cerebralis*, the causative agent of whirling disease in salmonid fishes, the infectivity of spores was revealed only after uncharacterized "aging" process in mud for several months (Hoffman and Putz, 1969; Uspenskaya, 1978), whereas Schafer (1968) reported unsuccessful transmission of *Ceratomyxa shasta* with the aged spores. Under natural conditions, the presence of the waterborne infective agent has been suggested by the exposure of susceptible fish to contaminated water (Ching and Munday, 1984; Foot and Hedrick, 1987; Ratliff, 1983), but the agent has not been identified.

Recently, however, a series of studies showed that *M. cerebralis* had a two-host life cycle involving fish and an invertebrate and alternated two different sporogenic stages in the life cycle (Markiw and Wolf, 1983; Wolf and Markiw, 1984; Wolf *et al.*, 1986; Markiw, 1986, 1989a, 1989b). They demonstrated that *M. cerebralis* spores were ingested by the tubificid oligochaete *Tubifex tubifex*, and transformed into the actinosporean *Triactinomyxon gyrosalmo*,

which belongs to another class of the phylum Myxozoa, in *T. tubifex* within 3 months, then the waterborne triactinomyxon spores released from *T. tubifex* became infectious to fish. This hypothesis was once confuted by Hamilton and Canning (1987), but reinforced by El-Matbouli and Hoffmann (1989).

However, whether or not the life cycle generally applies to the other myxosporeans is unknown. El-Matbouli and Hoffmann (1989) showed that *Myxobolus cotti* infecting the nerve tissue of bullhead, *Cottus gobio*, transformed into *Triactinomyxon* in tubificids. Hedrick *et al.* (1989)* also presented that triactinomyxons developed in the oligochaetes which had been exposed to spores of *Myxobolus* sp. from cranial tissues of rainbow trout, *Oncorhynchus mykiss*, and *Henneguya* sp. from the skin. Chilmonczyk *et al.* (1989) reported that PKD (proliferative kidney disease caused by the myxosporean PKX) transmitted in an indoor recirculating unit with sediments containing invertebrate fauna including some tubificid worms. But they did not show the involvement of actinosporeans in the worms. More recently, Kent *et al.* (1990) suggested that the infection with *Myxobolus arcticus* in the brain of sockeye salmon, *Oncorhynchus nerka*, involved the aquatic oligochaete, *Eclipidrilus* sp. (Lumbriculidae).

We have been studying myxosporean infections of goldfish, *Carassius auratus*. *Branchiura sowerbyi* is a dominant species of oligochaete in the goldfish ponds, and has been found infected with several species of actinosporeans (Yokoyama, Ogawa and Wakabayashi, unpublished). However, the infection in the oligochaete was not visible from outside, and we had difficulty in collecting enough amount of intact actinosporean spores from the oligochaete for infection experiments. In this study, we introduced a new collection method of actinosporean spores: by this method, infected tubificids were separated easily, and pure and intact spores were collected in great numbers. We, then, tried several transmission experiments using myxosporean-free goldfish.

* Hedrick, R. P., A. Wishkovsky, J. M. Groff and T. McDowell (1989): Int. Conf. Eur. Ass. Fish Pathol., 24–25 Sep., 1989, Spain, p. 38.

Materials and Methods

The tubificid oligochaete, *Branchiura sowerbyi*, was collected from two goldfish farms in Tokyo Metropolis and Saitama Prefecture. Preliminary surveys revealed that those farms were contaminated with several species of myxosporeans in the goldfish and several species of actinosporeans in the oligochaete. Goldfish fry (average body weight 0.2 g), reared in myxosporean-free well water, were used for three exposure tests described below.

"Squash Method" and "Filter Method"

Tubificids were anaesthetized by 0.1% chloretone liquid, and squashed individually on a slideglass, and actinosporeans were harvested after the detection of infected tubificids under phase-contrast microscopy ("squash method").

A stock of *B. sowerbyi* was kept in a 20 *l* running water aquarium. Continuous flow of tapwater was dechlorinated by active carbon and gently supplied at a rate of 20–80 m*l*/min. Waterborne actinosporeans released from *B. sowerbyi* were collected by filtration of effluent from the tank on a 50 μm-mesh screen according to Markiw (1986) ("filter method").

Then, the suspension of actinosporeans harvested by "squash or filter method" were added to goldfish aquaria. The dose of actinosporean was 2.7×10^3–1.5×10^5 spores/*l* in several trials.

Coexistence of a Stock of Oligochaetes with Goldfish

A population of *B. sowerbyi* shedding several species of actinosporeans was placed in a 20 *l* running water tank. Subsequently, 20 myxosporean-free goldfish were held in a netcage in the same tank, and exposed to waterborne actinosporeans.

"Cell-Well Plate Method"

Tubificid worms were individually kept in cell-well plates with 24 2 m*l*-wells (Corning). Each well was filled with dechlorinated tapwater. The cell-well plates with worms were held overnight in an incubator at 20°C. Waterborne actinosporeans were examined under inverted phase-contrast microscopy.

Infection experiments were performed in two

ways. First, myxosporean-free goldfish and tubificid worms harbouring a species of actinosporean, which were separated by the cell-well method, were kept in the same running water tank. The tubificids were held in a glass-dish (28 m*l*) containing mud substrate sterilized at 70°C for 1 hour. The goldfish had been placed in a netcage for 1 or 21 days of exposure, and then were transferred to parasite-free aquaria. Negative controls were goldfish exposed to actinosporean-free tubificids judged by "cell-well plate method". Water temperature was 21–26°C.

Another way of infection experiments was exposure of goldfish to actinosporean spores in a 100 m*l* glass-beaker for 1 hour. Actinosporeans were harvested from infected worms kept in the cell-wells. After the exposure, the goldfish were moved to parasite-free aquaria, and kept at 20°C. Unexposed goldfish were served as negative control.

Examinations of myxosporean infections in goldfish were periodically conducted during 1 to 6 months post-exposure. When dissected, each fish was divided into viscera and other body parts, and minced separately. Wet mounts of minced body parts, and kidney smear preparations stained with May Grünwald-Giemsa were observed under light microscopy (200 or 400× magnifications).

Results

Harvested Actinosporeans

"Squash Method"—The suspension of actinosporean spores contained immature stages and tissue residue of squashed worms. Immature spores were quite fragile and spontaneously lost their sporoplasm in a short time.

"Filter Method"—Only mature spores were collected, but phyto- or zooplankton and undefined matters were contaminated and attached to the spore processes. Almost all actinosporean spores were physically damaged and some entangled each other. In many cases, mixed species of actinosporeans were collected.

"Cell-Well Plate Method"—Mature spores were released from infected tubificid worms in the wells. Prevalence of infection with actinosporeans was 0.1 to 4.1%, and mixed infections

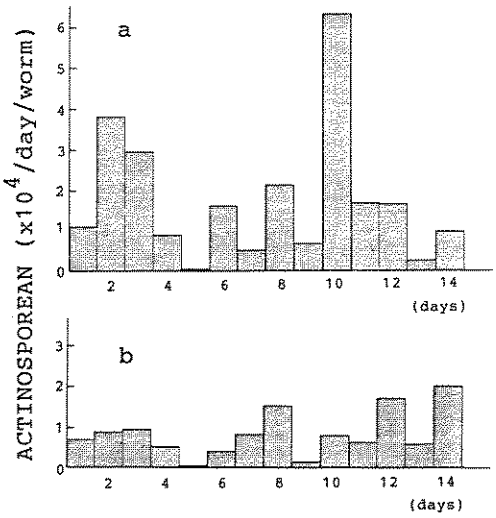


Fig. 1. Daily harvests of the waterborne actinosporean *Raabeia* from two individuals (a, b) of *Branchiura sowerbyi* in the cell-well at 20°C.

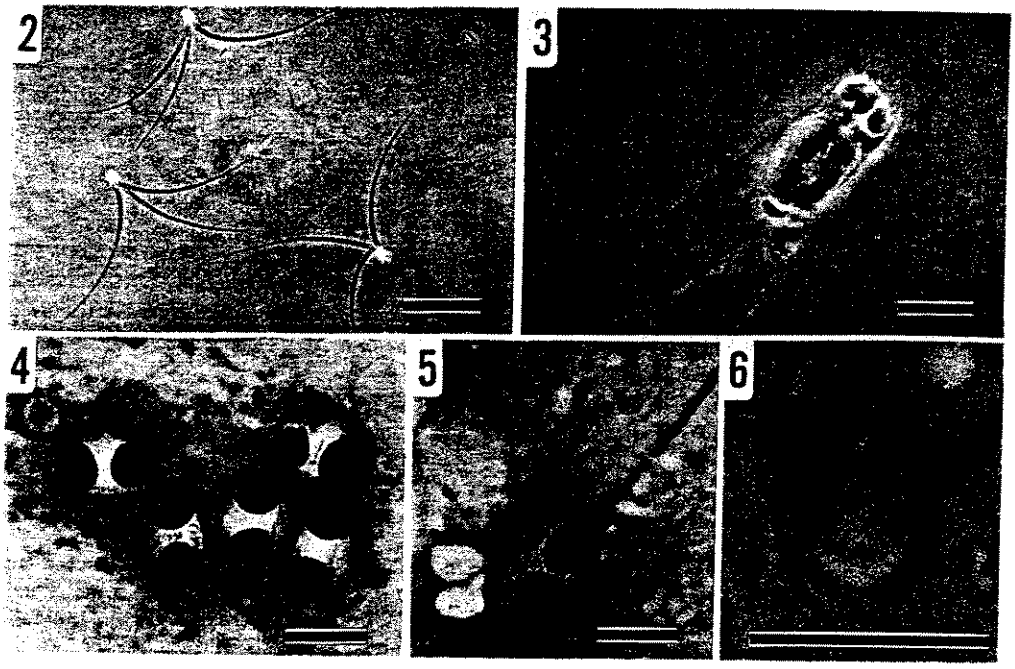
with different species in one individual were very rare. Emergence of actinosporean spores from infected tubificids continued for at least 2–3 weeks. Daily harvests of released spores per worm varied considerably and were 1–2× 10^4 spores/day/worm on average (Fig. 1). *B. sowerbyi* placed in the wells became gradually weakened, but survived for at least 3 weeks without being fed. Collected spores were intact and purely concentrated (Fig. 2).

Transmission Experiments

"Squash or Filter Method"—A total of 530 fish exposed to actinosporean spores harvested by "squash method" or "filter method" and unexposed fish (total of 35 fish) were negative for infection 1 to 6 months after the initial exposure.

Coexistence of a Stock of Oligochaetes with Goldfish—2.5 to 4 months after initial exposure, three species of myxosporeans—*Zschokkella* sp. (Fig. 4) from the gallbladder, *Myxobolus* sp. and *Thelohanellus* sp. (Fig. 5) from the minced body parts excluding visceral organs—were detected in the goldfish. Prevalences of infection with the three myxosporeans were 15–20% (Table 1).

"Cell-Well Plate Method"—In the goldfish



Figs. 2–6. 2. Phase-contrast photograph of waterborne *Raabeia* released from *B. sowerbyi* in the cell-well plate. Note the intact and purely concentrated spores. Scale bar=100 μm. 3. Epispore of *Raabeia* topped with three polar capsules. Scale bar=10 μm. 4 and 5. Myxosporeans from goldfish held with a stock of *B. sowerbyi* shedding several species of actinosporeans in the same tank. 4; *Zschokkella* sp. 5; *Thelohanellus* sp. May-Giemsa stain. Scale bars=10 μm. 6. *Myxobolus* sp. detected from the goldfish exposed to *Raabeia* suspension. May-Giemsa stain. Scale bar=10 μm.

Table 1. Results of coexistence of goldfith with a stock of *B. sowerbyi* shedding several species of actinosporeans in the same tank

Detected myxosporeans in goldfish	Prevalence of infection at				
	2.5 Mo	3.0 Mo	3.5 Mo	4.0 Mo	Total
Zschokkella sp.	1/5*	0/5	0/5	2/5	3/20
Myxobolus sp.	1/5	2/5	1/5	0/5	4/20
Thelohanellus sp.	0/5	1/5	3/5	0/5	4/20

* No. of fish infected/No. of fish examined.

kept with the tubificids harbouring an actinosporean species in the same tank for 3 weeks, and in the goldfish exposed to the actinosporean suspensions, *Myxobolus* spores were detected from minced body parts excluding viscera organs and kidney smear preparations 3–4 months after exposure. Prevalences of infection with *Myxobolus* were 20–60% (Tables 2 and 3).

Spores of the actinosporean consist of three long epispore processes, about 200 μm in length, and the epispore which is cylindrical and about 9 μm in diameter, topped with three polar capsules, and a style is lacking between the epispore and the processes (Fig. 3). Therefore, we identified this actinosporean as a member of the genus *Raabeia* according to Janiszewska (1955, 1957).

Spores of the myxosporean *Myxobolus* were oval in shape, a mean of 10.0 (a range of 8.7–10.7) μm in length, 6.2 (5.3–6.7) μm in width. Polar capsules were equal in size, 3.3 (3.0–3.7 μm in length (Fig. 6). The site of infection was not determined. This species was the same as the one recovered in the goldfish, which

Table 2. *Myxobolus* sp. infection in goldfish kept with *B. sowerbyi* harbouring *Raabeia* in the same tank

Duration of exposure	Prevalence of infection in goldfish after initial exposure		
	2 Mo	3 Mo	4 Mo
1 day with worms harbouring *Raabeia*	0/10*	0/5	0/5
21 days with worms harbouring *Raabeia*	0/10	1/5	1/5
1 day with actinosporean-free worms	NC**	0/5	0/5
21 days with actinosporean-free worms	NC	0/5	0/5

* No. of fish infected/No. of fish examined.
** NC: Not Checked.

Table 3. *Myxobolus* sp. infection in the goldfish exposed to *Raabeia* suspension for 1 hour

Concentration of *Raabeia* sp. (spores/*l*)	Prevalence of infection in goldfish after exposure			
	1 Mo	2 Mo	3 Mo	4 Mo
9.1×10^6	0/5*	0/5	2/4	2/5
1.0×10^7	NC**	NC	1/5	1/5
6.0×10^5	NC	NC	1/5	3/5
Unexposed	NC	NC	0/5	0/5

* No. of fish infected/No. of fish examined.
** NC: Not Checked.

had been held with a stock of *B. sowerbyi* (p. 135).

Discussion

Actinosporean infections in *Branchiura sowerbyi* were not recognizable from the outside, whereas infected *Tubifex tubifex* was characterized by its abnormal coloration and swelling appearance (Wolf *et al.*, 1986). This difference seems to be due to difference in size of tubificids; *B. sowerbyi* was much larger than *T. tubifex*, and grew more than 10 cm at maximum. Therefore, so far, we had to squash all tubificids or trap waterborne actinosporeans by filter mesh in order to collect actinosporeans. However, actinosporean spores collected by "squash method" or "filter method" were not suitable for infection experiments to goldfish; they were immature, damaged and/or unclean. Furthermore, we could not harvest spores smaller than 50 μm by "filter method".

The infection method of coexistence with a stock of tubificids in a same tank could avoid the physical damages during experimental procedures, but wild populations of tubificids harboured several species of actinosporeans, so we could not determine the corresponding infective stages of the three species of myxosporeans in goldfish.

"Cell-well plate method", on the other hand, has many advantages for studies of actinosporeans as follows.

1) Pure, intact, and mature spores, even though they are small enough to pass through filter meshes, are easily available in large quantity.

2) Intact oligochaetes infected with a known species of actinosporean are available.

3) Detection of actinosporean infections are easy and rapid.

Prevalence of actinosporean infections in oligochaetes under natural environments has been known to be very low: only 0.3–2.0% (Mackinnon and Adams, 1924; Markiw, 1986). The conditions for the experimental production of actinosporean spores have not yet been established. However, the "cell-well method" makes it possible to collect pure spores even from natural sources.

The spores of *Myxobolus* in the goldfish exposed to *Raabeia* were detected from the kidney as well as minced body parts after removal of viscera, and probably spores in the kidney were transported from the site of infection to melanomacrophage centers in the kidney by the host's defence reactions (Dykova, 1984). Determination of the site of infection and more detailed description of this *Myxobolus* sp. needs further study.

We speculate that the infections with *Zschokkella* and *Thelohanellus* induced by holding *B. sowerbyi* and goldfish in the same tank may also be the results of transformation of other actinosporeans into the myxosporeans.

In conclusion, we improved and developed the collection method of actinosporean spores, and demonstrated that the actinosporean *Raabeia* infecting the oligochaete *Branchiura sowerbyi*

was transformed into *Myxobolus* in goldfish.

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Whirling Disease in Utah Salmonids

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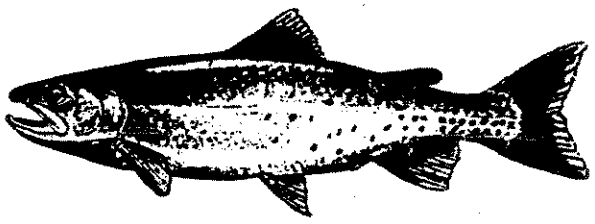
During 1991 whirling disease, caused by Myxobolus cerebralis, was reported for the first time in Utah. This protozoan (myxosporidan) has been found in salmonids in states surrounding Utah but had not been detected in the beehive state until June 1991 when it was reported at a private aquaculture facility in the Bicknell-Loa region. The Ichthyogram a newsletter of the Utah Fisheries Experiment Station (Logan) has published excellent articles pertaining to the eradication and control of whirling disease. Several articles in local newspapers have informed the public of the parasitic protozoan in Utah. Our laboratory at Brigham Young University was contacted when the disease was found at the Rainbow Pond, Tooele Army Depot for confirmation of M. cerebralis presence. A definitive diagnosis of infected fish requires killing the suspect host and removing a core of bone from the cranium. During the past two years we have investigated non-destructive techniques of detecting M. cerebralis. The purpose of this article is to review the biology of M. cerebralis and then list preliminary data for the non-destructive detection methods.

Myxobolus cerebralis a myxosporidan protozoan which was discovered in Europe in 1903 and has spread to other countries including the United States is the causative organism of whirling disease.

The development of the parasite takes approximately four months at which time the spores can be demonstrated in wet mounts made from scrapings of cranial skeleton and auditory capsule (organ of equilibrium). The spores remain in the cranial tissue until the infected fish dies. The spores are used as a definitive diagnosis for the disease.

This disease derives its name from the rapid, tail-chasing type of whirling which is often seen when infected fish are frightened or try to feed. The whirling symptom is associated with lesions and disintegration of the cartilaginous skeletal support of the organs of equilibrium caused by invasion by M. cerebralis into the tissue. The damage to the cranial skeleton is evident in older trout as a depression in the head or as misshapen jaws. Sometimes the spinal column is affected resulting in a change in the spinal curvature. Pressure on the nerves which control the caudal pigment cells results in "blacktail" in many fish. Symptoms may appear as early as two weeks after the salmonid fry start feeding and in heavily infected groups of fish there may be many mortalities. High mortalities due to M. cerebralis are under question and investigation.

Trout usually become infected during the first few weeks of feeding, mortalities ensue and most of the survivors exhibit disease symptoms for 3 or more years. The spores gain entrance to the fish, presumably through accidental ingestion, and the sporoplasm of the spore emerges and migrates to the cartilage, mainly that of the head. The very small sporoplasm, now called a trophozoite, grows and its nuclei divide repeatedly to form a much larger organism which finally produces the spores. During the growth of the parasite much host cartilage is eroded and the skeleton becomes weakened, resulting in the symptoms - whirling, black tail, gaped jaws, misshapen heads and trunks. Whirling disease was diagnosed in the U.S. in 1958 by S.F. Snieszko. Whirling disease primarily affects fry and fingerling fish. Older fish are more resistant to the pathogen due to osteogene-



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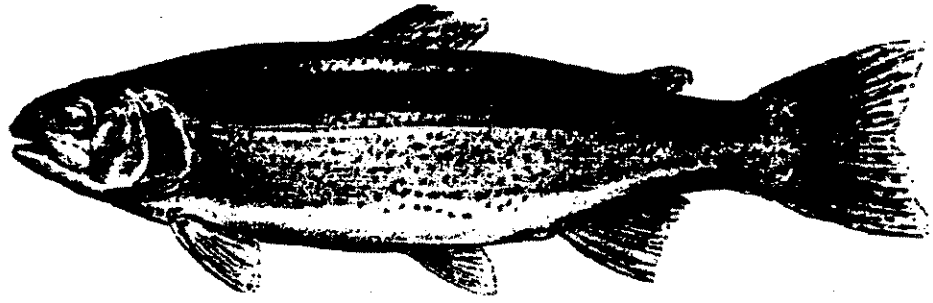
sis. Age of fish, number of infective agents, and water temperature are important factors in susceptibility to whirling disease. Older fish become carriers for the disease. Spores are environmentally persistent which makes the disease very difficult to control. Identification of the pathogen is based on the size and shape of the spores. Myxobolus cerebralis spores can be confused with other myxosporidans, notable; M. squamalis (found in scale pockets), M. kisutchi (found in the central nervous system), M. nevrobius, and Myxobolus sp. (found in brain tissue). Enhancement of finding spores in prepared sections is through the sue of suitable stains.

The life cycle of the pathogen is not well understood though it was proposed by Wolf and Markiw 1985 that a tubificid (Tubifex sp.) worm serves as an intermediate host. Experiments involving transfer of spores to tubificid worms from nonenzootic waters have produced Triactinomyxon gyrosalmo in the tubificid worms following a 104 day incubation period. Feeding tubificid worms harboring the Triactinomyxon gyrosalmo to "clean" salmonids produces whirling disease in trout following a 45 day period. Researchers in Germany (El-Mathbouli and Hoffman) have corroborated this proposed whirling disease life cycle. Additional research at the University of California, Davis has failed to corroborate this life cycle. No Triactinomyxon gyrosalmo could be isolated from the California studies. Additionally, no Tubifex worms could be located in sites in the wild that harbored fish positive for whirling disease in California.

Infected fishes are commonly found in facilities which have earthen ponds or raceways, or open water sources that can be easily infected with the M. cerebralis. Where the disease occurs in the wild, conditions are often typified by low stream gradient and high alkalinity.

Treatment has ranged from the severe (destruction of fish and depopulation of facility including abandonment and burial of facility sites) to managing with and around the pathogen. Hundreds of tons of fish have been destroyed and millions of dollars spent in attempts to eradicate the pathogen with limited success. These efforts have shown that enclosing open spring sources and using concrete fish rearing facilities offers protection against whirling disease. The environmentally persistent spores make the disease virtually impossible to eradicate in the wild once it is established without eliminating the intermediate and definitive hosts. The mud bottom is conducive to spore survival and disease spread.



Losses at fish cultural facilities directly attributable to whirling disease are minimal. Much of the reported mortality appears to be associated with other pathogens and secondary infection. Whirling disease lowers resistance of fish towards other diseases.

Reported impacts on wild populations are thought to be minimal. However, there has been no detailed study of susceptible young fishes in areas where whirling disease has been discovered in the wild. It is important that such work be launched because the introduction of whirling disease in wild stocks represents an irreversible decision permitting environmentally persistent spores to become established in the wild. Also the spread of this disease can be maintained in wild populations representing a "reservoir" for cultured fish.

Most state regulating agencies have regulations governing import of fish into the State but do not have regulations governing intrastate movement of live fish. There is no mechanism in place to detect and contain the disease if it should be introduced into a State and it may spread over a considerable range before detection. It has been proposed by numerous experts that Whirling Disease is not as

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serious a disease as once thought.

Non-Destructive Detection Methods: Preliminary Results.

Our laboratory assumed that it may be feasible to detect Whirling Disease (presumptive diagnosis) without killing the suspect host. The accepted method of examining salmonid fishes for whirling disease requires the death of the host. This represents a substantial loss to fish farmers when samples from each fish lot are examined for fish certification. Samples of infected and non-infected fish from Idaho were examined with SEM and X-ray microprobes. Data from the latter technique showed a marked reduction in potassium and calcium ions in infected fish. This was the reason for considering non-destructive testing of fish. Blood samples, from 10 infected and 10 non-infected fish were obtained using standard methods, were stored in microtainer and sarstedt phosphatase tubes and analyzed with a Adam automated analyzer, Hitachi EMD 737 which registers results for 14 blood parameters. The average acid phosphatase is higher for infected fish (1.6 IU/L) than non-infected fish (1.12 IU/L) while alkaline phosphatase, inorganic phosphate, blood calcium, magnesium and sodium are lower (53.0/103.30 IU/L; 15.7/20.4 MG/DL; 10.7/15.3 MG/DL; 3.2/4.0 MG/DL; 153/161 MEQ/L infected/non-infected). The Adam Analyzer requires 0.1 ml of blood which is feasible for non-destructive analysis of brood stock in a fish hatchery.

We are continuing with the analysis of blood form infected and non-infected fish from Idaho through the cooperation of the Eagle State Fish Disease Laboratory (K. Hauck) and hope to see a blood profile unique for Myxobolus cerebralis. These data will be compared with blood profiles for related parasitic, bacterial and viral fish diseases to see if it is unique.

Editors Note: Dr. Heckman can be reached at (801) 378-2495. His address is 109 WIDB, Zoology Department, Brigham Young University, Provo, Utah, 84602.

Heckmann, R.A. 1993. Characteristics of two fish parasites introduced into Rocky Mountain region (United States): Effect on fish. Fish Health Section: Asian Fisheries Society Newsletter. 4: 2-3.

CHARACTERISTICS OF TWO FISH PARASITES INTRODUCED INTO ROCKY MOUNTAIN REGION (UNITED STATES): EFFECT ON FISH

During the past decade, parasites previously not present in the Rocky Mountain Region have been found in fish. These symbionts have had a detrimental effect on the host. Two examples are : whirling disease caused by *Myxobolus cerebralis* and the Asian fish tapeworm *Bothriocephalus acheilognathi*. The origin of these parasites can be attributed to new fish introductions like grass carp and minnows or to poor management of aquaculture facilities. Fish introduced into a new area should be quarantined or certified parasite free before being released into the aquatic environment.

WHIRLING DISEASE: *Myxobolus cerebralis*

Myxobolus cerebralis is the causative organism of whirling disease, a myxosporidian protozoan which was discovered in Europe in 1903 which has spread to other countries including the United States, is the causative organism of whirling disease. The development of the parasite takes approximately four months at which time the spores can be demonstrated in wet mounts made from scrapings of the cranial skeleton and auditory capsule (organ of equilibrium). The spores remain in the cranial tissue until the infected fish dies. The spores are used as a definitive diagnosis for the disease.

This disease derives its name from the rapid, tail-chasing type of whirling which is often seen when infected fish are frightened or try to feed. The whirling symptom is associated with lesions and disintegration of the cartilaginous skeletal support of the organs of equilibrium caused by invasion by *M. cerebralis* into the tissue. The damage to the cranial skeleton is evident in older trout as a depression in the head or as misshapen jaws. Sometimes the spinal column is affected resulting in a change in the spinal curvature. Pressure on the nerves which control the caudal pigment cells results in "blacktail" in many fish. Symptoms may appear as early as two weeks after the salmonid fry start feeding, and in heavily infected groups of fish there may be many mortalities. High mortalities due to *M. cerebralis* are under question and investigation.

During 1991, whirling disease, caused by *Myxobolus cerebralis*, was reported for the first time in Utah. This protozoan (myxosporidan) has been found in salmonids in states surround Utah but had not been detected in the Beehive State until June 1991 when it was reported at a private aquaculture facility in the central region of Utah. The definitive diagnosis of infected fish required killing the suspect host and removing a core of bone from the cranium to find the spores.

Infected fishes are commonly found in facilities which have earthen ponds or raceways or open water sources that can be easily infected with the *M. cerebralis*. Where the disease occurs in the wild, conditions are often typified by low stream gradient and high alkalinity.

Trout usually become infected during the first few weeks of feeding, mortalities ensue, and most of the survivors exhibit disease symptoms for three or more years. The spores gain entrance to the fish, presumably through accidental ingestion, and the sporoplasm, now called a trophozoite, grows and its nuclei divide repeatedly to form a much larger organism which finally produce the spores. During the growth of the parasite, much host cartilage is eroded and the skeleton becomes weakened, resulting in the symptoms - whirling, black tail, gaped jaws, misshapen heads and trunks. Whirling disease was diagnosed in the U.S. in 1958 by S.F. Snieszko. Whirling disease primarily affects fry and fingerling fish. Older fish are more resistant to the pathogen due to osteogenesis. Age of fish, number of infective agents, and water temperature are important factors in susceptibility to whirling disease. Older fish become carriers for the disease. Spores are environmentally persistent which makes the disease very difficult to control. Identification of the pathogen is based on the size and shape of the spores. *Myxobolus cerebralis* spores can be confused with other myxosporidians, notably *M. squamalis* (found in scale pockets), *M. kisutchi* (found in the central nervous system), *M. nevrobius*, and *Myxobolus* sp. (found in brain tissue). Enhancement of finding spores in prepared sections is through the use of suitable stains.

Treatment has ranged from the severe (destruction of fish and depopulation of facility including abandonment and burial of facility sites) to managing with and around the pathogen. Hundreds of tons of fish have been destroyed and millions of dollars spent in attempts to eradicate the pathogen with limited success. These efforts have shown that enclosing open spring sources and using concrete fish rearing facilities offers protection against whirling disease.

The environmentally persistent spores make the disease virtually impossible to eradicate in the wild once it is established without eliminating the intermediate and definitive hosts. The mud bottom is conducive to spore

survival and disease spread. A tubificid worm has been shown as an intermediate host in the life history of whirling disease.

Losses at fish cultural facilities directly attributable to whirling disease are minimal. Much of the reported mortality appears to be associated with other pathogen and secondary infection. Whirling disease lowers resistance of fish toward other diseases. The appearance of infected fish (misshapen heads, curved and blunted tails) negates their commercial value.

Reported impacts on wild populations are thought to be minimal. However, there has been no detailed study of susceptible young fishes in areas where whirling disease has been discovered in the wild. It is important that such work be launched because the introduction of whirling disease in wild stocks which represents an irreversible decision permitting environmentally persistent spores to become established in the wild. Also, the spread of this disease can be maintained in wild populations representing a "reservoir" for cultured fish.

Most state regulating agencies in the United States have regulations governing import of fish into the State but do not have regulations governing intrastate movement of live fish. There is no mechanism in place to detect and contain the disease if it should be introduced into a state, and it may spread over a considerable range before detection. It has been proposed by numerous experts that Whirling Disease is not as serious a disease as once thought.

ASIAN FISH TAPEWORM: *Bothriocephalus acheilognathi*

The Asian fish tapeworm *Bothriocephalus acheilognathi* (Yamaguti, 1934) has been introduced into the United States through shipments of grass carp *Ctenopharyngodon idella* which were brought into this country from China to control aquatic vegetation. The Asian fish tapeworm has spread from its initial introduction in the southern part of the United States to the western part due to infected fish introductions. *Bothriocephalus acheilognathi* is considered one of the most dangerous pseudophyllidean cestodes for cultured carp in Europe, and now it is the western states of the United States. This species, first described from fish in Japan, is common in the intestine of grass carp cultured in South China. From China, cestode infections have followed grass carp imports into Europe, Russia, and the United States.

Bothriocephalus acheilognathi appeared in introduced and endemic fishes in the Virgin River in Utah. Many fish in this river are considered endangered species. Presence in the Virgin River is attributed to the use of live bait minnows by fishermen in a lake receiving the Virgin River drainage.

The Asian fish tapeworm is also found in states bordering Utah due to the introduction of grass carp, *Ctenopharyngodon idella*, and the use of live minnows for fish bait.

The best known carp parasite transported to the fish ponds of many countries with the Chinese carp is *Bothriocephalus acheilognathi* (= *B. gowkongensis* = *B. opsalichthydis*). All European countries that culture carp in large quantities now have this pathogen.

The spread of this parasite to new localities usually results in heavy infection of young fishes during the first year after it appears. *Bothriocephalus acheilognathi* can infect many fish species. Presumably it travelled to the United States by airplane in grass carp shipped from Asia.

Bothriocephalus acheilognathi is characterized by viper-like or arrow shaped scolex and numerous microtriches. This parasite, spreading into new localities, results in heavy infections of fishes, especially cyprinids.

In Europe, it has also been found in European catfish, guppies, mosquito fish, and other species. In the United States it has been found in golden shiners and fat-head minnows, as well as in grass carp, Colorado squawfish, and mosquito fish. In the western United States, *B. acheilognathi* has been found in *Notemigonus crysoleucas, Pimephales promelas, Ctenopharyngodon idella, Gambusia affinis, Gila robusta, Rhinichthys osculus, Lepidomeda mollispinis, Plagopterus argentissumus* and *Ptychocheilus lucius* and recently in *Cyprinella lutrensis.*

Praziquantel (Droncit, Bayvet Div., Cutter Lab) has been successfully used in veterinary and medical experiments against cestodes and trematodes in mammals and larval Digenea in fishes. This drug can be used for treating fishes infected with the Asian fish tapeworm. Fish can be purged with a treated bath of praziquantel (2 ml., injectable per 50 gallons water).

The major management problem for fish infected with *B. acheilognathi* is that this cestode is non-host specific and uses two host (Copepod, Fish) in its life history instead of the usual three hosts for cestodes. The adult stage occurs in fish and the larval state in copepods. There are other cestodes in fish that look like the Asian fish tapeworm. Suspected infections of the Asian fish tapeworm should be confirmed by a qualified fish parasitologist.

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WHIRLING DISEASE

Dizzying disease makes life difficult for DWR Hopes of containing it to a small area dashed

Y LAVARR G. WEBB

anaging Editor

At least Mark Leavitt can still crack a wry joke.

"We're telling people to come on down to the Road reek Ranch and give us a whirl," he deadpanned.

What is not so funny is that a dreaded fish sickness amed whirling disease has cost Leavitt's Road Creek anch trout-raising operation hundreds of thousands of ollars and could yet cost ıore in fines and sanctions – and the operation could ven be shut down.

The disease is no joke, ither, to the Utah Division f Wildlife Resources. The gency spent some $100,000 ı a massive chemical reatment program to try to ontain the disease, only to iscover it recently outside he treatment area in Mill Meadow Reservoir, an mportant trout water in Wayne County.

"This is just a long saga, it keeps going on and on," aid DWR fisheries chief Bruce Schmidt. No plan has een developed yet to combat the disease in Mill leadow. Tests must be done on all waters in the urrounding area, including Fish Lake and Johnson Reservoir, to determine how widespread the disease is efore settling on a plan of action. So far, containment ıas failed.

"We have a lot more studying to do," said Schmidt, bviously agitated over the matter. "We're just trying to ssess the facts." The disease could also have serious onsequences for Utah's sport fishing community.

Initially, the disease had been discovered in a number f private fish runs, most of which had received fish from he Road Creek Ranch operation in Wayne County.

One big question is where the disease came from. Utah isheries managers have prided themselves for years ecause Utah was free of the disease, a chronic, lebilitating conditions that can cause deformities and equilibrium losses in trout and salmon.

But now the disease is here, apparently in a fairly big way, and DWR officials would like to find out where it came from. At first, most fingers were pointing at the Road Creek operation, where the disease was first discovered. But Leavitt and his brother Dane, a co-owner of the fish farm, have been adamant all along that they did not bring the disease to Utah. They say they are victims of the disease like everyone else.

The implication is that the disease could have been in Utah for some time and the DWR would not have known it.

The fact that the disease has now been found in Mill Meadow, well above their fish-raising business, is clear proof that the disease did not originate at Road Creek, Dane Leavitt said. It would be physically impossible for a Road Creek trout to get up the stream and into Mill Meadow, he said.

He also said the disease appeared to be much further advanced in some of the Mill Meadow fish than it was in the private hatchery fish, indicating the disease has been in Mill Meadow longer. Schmidt said the length of time the disease has been in Mill Meadow is pure speculation.

At least some DWR officials are apparently coming around to that view that Road Creek didn't bring the disease to the state. DWR director Tim Provan said the Road Creek Ranch people have cooperated well with the investigation and biologists are now assuming that Road Creek was not the source of the disease.

However, that does not mean that Road Creek is off the hook. The operation is still being investigated for violating regulations governing the movement of fish from one hatchery facility to another. If the Wildlife Board determines that regulations were clearly and flagrantly violated, then Road Creek could come under some severe sanctions, even losing its ability to operate.

Mark Leavitt is hopeful that won't happen. He acknowledges that fish were moved, but he feels any violations were technical. He would have had to kill

hundreds of thousands of dollars worth of fish if he didn't move some of them, he said. As it turned out, many fish had to be destroyed anyway.

The Leavitts want the DWR to proceed quickly with whatever citations are to be issued, so the matter can be resolved. The investigation has been going on for four months.

"This has really wrenched our lives and emotions," Mark Leavitt said. "It has cost us hundreds of thousands of dollars, but it is our reputations that we are most concerned about. We really want to get it resolved and behind us."

Meanwhile, the discovery of the disease in Mill Meadow dashes the hopes of DWR officials who had hoped to contain it to a small area.

Dane and Mark Leavitt believe the disease might have been in the state for a number of years. They said the DWR testing method didn't find the disease and it took a private fish pathologist using a different method to first detect the disease. The implication is that the disease could have been in Utah for some time and the DWR would not have known it.

Schmidt said that is simply not true. A DWR fish pathologist failed to detect the disease in one test because of human error, he said, but there has never been anything wrong with the testing method. The disease would have been readily detected had it been around for any period of time in any of the state's hatcheries or waters where eggs are taken, he said.

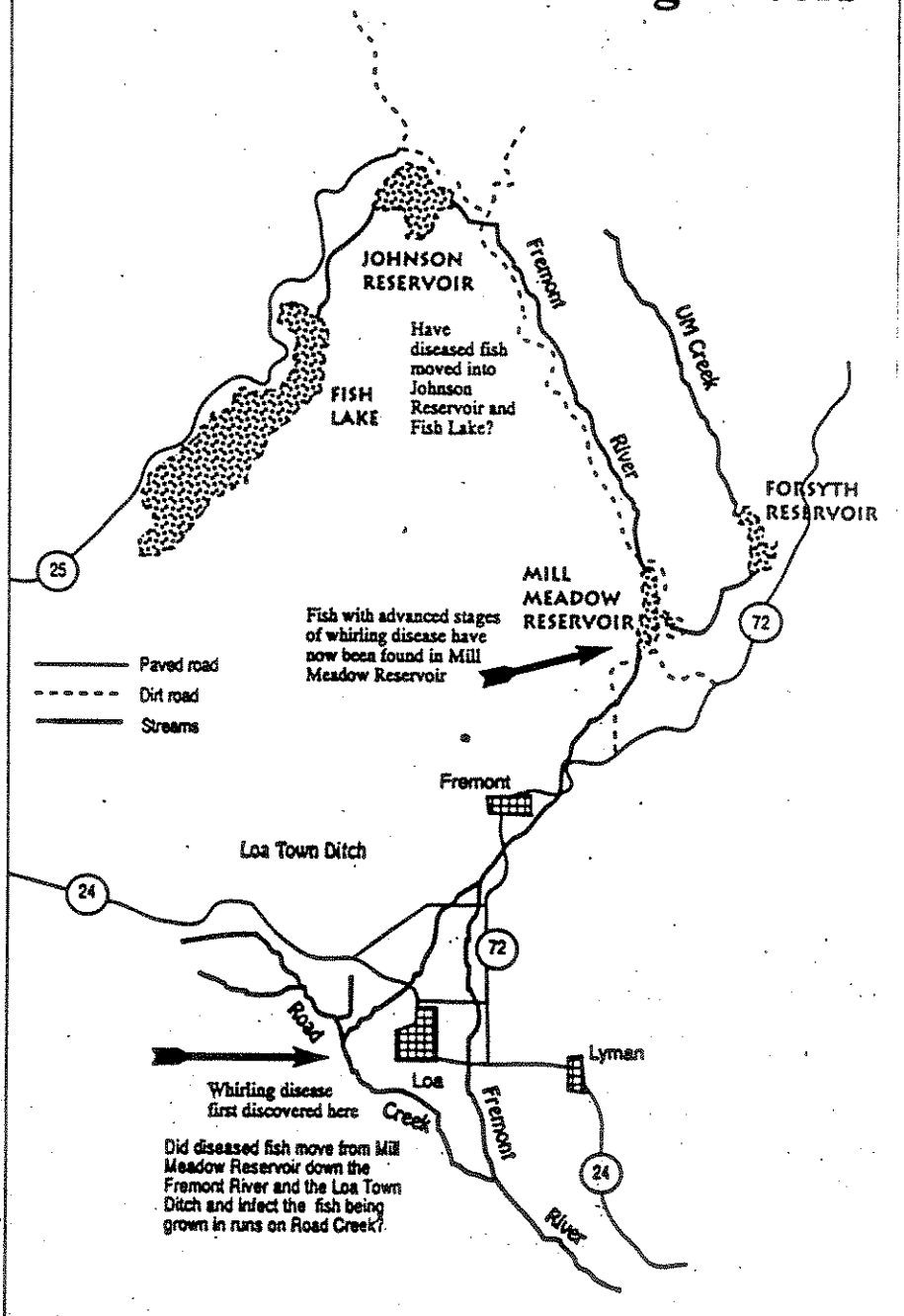
The Road Creek officials suspected all along that the disease had come down from above their operations. Dane Leavitt said they suggested months ago to the DWR that Mill Meadow be checked for the disease.

The Road Creek operators have long held that whirling disease is not as serious as DWR officials say it is. "They treat it like it's the bubonic plague when it's more like the common cold," Mark Leavitt said. "It might have been around for 10 years with no real impact on the fishery."

If the disease is widespread, then Utah is going to have to learn to live with it, Leavitt said, as many other states are doing.

A bigger issue that will have to be addressed later, he said, is how the private aquaculture business is regulated. Some regulations being proposed by the DWR would destroy the industry, he said. "If Utah wants to have an aquaculture industry, then we're going to need to have some dialogue on how it is regulated."

Area map of the Mill Meadow Reservoir and surrounding waters



The Ichthyogram

Volume 4 Issue 1 — STATE OF UTAH NATURAL RESOURCES — April 1993

◆ **Fish Health in Utah**

Whirling Disease Discovered at New Sites in Utah!

A recent examination of rainbow trout from a commercial aquaculture facility in Wayne County led to the discovery of a new occurrence of whirling disease in Utah. The fish were purchased in December, 1992 from Trout of Paradise in Cache County in northern Utah. The whirling disease parasite was discovered in January 1993. Since it takes three to four months for detectable spores of the parasite to develop, it was concluded that the fish were infected before shipment to Wayne County. Whirling disease was later confirmed in the operation near Paradise on the Little Bear River and a satellite operation near Amalga, Utah on the Bear River. Trout of Paradise had been inspected and found to be free of all prohibited pathogens for several years (as recently as October 1992) through the combined efforts of the Utah Division of Wildlife Resources (DWR) and the U. S. Fish and Wildlife Service. They had made substantial efforts to maintain a disease-free status.

Deformed brown trout with whirling disease from Fremont River in southern Utah. Similar lesions were seen in 8% of brown trout from that area.

The hatchery at Paradise, Utah uses water from the Little Bear River and returns water back into the river. UDWR biologists from the Fisheries Experiment Station and the Northern Region decided that substantial sampling was needed in the Little Bear River drainage. Work in the Fremont River drainage in southern Utah has shown the presence of deformities and spores in various species and year classes of trout. It was hoped sampling might permit some assessment of when and where the parasite first appeared. In the Fremont drainage it was found that whenever the parasite was found in waters with naturally reproducing trout, deformed fish were present. Deformity ranged from 8 to 22% of the trout, depending upon the species and water. Where there was no natural reproduction and only stocked fish were present, there were no deformities though the parasite was found in many of the fish.

In Cache Valley, it was also decided to determine the general health and condition of the sampled fish. This was done to establish base line data needed to determine impacts of the parasite on wild populations. This involves use of a system developed in Utah for assessment of the health/condition profile (HCP) of populations of fish.

Sampling began between Hyrum Reservoir and Trout of Paradise effluent and proceeded upstream and downstream through various sites.
(continued - page 5)

Whirling Disease - *continued*

These included the Little Bear River, East Fork and South Fork of the Little Bear, an irrigation canal carrying water from the South Fork to the East Fork, Porcupine Reservoir, Hyrum Reservoir, Blacksmith Fork River, Logan River, Spring Creek and Summit Creek. Aquawest Inc., another commercial aquaculture facility in Cache Valley, was tested. Sampling was difficult because of snow and ice.

Infected fish were found upstream in the Little Bear River drainage. About 75% of the sampled fish were brown trout. All of them were free of the parasite. Only a few cutthroat trout were found infected. Most of the contaminated fish were rainbow trout. No infected fish were found in the South Fork of the Little Bear. Most were found below the confluence of the East and South Forks of the Little Bear. No deformed fish were found anywhere in these streams. The largest group of infected fish were found immediately above Trout of Paradise. Porcupine Reservoir upstream and Hyrum Reservoir below the contaminated facility were found free of the parasite. Biologists were surprised all fish immediately below Trout of Paradise were free of the parasite, except one small rainbow trout. Considering the life cycle of the parasite, this area should have shown the highest rate of infection. Fish in the Logan River, Summit Creek and Spring Creek were free of the parasite. Fish at Aquawest Inc. and at one site of the Blacksmith Fork have tested positiver. The pattern of infection and the lack of deformity would suggest that the parasite has not been in the river very long.

HCP profiles were determined for many of the sampled fish. The fish had little or no fat storage, but in all other aspects they appeared normal. The low fat reserves were most likely due to the winter and loss of habitat due to the drought. A big surprise was the discovery of two atypical groups of rainbow trout in the Little Bear River drainage. These two groups were more consistent with fish living in a hatchery. When captured, biologists immediately noticed that these fish were physically much more robust than the other fish they had been collecting. The fish showed a pale coloration, quite unlike wild trout. Independently in the laboratory, DWR's fish pathologists found that these fish had heavy deposits of stored fat compared to other fishes in the river. The fins showed active erosion commonly found in hatcheries, especially cement raceways. There were abnormal gills and abrasions on the noses. The profile also showed that the fish had not eaten for at least a couple of weeks. This would suggest that the fish had not learned to eat in the stream. In both cases, these fish were found only in culverts at road accesses.

It was concluded that these questionable fish had not been in the river for more than about two or three weeks. The most recent plants by the DWR were August 1992. Fish having lived in the stream that long would no longer display these characteristics. These fish were also found to be infected with whirling disease. Since it takes three to four months for detectable spores to develop, these fish were probably infected when they were planted. Since these fish were found positive, the DWR hatchery that stocked the stream was retested and found negative for the parasite. DNA testing is being planned in an effort to determine the source of the infected fish .

The source of whirling disease at Trout of Paradise and in the Little Bear River drainage remains a mystery. The finding of infected fish upstream of the hatchery implies the possibility that it may have been contaminated from the river water used in their operation. The locations and appearance of the infected fish raise the distinct possibility of deliberate eco-sabotage of the downstream facilities. In some states, "zone regulations" have been instituted to permit transfer of infected fish only to areas where whirliing disease exists. Wildlife officials in these states have expressed concerns that infected fish have been deliberately stocked by malefactors in uncontaminated areas to expand the range of permissible sales. Biologists are concerned that this scenario may have already occured in Utah. Because of this concern, several private hatcheries in Utah are being tested earlier than usual to ensure they have not been contaminated with the parasite.

Chris Wilson

Hoffman, G.L. 1970. Intercontinental and transcontinental dissemination and transfaunation of fish parasites with emphasis on whirling disease (*Myxosoma cerebralis*). *In*: Symposium on Diseases of Fishes and Shellfishes. Snieszko, S. F. (ed.) American Fisheries Society. p. 69-81.

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INTERCONTINENTAL AND TRANSCONTINENTAL DISSEMINATION AND TRANSFAUNATION OF FISH PARASITES WITH EMPHASIS ON WHIRLING DISEASE (*MYXOSOMA CEREBRALIS*)

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ABSTRACT: The parasites of North American freshwater fishes are very similar to those of Europe and Northern Asia. Of 250 genera and 1000 species in North America, 66 identical species and 118 identical genera are represented in Europe and Northern Asia. The fish parasites of South America are unlike those of N. America but are more nearly like those of Africa and India.

At least 48 species (5 protozoa, 31 monogenea, 5 digenea, 3 nematodes, 1 acanthocephalan, 3 copepods) of freshwater fish parasites have become established on other continents through the transfer of infected live and frozen fish. Five, and probably more, very harmful parasites have been transferred to fishes in foreign countries through the indiscriminate transfer of their hosts. Most notable is *Myxosoma cerebralis* the very devastating agent of whirling disease. Many fishes have been involved in the transcontinental spread of fish parasites but only a few cases are well documented.

Suggestions are made concerning improvements in the fish diagnostic and reporting services. The adoption of methods which are effective in the control of communicable diseases of domestic animals and plants would be appropriate for control of the spread of fish parasites and diseases.

INTRODUCTION

The origin and spread of a fish disease is of great academic and economic interest. There is geological and biological evidence (Lindroth, 1957; Manter, 1963) that the land-mass of the Americas was connected with European and African landmasses. Also, there is parasitological evidence of a circumpolar connection of all arctic land-masses(Van Cleave and Lynch, 1950). Further indication of these former land connections will be presented here. In spite of these land junctions which provided some genera and a very few species of freshwater fish parasites in common, there are many specific and generic differences. In addition, the fish and fish parasite fauna of the southern hemisphere are quite distinct from that of the northern hemisphere and there are evident relationships between South American, African and Indian parasite faunas (Manter, 1963).

WHIRLING DISEASE

The history of dissemination and transfaunation of *Myxosoma cerebralis* can be especially well documented. It is an internationally important disease and the symptoms are usually so spectacular that even nontechnical workers can easily recognize it presumptively in trout and salmon hatcheries.

Although the organism causes no apparent disease in *Salmo trutta,* the effect on *S. gairdneri* is often quite spectacular and devastating. It is well established that *M. cerebralis* is an enzootic European parasite, therefore the rainbow trout, a North American salmonid, is very susceptible to infection and disease. Small trout are most severely affected and if heavily infected many may die. The parasite invades cartilage, erodes it, and weakens the skeletal structure. When the cartilaginous auditory-equilibrium capsule is weakened, the fish go into an erratic, tail-chasing whirl. If a lesion in the

vertebral column pinches the caudal nerves, the posterior one-third of the fish appears nearly black because the pigment cells are not properly controlled. The head skeleton may be damaged badly, causing greatly misshapen heads (Figure 1) which persist for the life of the fish. More rarely spinal curvature may develop and persist. Because of these spectacular symptoms in the rainbow trout it has been possible to follow some of the dissemination of this disease from its probable original loci to many other sites.

Plehn (1924) assumed that the disease organism originated from uncooked codfish fed to the trout. However, it is probable that the spores she saw in the marine fish were *Myxobolus aeglefini*, a common cartilage parasite of Gadidae (Kabata, 1957; Schäperclaus, 1954). Following the export of the rainbow trout (*S. gairdneri*) from the U.S.A. to Germany about 1900, they became infected and whirling disease became first known.

From 1903 until 1952 whirling disease was known only from Germany, France (Schäperclaus, 1931, 1969 [1]) and Denmark (Bruhl, 1926). The disease became known because of the spectacular symptoms in the cultured American rainbow trout. It has since been learned that *Salmo trutta* becomes infected but not diseased, suggesting that *S. trutta* is the original natural host (Bogdanova, 1968; Schäperclaus, 1954, 1969 [1]; Hoffman, Dunbar and Bradford,

[1] Schäperclaus, W. 1969. Personal Communication. 1162 Berlin-Friedrichshagen, Muggelseedamm 310, Institut für Binnenfischerei, Berlin, Germany.

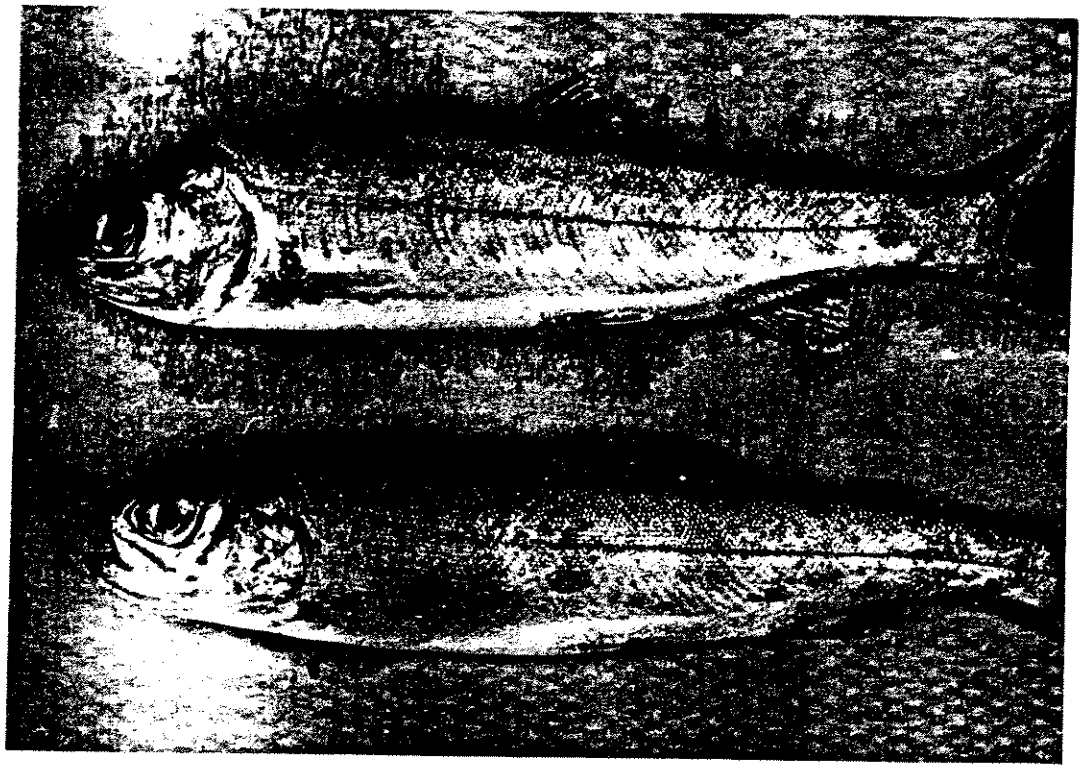

FIGURE 1. Two 13-month-old rainbow trout from a typical epizootic of whirling disease. Note the "sunken head" of the top fish.

1962; Ghittino, 1969 [2]; and Bauer, 1969 [3]). The following recent reports further substantiate this: Uspenskaya (1957) reported whirling disease from rainbow trout near Leningrad where there had been no recent shipments of trout from the west. She found spores of *M. cerebralis* in native *S. trutta* and cultured *S. gairdneri* near the Black Sea. Bogdanova (1960) found trophozoites and spores in *Oncorhynchus* spp. and *Salvelinus malma* on Sakhalin Island (Far East U.S.S.R.). Margaritov (1960) found *M. cerebralis* in Bulgaria. Tinkina (1962) reported it from Korea. Bogdanova (1964) found it in *S. salar* and *S. trutta* near the White Sea (Northwest U.S.S.R.). Bogdanova (1966, 1968) reported finding *M. cerebralis* in trout farms near the Black Sea (Southwest U.S.R.), in trout in Lake Issik-Kul (Southcentral U.S.S.R.) and at trout farms in the Caucasus and Transcarpation Mountains near the Caspian and Black Seas (Southwest U.S.S.R.). Thus, it appears that the original range of *M. cerebralis* covered, at least, a rather large area from Central Europe to Northeast Asia. There is a possibility that it was transferred to some of these places in live rainbow trout or contaminated trout eggs.

It is possible that other European and Asian salmonids are also natural native hosts of *M. cerebralis* because, as listed above, it has been found in *Oncorhynchus* spp., *Salmo salar* and *Salvelinus malma* (Bogdanova, 1960, 1964, 1968).

It is very likely that the range of *M. cerebralis* would have remained stationary for thousands of years because the catadromus trout of various watersheds seldom intermingle. However, following World War II, live rainbow trout were transferred freely in Europe, and later a market for frozen table trout was established and even reached other continents. The viable spores can be transferred in live and frozen trout (Hoffman and Putz, 1969) and thus the disease was disseminated widely. There is also the possibility that viable spores were transferred as a contaminant with egg shipments (Schäperclaus, 1931). Some of the following records clearly indicate a man-made transfer of *M. cerebralis* to cultured trout because *S. trutta* was not native to North America and no trout are native to Africa. It is improbable that *M. cerebralis* was native to Great Britain because the disease did not appear there until a frozen table trout market had been established in Scotland. However, some of the European and Asian fish farms might have gotten spores from wild native *S. trutta*. Following is a list of locations where it is probable that whirling disease was transferred by fish shipments: France (Vanco, 1952); Poland (Kocylowski, 1953); Czechoslovakia (Dyk, 1954; Volf, 1957); Italy (Scolari, 1954; Ghittino, 1962); Bulgaria (Margaritov, 1960); U.S.A. (Hoffman, Dunbar and Bradford, 1962); Sweden (Johansson, 1966); Scotland (Elson, 1968[4]); South Africa (van Wyk, 1966) (Figure 2).

Further indication that the disease was transferred with fish is the fact that it has not been found in those countries which do now prohibit the importation of salmonids—Australia, New Zealand, Japan, England and Canada.

M. cerebralis has rarely been detected in wild salmonids but any salmonid, wild or cultured, which is found in an enzootic area is a suspect carrier and should not be transferred, alive, fresh, or frozen to an area which does not have the disease.

Bogdanova (1960) found that salmon on Sakhalin Island became infected when very young but no spores were present while the small salmon were still in the cold fresh water (3° to 7° C.). However, after the marine sojourn, spores were present in the salmon and could contribute to the infection

[2] Ghittino, P. 1969. Personal Communication. Instituto Zooprofilattico Sperimentale del Piemonte e della Liguria, Via Bologna, 148, Torino, Italy.

[3] Bauer, O. N. 1969. Personal Communication. State Institute of Freshwater Fisheries (GOSNIORKh), Smolnoja 3, Leningrad C-124, USSR.

[4] Elson, K. G. R. 1968. Personal Communication. Marine Laboratory, Dept. Agr. and Fish., Scotland, Torry, Aberdeen.

FIGURE 2. Appearance and dissemination of whirling disease. Countries are numbered in chronological order of first published record of the disease in each country. Solid lines indicate probable route of transfer; broken lines indicate possible route. Numbered locations without arrows indicate probable natural loci of *M. cerebralis* although even here, there is the possibility of man-made transfer. 1. Germany (1904); 2. Denmark (1926); 3. France (1952); 4. Poland (1953); 5. Czechoslovakia (1954); 6. Italy (1954); 7. U.S.S.R., near Leningrad (1957); 8. U.S.S.R., near Black Sea (1957); 9. U.S.A. (1962); 10. U.S.S.R., Sakhalin Island (1960); 11. Bulgaria (1960); 12. Yugoslovia (1960); 13. N. Korea (1962); 14. U.S.S.R., near White Sea (1964); 15. Sweden (1966); 16. U.S.S.R., Lake Issik-Kul, South Central U.S.S.R. (1966); 17. U.S.S.R. Caucasus and Transcarpts (1968); 18. Scotland (1968); 19 South Africa (1968).

of salmon fry. There is no other evidence, except morphological characteristics, showing that the East Asiatic form of *M. cerebralis* is identical with the European form.

OTHER DISEASES AND PARASITES

Whirling disease serves well as an example of parasite origin and the trans- and intercontinental spread of a freshwater fish parasite. The origins of other intercontinental fish parasites are more difficult to trace and their dissemination has not been as well documented. However, the following will show some origins, and some of the intercontinental transfers that have been documented.

I. Intercontinental Origin

A. *North America and Europe*

The similarities in N. American and European fauna and plants are well known (Lindroth, 1957). Similarly, the parasites of the fish of both continents are closely related and some are even morpohologically identical.

Published records show that many genera and species of N. American freshwater fish parasites are also found in Europe, giving

support to a hypothesis of a former land connection (Figure 3).

A few species, *Neoechinorhynchus rutili* and *Nanophyetus salmincola,* for example, have been reported from Asia and North America (see section on circumpolar distribution). I am not aware of any evidence supporting a parallel evolution theory for these parasites. Conversely, there are many genera and species not common to both temperate land masses which indicates that the geologic separation took place a long time ago.

In North America there are approximately 1000 known species of freshwater fish parasites representing 250 genera. Of these, 66 species and 118 genera representing 6 phyla, 12 subphyla or similar, 20 orders and 44 families are found in Europe and northern Asia. Thus, nearly half of the North American genera are represented in Europe and North Asia (Hoffman, 1969). Similarly, of the many genera and 1211 species of European and northern Asia freshwater fish parasites, only 84 genera are not represented in North America. Most noable of these are Coelenterate—*Polypodium;* Trematoda — *Apharyngostrigea, Diplozoon, Metagonimus, Nitzschii, Palaeorchis* and *Paracoenogonimus;* Hirudinea — *Acanthobdella;* Copepods — *Coregonicola, Lamproglena, Pseudotracheliastes* and *Tracheliastes;* Isopoda—*Livoneca;* Acarina — *Hydrachna* and *Hydraphantes* (Bykhovskaya—Pavlovskaya et al., 1962). The parasites show a greater similarity than do the fishes. There are only 16 genera and 21 species of fishes common to these continents. They are *Acipenser medirostris, Anguilla, Coregonus au-*

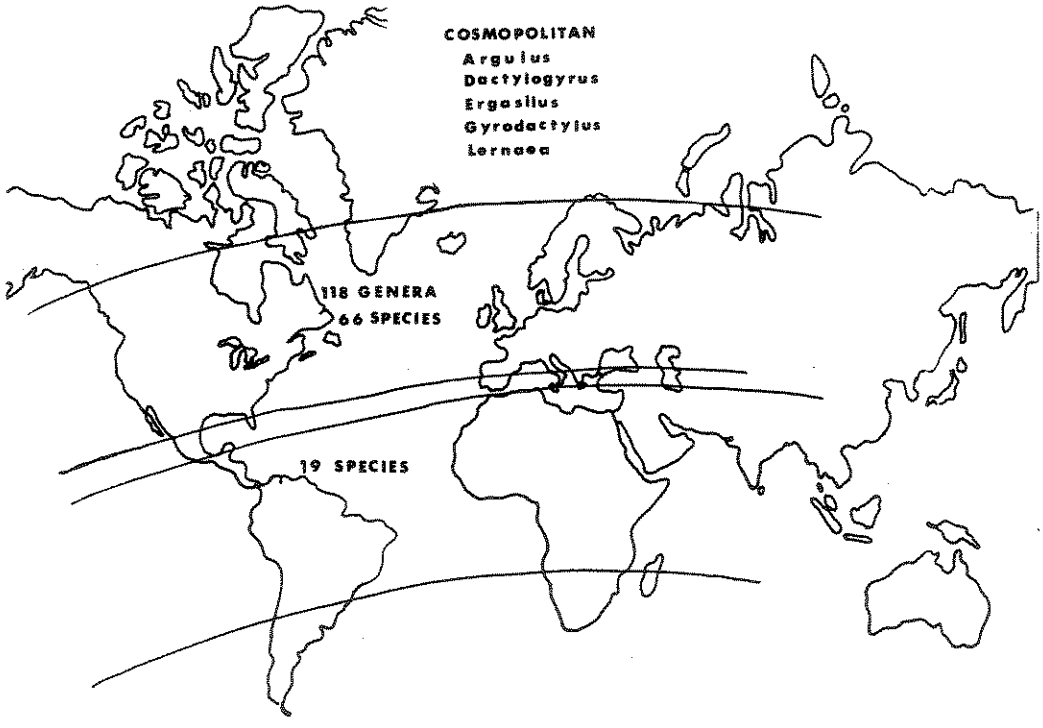

FIGURE 3. Intercontinental origin of some freshwater fish parasites. *Argulus, Dactylogyrus, Ergasilus, Gyrodactylus* and *Lernaea* occur on every major land mass; 118 genera and 66 species are found in common in North America and Europe; at least 19 species are found in common in South America, Africa and India.

tumnalis, C. nasus, C. sardinella, Cottus, Dalla pectoralis, Esox lucius, Gasterosteus aculeatus, Hypomesus olidus, Lota lota, Myoxocephalus quadricornis, M. scorpius, Oncorhynchus gorbuscha, O. keta, O. kisutch, O. nerka, O. tshawytscha, Prosopium, Salmo salar, Salvelinus alpinus, S. malma, Stenodus leucichthys, and *Thymallus arcticus.*

These data further substantiate, as Manter (1963) has suggested, that there was probably a land connection between North America, Europe and North Asia. Studies in entomology (Lindroth, 1957) and paleontology further confirm this hypothesis.

B. *North America, Europe and Asia* (Circumpolar Distribution)

Six species of trematodes, one cestode, three nematodes, one acanthocephalam and one copepod have been reported from all three land masses (Hoffman, 1969).

C. *North America and Asia*

Three nematodes have been reported from North America and Asia but not Europe (Hoffman, 1969).

D. *North America and South America*

Seven species of trematodes have been reported from both continents (Manter, 1963; Price, 1967a, 1967b; Price and Schlueter, 1967).

E. *South America, Africa and India*

Nineteen species of trematodes have been reported from these three land masses (Manter, 1963). *Nesolecithus* (Cestoda: Amphilinidea) is represented in S. America and Africa (Dönges, 1967). There is also geological evidence that these two land masses were connected at one time (Dönges, 1967).

F. *Cosmopolitan*

Ten genera, but no individual species, have been reported from nearly all major land masses (Hoffman, 1969). These include the dangerous parasites *Argulus, Dactylogyrus, Ergasilus, Gyrodactylus* and *Lernaea.*

II. Intercontinental Dissemination

Without the assistance of man there is probably very little continental and intercontinental spread of fish parasites. There is evidence for some minor spread of larval helminths of fish by birds in which the worms become adult, e.g., *Bolbophorus confusus* and possibly *Cryptocotyle concavum* and *Clinostomum* spp. Most of the spread, as in the case of terrestrial animals, has been due to the activities of man (Lindroth, 1957; Reichenbach-Klinke, 1960).

During the last 70 years, fish have been transferred from place to place for several reasons: (1) to attempt to establish highly desirable species in new locations, (2) to rear edible species such as trout, catfish, carp, *Tilapia,* etc. in foreign countries and (3) for the fish fanciers' trade (goldfish, tropicals, etc.). Unfortunately, too little thought has been given to the simultaneous transfer of fish parasites and diseases. It would be best if absoutely no parasites were transferred with fish or fresh fish products; however there are alternatives due to the different requirements of various parasites: (a) if parasitized fish are being transferred to a location which already has those same parasites it would probably cause no harm, (b) if parasitized fish are being transferred to a location which has different fish species which are not susceptible to the introduced parasites it would probably cause no harm and (c) if parasitized fish are being transferred to waters which do not have the necessary intermediate or final hosts of the parasites it could cause no harm. This assessment assumes mandatory inspection and complete understanding of the parasites and diseases involved.

At least 48 species of fish parasites are known to have been transferred to other continents with the transfer of live fish in addition to *Myxosoma cerebralis* which was probably also transferred in frozen table trout. Of these 48 species, there were 5 protozoa, 31 monogenetic trematodes, 5 digenetic trematodes, 3 nematodes, 1 acanthocephalan and 3 copepods. Monogenetic forms (some protozoa, Monogenea and copepods) probably become more easily established because no intermediate host is

required. Of these transferred parasites it is well known that the following have caused great loss to fish culturists and fishermen: *Ichthyophthirius multifiliis, Myxosoma cerebralis, Oodinium* sp., *Dactylogyrus anchoratus, D. extensus, D. vastator, D. wegeneri, Gyrodactylus elegans, Philometra carassii and Lernaea cyprinacea.* Following is a list of known transfers:

A. *Fungi*

Ichthyophonus hoferi (*Ichthyosporidium h.*) Plehn and Mulsow, 1911. Probably spread to freshwater by feeding raw marine fish to trout and salmon.

B. *Protozoa*

Ichthyophthirius multifiliis Fouquet, 1876 —many fishes, skin. Origin possibly Asia— to U.S., England, France, Sweden, Russia, Norway, China, Japan, India, Australia; now cosmopolitan.

Myxosoma cerebralis (Plehn, 1904)— Salmonidae, cartilage. Alive and frozen. Central Europe to Sweden, U.S.A., Scotland, South Africa, (Hoffman, Dunbar and Bradford, 1962; Bogdanova, 1968).

Oodinium pillularis—many fishes, cospolitan, origin unknown (Reichenbach-Klinke, 1960).

Trichodina reticulata Hirschmann and Partsch, 1955 — *Carassius auratus,* skin. Asia (?) to Europe and U.S.A. (Lom and Hoffman, 1964).

Trichodinella (Foliella) *subtilis* Lom, 1959—*Carassius auratus,* gills. Asia (?) to Europe and U.S.A. (Lom and Hoffman, 1964).

C. *Trematoda:Monogenea*

Acolopenteron ureteroecetes Fischthal and Allison, 1941. *Micropterus salmoides,* in ureters. U.S.A. to South Africa (DuPlessis, 1948).

Anacanthorus anacanthorus, A. brazielensis and *A. neotropicalis.* On gills of the redbreasted piranha (*Serrasalmus nattereri*) South America to U.S.A. (Mizelle and Price, 1965).

Cichlidogyrus arthracanthus Paperna, 1960. On gills of *Tilapia zilli, Tristramella simonis* and *T. sacra.* Africa to Israel (Paperna, 1964).

Cichlidogyrus bifurcatus Paperna, 1960. On gills of *Haplochromis flavii-josephi* and *Tilapia nilotica.* Africa to Israel (Paperna, 1964).

Cichlidogyrus cirratus Paperna, 1964. On gills of *Tilapia galilaea.* Africa to Israel (Paperna, 1964).

Cichlidogyrus tiberianus Paperna, 1960. On gills of *Tilapia zilli.* Africa to Israel (Paperna, 1964).

Cichlidogyrus tilapiae Paperna, 1960. On gills of *Tilapia galilaea, T. nilotica* and *Tristramella sacra.* Africa to Israel (Paperna, 1964).

Cichlidogyrus sp. Price, 1969. On gills of *Tilapia mossambica.* Africa to U.S.A.

Cleidodiscus amazonensis, C. piranhus and *C. serrasalmus.* On gills of redbreasted piranha (*Serrasalmus nattereri*). From South America to U.S.A. (Mizelle and Price, 1965).

Cleidodiscus pricei Mueller, 1936. On *Ictalurus nebulosus.* U.S.A. to U.S.S.R. (Chechina et al., 1953).

Dactylogyrus anchoratus (Dujardin, 1845) Wegener, 1857. On *Carassius auratus* (goldfish). Probably originated in Asia and transferred to Europe, Israel and U.S.A. (Mueller, 1936; Monaco and Mizelle, 1955; Paperna, 1964; Price and Mizelle, 1964; Nowlin et al., 1967).

Dactylogyrus extensus Mueller and Van Cleave, 1932. On *Cyprinus carpio.* Europe to Israel and U.S.A. (Paperna, 1964).

Dactylogyrus vastator Nybelin, 1924. On *Carassius auratus* (goldfish). Probably originated in Asia and transferred to Europe, Israel and U.S.A. (Paperna, 1964; Price and Mizelle, 1964; Nowlin *et al.,* 1967).

Dactylogyrus wegeneri Kulwiec, 1927. On *Carassius auratus* (goldfish). Probably originated in Asia and transferred to Europe and U.S.A. (Price and Mizelle, 1964; Nowlin *et al.,* 1967).

Diplozoon (species?). Host? S.E. Asia to Germany (Reichenbach-Klinke, 1960).

Diplozoon tetragonopterini. Salmon. S. America to Germany (Sterba, 1957).

Enterogyrus cichlidarum Paperna, 1963. In intestine of *Talapia* spp. Africa to Israel (Paperna, 1964).

Gyrodactylus bullatarudis Turnbull, 1956. *Lebistes reticulatus* (guppy). Origin (?) to Canada and Germany (Reichenbach-Klinke, 1960).

Gyrodactylus cyprini Diarova, 1964. *Cyprinus* carpio (carp). Probably transferred with carp to North America (Rogers, 1968).

Gyrodactylus sp. *Haplochromis, Tilapia* spp., *Tristramella.* Africa to Israel (Paperna, 1964).

Gyrodactylus elegans. On *Carassius auratus.* Probably originated in Asia and transferred to Europe and U.S.A. (species in America not thoroughly studied; identifications possibly erroneous — Malmberg, 1962).

Macrogyrodactylus polypteri Malmberg, 1956. *Polypterus senegalus.* West Africa to Sweden (Aquarium only).

Metahaliotrema scatophagi Yamaguti, 1953. On *Scatophagus argus.* Trinidad to Capitol Aquarium, Sacramento, Calif. (Mizelle and Price, 1964).

Pseudacolpenteron pavlovskyi Bychowsky and Gussev, 1955. On *C. carpio.* Asia to Israel and U.S.A. (Paperna, 1964; Rogers, 1968).

Quadracanthus clariadis Paperna, 1961. On gills of *Clarias lazera.* Africa to Israel (Paperna 1964).

Urocleidoides reticulatus Mizelle and Price, 1964. On *Lebistes reticulatus* (guppy). Trinidad to Capitol Aquarium, Sacramento, Calif. (Mizelle and Price, 1964).

Urocleidus crescentis and *U. orthus.* On gills of redbreasted piranha (*Serrasalmus nattereri*). South America to U.S.A. (Mizelle and Price, 1965).

Urocleidus dispar (Muller, 1936). *Lepomis gibbosus* (common sunfish). U.S.A. to Rumania (Roman, 1953) and Czechoslovakia (Vojtek, 1958).

Urocleidus furcatus (Mueller, 1937). *Micrropterus salmoides* (largemouth black bass). U.S.A. to Germany (Reichenbach-Klinke, 1960).

Urocleidus helicis (Muller, 1936). *Micropterus salmoides* (largemouth black bass). U.S.A. to Italy (Ghittino, 1965).

Urocleidus principalis (Mizelle, 1936). *Micropterus salmoides.* U.S.A. to England (Maitland and Price, 1969).

Urocleidus similis (Muller, 1936). *Lepomis gibbosus* (common sunfish). U.S.A. to Rumania (Roman, 1953) and Czechoslovakia (Vojtek, 1958).

D. *Trematoda: Digenea*

Bolbophorus confusus (Krause, 1914) Dubois 1935. Common in Europe and recently reported from U.S.A. (Fox, 1962). Probably transferred to U.S.A. by a stray European pelican.

Clinostomum sp. In *Colisa labia.* India and Africa to Europe (Reichenbach-Klinke, 1960).

Crepidostomum farionis (Mueller, 1784) Nicoll, 1909. Probably transferred with trout from Europe to North Africa (Manter, 1963).

Cryptocotyle lingua. In coastal marine fish. Spread from eastern Atlantic to east coast U.S.A., then to west coast U.S.A. about 100 years ago probably by the European snail, *Littorina littorea,* on ships (Sindermann and Farrin, 1962).

Stephanostomum sp. Origin? to Europe. Did not survive because of lack of right intermediate host (Reichenbach-Klinke, 1960).

E. *Nematoda*

Capillaria sp. In *Pterophyllum,* S. America to Germany (Heinze, 1933).

Filaria ? In *Monocirrhus polyacanthus.* S. America to Germany (Geus, 1958).

Philometra carassii (Ishii, 1934). In tail of *Carassius auratus.* Japan to U.S.A. (personal observation).

F. *Acanthocephala*

Polyacanthorhynchus kenyensis Schmidt and Canaris, 1967. Juvenile forms found in the liver of *Micropterus salmoides and Tilapia* sp.; probably trans-

ferred from S. America to N. America (Schmidt and Canaris, 1967).

G. *Copepoda*

Argulus foliaceus Linn. Europe to Ceylon on mirror carp and trout; now on many freshwater fishes in Ceylon (Kirtisinghe, 1964).

Argulus japonicus Thiele, 1900. (Syn. *A. foliaceus* Nettowich, *A. pellucidus* Wagler). Reported from *Carassius auratus* and *Cyprinus carpio* and many other fishes from Japan, China, Europe, N. America, Africa, Israel. Possibly originated with goldfish and carp.

Lernaea cyprinacea Linnaeus, 1758. Has been reported from many species of freshwater fishes and frog and salamander tadpoles from Africa, Europe, Israel, Japan, Poland, Russia and U.S.A. Probably originated in Asia and spread with the goldfish trade (personal theory).

III. Transcontinental Dissemination

Because fish have been indiscriminately distributed across continents by fish culturists, fishermen, and fish fanciers, their diseases and parasites have likewise been distributed. There is little doubt that this list represents only a very small percentage of the total number of fish parasites which have been transferred to waters where they did not previously exist.

The following are known to have been spread transcontinentally:

A. *Protozoa*

Myxosoma cerebralis (Plehn, 1905). In salmonids.

a. Origin apparently Central Europe and North Asia. Possible transfer to Scandinavia, Poland, Italy, Bulgaria.

b. From Europe (probably) to Pennsylvania to Connecticut, Virginia, New Jersey, West Virginia, Ohio, Michigan.

c. From Europe (possibly) to California and Nevada.

Plistophora ovariae (Summerfelt, 1967). In golden shiners. From east coast range of shiners throughout the U.S. in bait minnow hatcheries and bait shops, possibly to Mexico and Canada.[5]

Sphaerospora carassii Kudo, 1920. Japan or China to North Europe (Bykhovskaya-Pavlovskaya, 1962).

B. *Trematoda*

Actinocleidus fergusoni Mizelle, 1938. From Midwest U.S.A. to California (Crane and Mizelle, 1967).

Actinocleidus fusiformis (Muller, 1934) Muller, 1937. From Midwest U.S.A. to California (Mizelle and Crane, 1964).

Clavunculus unguis (Mizelle and Cronin, 1943) Mizelle *et al.*, 1956. From Midwest U.S.A. to Caiforna (Mizelle and Crane, 1964).

Metagonimus yokogawai Katsurada, 1912. Spread from eastern Asia to western U.S.S.R. and Czechoslovakia with spread of the snail host (Vojtek, 1959).

Urocleidus dispar (Mueller, 1936). From Midwest U.S.A. to California (Crane and Mizelle, 1967).

Urocleidus ferox Mueller, 1934. From Midwest U.S.A. to California (Crane and Mizelle, 1967).

Urocleidus furcatus (Mueller, 1937) Mizelle and Hughes, 1938. From Midwest U.S.A. to California (Mizelle and Crane, 1964).

Urocleidus principalis (Mizelle, 1936) Mizelle and Hughes, 1938. From Midwest U.S.A. to California (Mizelle and Crane, 1964).

C. *Cestoda*

Khawia sinensis Hsii (Caryophyllaeid). Spread from Peking to western Ukraine with carp (Kulakovskaya and Krotas, 1961).

Proteocephalus ambloplitis (Leidy, 1887) Benedict, 1900. From East to Midwest to State of Washington in largemouth black bass (Becker and Brunson, 1968).

[5] R. C. Summerfelt, Oklahoma State University, Stillwater, Oklahoma.

Pomphorhynchus perforator Linstow, 1908. Spread from Central Asia to Kirgiz S.S.R. (Iksanov, 1962).

IV. Transfaunation

When a fish is introduced into an area not previously occupied by that species, it may acquire parasites possessing little host specificity or those from closely related fish species. Following is a list of freshwater fish parasites which have been acquired by such introduced fish.

Myxosoma cerebralis (Plehn, 1905). Apparently this myxosporidean existed in *Salmo trutta* (German brown trout) in Europe without pathogenesis. However, when imported *Salmo gairdneri* (rainbow trout) became infected, a very serious disease (whirling disease, Drehkrankheiten) was produced.

Diplostomulum spathaceum (Rud., 1819) Braun 1893. Transferred to *Ictalurus nebulosus*, Russia (Chechina, A.C. *et* al., 1953).

Bothriocephalus gowkongensis of Russian fish. Transfaunated to carp in Russia (Malevitskaya, 1958).

Capillaria eupomotis Ghittino, 1961. Adult in liver of fish (*Leuciscus and Phoxinus*) in Italy. Transfaunated to *Salmo gairdneri* and *Lepomis gibbosus*.

Raphidascaris acus (Bloch, 1779). Transfaunated to *Salmo gairdneri* in Italy and caused mortalities (Carrara and Grimaldi, 1960).

DISCUSSION AND CONCLUSIONS

Because of the similarities of the fauna of North America, Europe and northern Asia it is very likely that many more fish parasites will become transfaunated if live, infected fish continue to be transferred. Some of the newly acquired parasites, such as *Myxosoma cerebralis* and *Capillaria eupomotis* in rainbow trout, cause considerable losses in fish culture.

It is apparent that fish parasites, including some very harmful ones, are being transferred with their hosts from state to state and continent to continent. Trout, salmon, catfish, carp, bass, bluegills, bait minnows, *Tilapia*, grass carp, tropical fish, goldfish, pike, muskie, walking catfish and others are being transferred due to increased volumes in fish culture and sports fisheries. Some are being shipped frozen and some dangerous parasites are not destroyed by freezing. It would be very helpful if a system of fish inspection and parasite identification could be set up in each state and country to discover dangerous parasites before they are transferred to another state or country. Exports and imports of live and frozen fish should be examined for pathogens. Some states in the United States are developing diagnostic services, and the Bureau of Sports Fisheries and Wildlife provides identification assistance through a regionalized system of fish hatchery biologists; however, even more services are needed. The fish disease committee of the American Fisheries Society is also working on this problem. The recently enacted U.S. Import Law (Title 50-Wildlife and Fisheries, Part 13) restricts the import of trout from areas where two serious salmonid diseases, whirling diseases and viral hemorrahagic septicemia, are endemic. Proposed S.1151 woul provide protection for the fish resources of the United States including the freshwater and marine fish cultural industries against the introduction and dissemination of diseases of fish and shellfish. We should investigate the possibility of assisting fish farmers in a manner already in practice with domestic animals and we should urge increased inspection of exports and imports and the confiscation of fish carrying serious parasites.

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VI. Whirling Disease of Salmonids

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A. Name of Disease and Etiological Agent

Whirling disease is caused by the myxosporean parasite *Myxobolus (Myxosoma) cerebralis*. A synonyn for whirling disease is blacktail.

B. Known Geographical Range and Host Species of the Disease

1. Geographical Range

California, Colorado, Connecticut, Idaho, Massachusetts, Michigan, Montana, Nevada, New Hampshire, New Jersey, Ohio, Oregon, Pennsylvania, Utah, Virginia, and West Virginia. The agent has also been found in Europe, United Kingdom, New Zealand, South Africa, and the USSR.

2. Host Species

All species of salmon, trout, char, and grayling are susceptible to infection. Coho salmon *Oncorhynchus kisutch*, and brown trout *Salmo trutta*, may show no signs of the disease and spores may be difficult to find even after heavy exposure at an early age. Brook trout *Salvelinus fontinalis*, and rainbow trout *Oncorhynchus mykiss*, are very susceptible. Intensity of exposure and fish age affect the severity of the disease.

C. Epizootiology

Markiw and Wolf (1983) and Wolf and Markiw (1984) determined that the infective stage of whirling disease was an actinosporean. The life cycle involves tubificid worms, *Tubifex tubifex*, in the development of the infective stage (Wolf and Markiw 1985). Hamilton and Canning (1987a, b) have contested the life cycle proposed by Wolf and Markiw (1984): however they have more recently reported (1987b), that the transmission of whirling disease can occur when tubificids are added to test tanks with sterile mud and

Myxobolus cerebralis spores. In addition, El-Matbouli and Hoffman (1989) demonstrated with two *Myxobolus* species, one of which was *Myxobolus cerebralis*, that transmission via triactinomyxon formation in tubificid worms occurs. Regardless of the role of the tubificid, repeatable laboratory challenges have been developed to study various aspects of whirling disease in salmonids.

D. Disease Signs

Frenzied, tail-chasing behavior, particularly when being fed or when startled. Whirling behavior usually occurs 2 to 3 months after infection and may last for up to a year in cold water situations. The posterior trunk and tail of young fingerlings may turn dark, especially in fish exposed at an early age, (blacktail). As the infected fish grows, the primary signs of the disease can be skeletal changes such as misshapen skulls and twisted spines. Loss rate is dose and age dependent; most serious losses occur in young, heavily infected fish. In histological sections cartilage may appear heavily plaqued depending on the degree of infection.

E. Disease Diagnostic Procedures

1. Presumptive Diagnosis

Diagnosis of epizootic whiling disease depends upon the detection and identification *Myxosoma cerebralis* spores.

Remove the heads from five suspect fish and warm them in 45°C water for 1 to 3 minutes so the flesh will separate easily from the bone and cartilage.

Remove loose flesh and the brain to a waste container of 1:1 water and household bleach for disinfection.

Collect bone and cartilage samples from the brain case, otolith region, and gill arches.

Grind the sample with an equal volume of 10% formalin (to kill viable spores and prevent dissemination of the disease agent) in a mortar. If the fish are older than the desired 5 to 11 months of age, the skeletal parts should be softened with a volume of 1% hydrochloric acid sufficient to cover the sample for one to several hours.

Wash all grindings into a small beaker with water and allow the material to settle.

Sediment can be examined directly in wet mounts at 400X magnification. Alternatively, smears can be dried and stained according to the method of MacLean (1971). For this procedure, spread 5 to 10 drops of sediment onto a clean glass microscope slide and allow to air dry.

Stain the slide is with 1% aqueous malachite green for 5 min, rinse with tap water and destain by placing the slide for 30 s each into 70, 90, and 100% ethyl alcohol.

Coat air-dried slides entirely with a thin film of low viscosity immersion oil and examine at about 200X magnification (not under oil immersion).

Scan the entire smear. Spores will appear as green ovals with dark green polar capsules against a nearly colorless background. At 200X magnification, there is less chance of missing spores and a larger area is covered at each pass over the slide than at higher magnifications required to find unstained spores..

Diagnosis of epizootic whirling disease depends upon the detection and identification of *Myxobolus cerebralis* spores.

Myxobolus cerebralis is the only species of *Myxobolus* found in the cartilage of salmonids. *Myxobolus squamalis* occurs in the scales of western U.S.A. salmonids, is about the same size as *Myxobolus cerebralis* (about 9µm), but possesses a narrow, but obvious, ridge that parallels either side of the sutural ridge. *Myxobolus kisutchi*, another western salmonid parasite, occurs in the central nervous system and is about the same size as *Myxobolus cerebralis*. *Myxobolus neurobius*, more widespread geographically, is also found in the central nervous system, but is larger (10 - 13 x 7.5 - 8 µm).

2. **Confirmatory Diagnosis**

a. Histological Confirmation

1. Preserve heads in 10% neutral buffered formalin (frozen heads may be preserved and processed but the sections are of poorer quality).
2. Decalcify for about 3 d.
3. Rinse for 4-5 h in running water.
4. Dehydrate in an ethanol-xylene series and embed in paraffin.
5. Cut into 5 to 7 µm sections.
6. Stain with a Geimsa stain (May-Grunwald Giemsa works well).
7. Scan section at 200X magnification for spores and trophozoites; the presence of spores, as described above,or trophozoites in association with cartilage lesions confirms diagnosis.

b. Serological Identification of *Myxobolus cerebralis*

This may be accomplished by the direct FAT described below. This test works best with fresh spores. Spores that have been stored in formalin for a week or more show reduced specific fluorescence, and older specimens show little or none (Wolf and Markiw 1985).

1. Transfer the suspect wet sediment from step E1 above, or from steps F1f, F2g, F2i or F2k below to a labelled centrifuge tube and concentrate the residues by centrifugation at 1200 x g for 10 minutes at room temperature. Decant the supernatant and, by the use of wet mounts, adjust the concentration of the residues with water to a volume that permits the best observation of spores among the debris.
2. Thoroughly clean FAT slides with detergent and deionized water and rinse well with acetone. Label slides for known positive *Myxobolus cerebralis* (positive

control), one or more other known sporozoan (negative control), and for material to be tested (suspect/unknown).

3. Lightly coat slides with a commercial tissue bonding agent or 50% egg albumin. (Egg albumin can be prepared by mincing egg white with scissors, filtering it through gauze and mixing with an equal volume of glycerin. Several drops of chloroform are added as a preservative; the albumin is stored in refrigerator).
4. Apply small drops of suspect/unknown and control material to the coated slides, dry the slide at 50°-60°C for 15 to 20 min. Fix slides in absolute methanol for 5 min and air dry.
5. Apply fluorescein isothiocyanate-conjugated rabbit anti-*Myxobolus cerebralis* serum to each slide. Allow serum to react for 30-60 min in the dark at room temperature.
6. Gently rinse conjugated antiserum from the slides with pH 9.0-9.5 buffer (NaH_2CO_3, 33.6 g; plus Na_2CO_3, 10.6 g in 1000 ml water) then soak slide in buffer, with gentle agitation for 5 min. Careless or too vigorous washing may lead to spore loss.
7. Remove slide and gently blot dry on clean absorbent paper. Add a drop of immersion oil to each test spot and examine at 400X or 1000X magnification on a fluorescence microscope. Positive identification of *Myxobolus cerebralis* depends upon the detection of spore and trophozoite stages which fluoresce apple green. Cartilage debris and spores may exhibit autofluoresce under UV light. However, this fluorescence is quite yellow.

F. Procedures for Detecting Subclinical Infections

Samples should be weighted towards the most susceptible species and ages of fish available. For example, select brook and rainbow trout over brown trout or coho salmon if all are reared under the same conditions. Select fish about 5 months old if possible. However if fish are continuously exposed in water of 13°C or warmer, fish as young as 2 to 3 months of age may yield mature spores. In water below 12°C, fish may have to reach 8 to 10 months of age before mature spores can be found.

The following procedures are acceptable for detection of infection in carrier fish (It is common practice in many diagnostic laboratories to only inspect the gills of larger fish.):

1. Plankton Centrifuge Method (O'Grodnick, 1975)

a. Pool in 20 g batches, heads, dissected pieces of cranium, and all gill arches.

b. Thoroughly homogenize each batch in 200 ml of aqueous 10% formalin for 3 minutes in a high-speed blender.

c. Strain the homogenate through loose glass wool in a large funnel or through a fine screen or sieve. (Millipore XX40 047 04 support screen in an XX40 047 00 holder or Tyler sieves #60 {0.250 mm} and #80 {0.180 mm}).

d. Rinse any remaining sample through the glass wool or screen with water and save all washings. (Caution: Infective material may remain in discarded tissue and equipment).

e. Transfer the entire filtrate to a separatory feed line of a plankton centrifuge (026WA106 plankton centrifuge, Kahl Scientific Instrument Corp., PO Box 1166, El Cajon, CA 92022, or equivalent). Operate the plankton centrifuge on high speed and set the separatory funnel flow rate at the lowest level that gives a thin steady stream

f. Centrifuge until flow from the separatory funnel has been completed including at least one thorough rinse of the apparatus. The residues adhering to the inner walls of the centrifuge drum will contain spores and debris. With a rubber policeman, suspend this residue in the water that remains in the drum. Transfer this material to screw-capped, labelled tubes and store in a solution of 10% neutral buffered formalin until the sample can be examined.

g. Shake the sample well and transfer a drop of the suspension to a clean microscope slide and add a cover glass. Systematically search each test area for approximately 2-1/2 minutes or until spores are found.

2. Digest Method (Markiw and Wolf 1974a)

a.The following solutions are required:

1. Pepsin: to 1 L of distilled water add 5.0 g pepsin and 5 ml concentrated HCl.

2. Trypsin: to 1 L of distilled water add 0.2 g EDTA, 8.0 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4 and 5.0 g trypsin, 1.5 g NaH_2PO_4.

b. Remove the heads from fish to be analyzed. Heads may be frozen for future analysis.

c. Preserve at least five heads or half heads (cut longitudinally) for histology (if needed) in 10% buffered formalin.

d. Heat heads for approximately 10 min in 60°C water- The eyes will turn opaque when ready.

e. Deflesh heads and save bone and cartilage mainly from the cranial area. Samples from 5 fish may be pooled. For adult fish, homogenize bone and cartilage in an electric blender in a small amount of pepsin.

f. Place cleaned heads in a pepsin solution. Use about 20 ml of solution for each gram of head material. Stir at 37°C for 30 min.

g. Centrifuge pepsin digest at 1200 x g for 10 min. Dispose of the supernatant into disinfectant. Check for spores at 100 X and 400 X magnification at this step.

h. Add the trypsin solution. Use 20 ml for each g of undigested material. Adjust pH to 8.5 with NaOH. Stir at room temperature for at least 30 min.

i. Pour digested material through cheese cloth and save fluid. Centrifuge fluid at 1200 x g for 10 min. Resuspend the pellet in a small volume of buffered 10% formalin and examine for spores.

j. If no spores are found in the previous steps, layer a sample over a 55% glucose solution (1 cm depth of sample to 5 cm depth of glucose). Centrifuge in swinging bucket rotor at 1200 x g for 30 min. Aspirate off all liquid over the pellet.

k. Resuspend the pellet in a few drops of buffered formalin, mix and examine for spores.

3. Core Method- Sampling Adults

a. Sample fish with a borer similar to a cork borer (about 110 mm long and 19 mm in

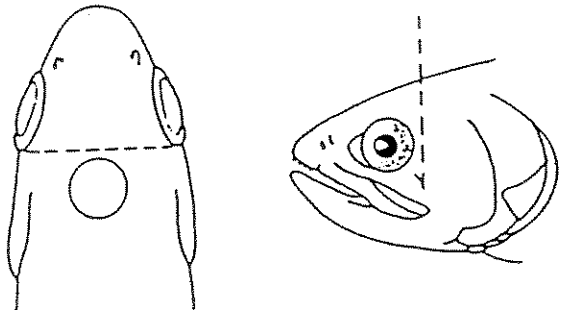

Figure 1. Dorsal and lateral views of an adult salmon head, indicating the location for obtaining a core sample for *Myxobolus cerebralis*.

diameter) by inserting the borer into the head, dorsally and perpendicular to the long axis of the body approximately behind the eyes and through the roof of the mouth (Figure 1). This sample should contain the semicircular canals, and the otoliths in smaller fish.

b. Remove the skin from the core.

c. Homogenize cores in small amount of pepsin (see F2a) in an electric blender.

d. Proceed with stirring step in procedure (F2f) in Digest procedure.

G. Prodedures for Determining Prior Exposure

No procedures have been reported.

H. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiological Agent

Samples collected from apparently normal, moribund, or dead fish should be packed on ice for shipment. Preservation of samples in 10% buffered formalin will eliminate infectivity.

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Whirling Disease Management in North American
An Emergency Conference
Denver, Colorado
April 12 - 14, 1988

Hosted by:

Fish Disease Subcommittee
Colorado River Wildlife Council

Sponsored by:

Arizona Game and Fish Department
California Fish and Game Department
Colorado Division of Wildlife
Nevada Department of Wildlife
New Mexico Department of Game and Fish
Utah Department of Natural Resources
Wyoming Game and Fish Department

Other Contributors:

U.S. Fish and Wildlife Service
Colorado Aquaculture Association
U.S. Trout Farmers Association
Sterling H. Nelson & Sons, Inc.

Wednesday, April 13

Experiences and Perspectives

8:00 - 8:15	Kent Hauk - Idaho
8:15 - 8:30	Steve Roberts - Washington
8:30 - 8:45	Don Manzer - California
8:45 - 9:00	Steve Henry - New Mexico
9:00 - 9:15	Ron Goede - Utah
9:15 - 9:25	Rick Cordes - South Dakota
9:25 - 9:35	Jim Gleim - Nebraska
9:35 - 9:45	Doug Mitchum - Wyoming
9:45 - 9:55	Gary Camenisch - Missouri
9:55 - 10:05	Roger Sorenson - Arizona
10:05 - 10:25	B R E A K
10:25 - 10:35	Jim Peterson - Montana
10:35 - 10:45	John Nickum - USFWS
10:45 - 10:55	CRWC - Bob Wiley
10:55 - 11:05	PNFHPC - Jim Warren
11:05 - 11:15	Great Lakes
11:15 - 11:25	New England - John Thoesen
11:25 - 11:35	Appalachian
11:35 - 11:45	Canadian
11:45 - 1:00	L U N C H
1:00 - 1:10	Analysis of Regulations - Paul Janeke (USFWS)
1:10 - 2:45	Panel Discussion - Ron Goede, Moderator Private Sector Perspectives: Dave Gann, Ken Cline, Dick Smith, Roger Ritzert, Anne Putnam
2:45 - 3:00	B R E A K
3:00 - 5:00	Panel Discussion - Ron Goede, Moderator Law Enforcement Considerations: Dave Croonquist, Monty Halcomb, Tim Barclough, Joe White, Ken Cline, Paul Woodbury/Craig Miya
6:00 - 7:00	Coctail Hour
7:00	Banquet

EMERGENCY WHIRLING DISEASE CONFERENCE

APRIL 12 - 14, 1988

DENVER, COLORADO

INTRODUCTION

Whirling disease has been classed as an Emergency Prohibitive disease from the beginning (1973) of the Colorado River Fish and Wildlife Council Fish Disease Control Policy and since its introduction into North America about 1958. In the last thirty years it has spread from coast to coast. The episodes in the past few years in California, Oregon, Idaho and Colorado indicated weakness in the regulations and enforcement of those regulations. Most regulating agencies have regulations governing import of fish into the State but do not have regulations governing intrastate movement of live fish. There is no mechanism in place to detect and contain the disease if it should "slip" into the State and it may spread over a considerable range before detection.

It has been proposed by numerous experts that whirling disease is not as serious a disease as once thought and that current status as Emergency Prohibitive is not justified.

The CRFWC Fish Disease Subcommittee has always based its decisions on consistent and defensible rationale and has always insisted that any changes in that policy be based on such rationale. The Committee, meeting in Salt Lake City in October, 1987, decided to recommend downlisting the disease from the Emergency Prohibitive to the Prohibitive category. It was felt that it might be possible to downlist further to Notifiable but it would be necessary to hold a special emergency meeting to gather a state of the art profile of the problem. That meeting was organized very quickly and each member State contributed a sum of money to support the effort. This permitted invitation of a broad range of experts with knowledge of and experience with the disease and to pay some expenses for those unable to obtain travel support.

The Emergency Conference on Whirling Disease was held in Denver on April 12 - 14, 1988 and was attended by about 70 persons (see appended roster) from the university and agency research communities as well as those from a variety of State., federal, and private fisheries programs. It represented, with very few exceptions, the most appropriate group available in North America to discuss this particular problem.

The CRFWC Fish Disease Subcommittee conducted the meeting and with the information generated, developed a conference statement which was presented to all fish health representatives of the member States for discussion and to the entire assembled group. Minor changes were suggested and incorporated by the Committee. The meeting was adjourned and, with very few exceptions, was considered a success and a giant step in the right direction.

Myxobolus cerebralis was downlisted to the Notifiable category which requires inspection but does not demand depopulation and disinfection. This classification is considered a caveat to "let the buyer beware". Each member State may further restrict but not liberalize. Utah, Wyoming, Arizona, Nevada, and New Mexico indicated that their borders and waters would remain closed to whirling disease. Colorado and California will likely have more complex regulations which will try to restrict movement of fish from contaminated stocks into waters with no history of the disease. The machinery is now in place to permit the States to be somewhat flexible in regulating the disease. The extreme resistance and persistence of the spores make it difficult (or impossible) to feel that the organism will be removed from wild systems once it has been introduced and established.

SUMMARY

Whirling disease was discussed according to several specific conference topics.

* Biological considerations.
* Experiences and perspectives from the agencies and the private sector.
* Analysis of agency regulations and policies and law enforcement considerations.
* Conference statement.

The following summary is presented according to discussion topic.

BIOLOGICAL CONSIDERATIONS

Whirling disease (caused by the pathogen Myxobolus cerebralis) was diagnosed in the U.S. in 1958 by Dr. S. F. Sniezko. Typically, the first indication of the disease is a black tail which can be accompanied by other clinical signs such as deformities in the head, jaw, and operculum. Tail chasing or "whirling" is caused by erosion of cartilage around the auditory organ and subsequent inflamation causing pressure on adjacent nerves. Whirling disease primarily affects fry and fingerling fish, older fish are more resistant to the pathogen due to osteogenesis. Age of fish, number of infective agents, and water temperature are important factors in susceptibility to whirling disease.

Whirling disease is typified by the presence of spores in cartilage in the head of affected fishes. Spores are environmentally persistent which makes the disease very difficult to control. Identification of the pathogen is based on the size and shape of the spores. Myxobolus

cerebralis (whirling disease, found in cartilage) spores can be confused with other myxosporidian diseases, notably; M. squamalis (found in scale pockets), M. kisutchi (found in the central nervous system), M. nevrobius, and Myxobolus sp. (found in brain tissue). Whirling disease can be confirmed by finding spores in histological sections of cartilaginous tissue from the head, gill arches and vertebral column.

Life cycle, Myxobolus cerebralis. The life cycle of the pathogen is not well understood though it has been proposed (Wolf and Markiw 1985) that a tubificid (Tubifex sp.) worm serves as intermediate host. Experiments involving transfer of spores to tubificid worms from nonenzootic waters have produced Triactinomyxon gyrosalmo in the tubificid worms following a 104 day incubation period. Feeding tubificid worms harboring the Triactinomyxon gyrosalmo to "clean" salmonids produces whirling disease in trout following a 45 day period. Researchers in Germany have corroborated this proposed whirling disease life cycle.

Additional research at the University of California, Davis has failed to transmit whirling disease. No Triactinomyxon gyrosalmo could be isolated from these studies. Additionally, no Tubifex worms could be located in sites in the wild that harbored fish positive for whirling disease. Attempts to infect experimentally with any method have been unsuccessful. Dr. Ron Hedrick (U.C. Davis) has been leading this research and is currently comparing similarities in DNA of Triactinomyxon and spores of M. cerebralis.

Environments suitable for Myxobolus cerebralis. Infected fishes are most commonly found in facilities having earthen ponds or raceways, or open water sources that can be easily infected with the whirling disease pathogen. Where the disease occurs in the wild, conditions are often typified by low stream gradient and high alkalinity. The intermediate host is suspected to be a tubificid worm but there is question as to species.

Treatment of whirling disease. Treatment has ranged from the severe (destruction of fish and depopulation of facility including abandonment and burial of facility sites) to managing with and around the pathogen. Hundreds of tons of fish have been destroyed and millions of dollars spent in attempts to eradicate the pathogen with virtually no success. These efforts have shown that enclosing open spring sources and concreting fish rearing facilities (eg; concrete ponds and raceways) offers protection against whirling disease. The environmentally persistent spores make the disease virtually impossible to eradicate in the wild once it is established. Chemicals used in attempts to control whirling disease have included HTH and hydrated lime. Ultra violet light may offer a means of treatment although success has been variable.

Impacts of whirling disease. The disease affects young fishes and has a potentially devastating effect on hatchery production. However, careful management (use of concrete ponds and raceways, enclosed spring sources, and treatment using ultra violet light) can reduce whirling

disease to limited concern in fish culture. Losses at fish cultural facilities directly attributable to whirling disease are minimal. Much of the reported mortality appears to be associated with other pathogens and secondary infection.

Reported impacts on wild populations are thought to be minimal. However, there has been no detailed study of susceptible young fishes in areas where whirling disease has been discovered in the wild. It is important that such work be launched because the introduction of whirling disease in wild stocks represents an irreversible decision permitting environmentally persistent spores to become established in the wild. Studies should include effects on population parameters such as fecundity, growth, performance, and so on.

Susceptibility of salmonids to whirling disease. Though irrefutable confirmation is needed, evidence suggests that the following species are susceptible to whirling disease in the approximate order presented (lists are separate for trout and salmon and indicate descending order of susceptibility).

Trout	Salmon
Golden	Kokanee
Rainbow	Chinook
Cutthroat	Coho
Brook	
Brown	
Lake	

Following this discussion, it was generally agreed that depopulation and disinfection of fish cultural facilities could not guarantee control of whirling disease. Use of concrete (to eliminate habitat for tubificid worms) at rearing facilities offers a potential means of reducing or controlling the pathogen. Managers at a private facility in Ohio enclosed spring sources and constructed concrete raceways (1968) with the result that whirling disease has not occurred at the facility since 1974.

HISTORY OF WHIRLING DISEASE IN THE U.S.

Dr. Glen Hoffman presented information pertaining to history in this country. Movement across the country can be traced to shipment of infected fishes whether processed, frozen, or live.

* 1956. Probable arrival of whirling disease in Pennsylvania in frozen, processed fish.
* 1957. Indications that M. cerebralis is in Pennsylvania.
* 1958. Discovered in earthen trout ponds in Pennsylvania.
* 1961. Discovered in Connecticut.
* 1961. Strong letter cautioning federal hatchery system regarding the potential serious nature of the pathogen.

* 1963. Pennsylvania, new site.
* 1964. Pennsylvania, new site.
* 1965. Earthen ponds in Virginia, fish heavily infected.
* 1965. Whirling disease discovered in California.
* 1966. Hatcheries in Pennsylvania.
* 1966. Nevada. Verdi Hatchery, cutthroat and rainbow trout, probably imported with frozen, processed fish from Denmark.
* 1966. Whirling disease discovered near Monterey, California.
* 1966. Suspected in Massachusetts, confirmed in 1973.
* 1966. New Jersey in raceways with gravel bottom.
* 1967. Pennsylvania, private hatchery.
* 1968. Ohio, private facility.
* 1968. West Virginia, earthen ponds.
* 1968. Michigan, private facility.
* 1969. Nevada, Lahontan NFH (cutthroat trout), Washoe State Rearing facility (rainbow trout), Gallagher State Hatchery (rainbow and brook trout).
* 1973. California, new site.
* 1977. Lamar National Fish Hatchery, Pennsylvania.
* 1980. New Hampshire.
* 1982. California, new site.
* 1983. California (20 tons of fish destroyed).
* 1984. New York.
* 1986. Oregon
* 1987. Idaho, Colorado.
* 1988. Wyoming, North Platte River Drainage.

Observation and research through this period has confirmed that the parasite can be transmitted through shipment of live infected fishes and through frozen processed fish. General consensus suggested that transmission via birds was possible though not probable. Dr. Hoffman stressed that infected fish have commercial value and could be used given careful precautions to preclude movement of spores.

EXPERIENCES AND PERSPECTIVES

Management experiences with whirling disease.

California: California has, perhaps, most experience on the largest scale of the western states. Whirling disease has been in California since 1965. There have been very large efforts to eradicate the disease . . . . . including literal burying of fish cultural facilities and burial of tons of fish. About 185 tons of salmonids have been buried in 22 years of dealing with whirling disease in California.

Through 22 years of dealing with the disease, experiences have been varied and perplexing. Whirling disease has been transported to several areas in California through intrastate commerce. Varied experiences with whirling disease in California have included identification in wild fish where no known infected stocks have been planted, failure to identify pathogen after stocking infected fishes in the wild, wild stocks exposed to the pathogen don't appear to show symptoms of the

disease (young infected fishes may have died before adulthood), and whirling disease has been found in parts of drainages while contiguous portions of the same drainage remain disease free.

Michigan: The State of Michigan has invested heavily in efforts to eradicate whirling disease. It is estimated that about $18,900,000 has been spent in attempts to eradicate the disease. Some of the costs identified were;

* $51,000 in federal funds used to reimburse for destruction of privately owned stocks of fish.
* $300,000 for the fish loss at Sturgeon River Fish Hatchery.
* $3,000,000 to build a new facility at Sturgeon River.
* $140,000 invested in cleanup effort on Tobacco River.
* $15,000,000 estimated recreational value of fish destroyed at the Sturgeon River facility.

The result of all this is that fish culture facilities remain free of whirling disease but the pathogen remains at very low levels in the Tobacco River system. This confirms that whirling disease can be managed at fish cultural facilities but that establishment in the wild is probable.

Nevada: Whirling disease was first confirmed in northern Nevada in 1966, although spores found in preserved specimens indicate its presence as early as 1957. Three State and one federal facility in Nevada have experienced confirmed occurrences of whirling disease since 1966. Currently, the disease exists only in the Gallagher Hatchery in northeastern Nevada. Periodic sampling of wild populations has demonstrated varied rates of infection among trout in waters stocked with infected fish.

Over $150,000 has been spent at the Gallagher facility to enclose the spring source. Recurrence of whirling disease at three other facilities in Nevada has been prevented through use of a combination of management measures including enclosed water systems, concrete lining of earthen ponds, treatment with ultra violet light, application of lime, disinfection with chlorine, and disinfection of eggs. Additionally, more than 750,000 cutthroat trout have been destroyed at Lahontan National Fish Hatchery in efforts to prevent the spread of the disease. Moreover, Nevada has developed importation and inspection regulations and intrastate transfer policies. Such management and control procedures have helped confine whirling disease to northern Nevada and prevent its spread south into the Colorado River Basin.

Ohio: Castalia Farms (Roger Ritzert, Manager) is a private operation serving as a guest facility with a large business in catch-out fishing. Annually, 200,000 pounds of fish are supplied to the recreational fishing industry. Whirling disease was isolated at the facility in 1968. The facility was treated several times with chlorine or chlorinated products and raceways and ponds converted to concrete. Cost of this work totalled about $200,000 and there has been no evidence

of whirling disease at the facility since 1974. Treatments were judged worth the investment.

Experiences and perspectives of agency people. The following information is presented as summaries of thoughts expressed by the presenters and any inaccuracies that may have crept into the information are entirely the responsibility of the Fish Disease Subcommittee.

Dr. Glen Hoffman. Dr. Hoffman indicated that treatment of water supplies with ultra violet light is effective in controlling whirling disease. It has been demonstrated that healthy fish can be raised in the presence of the disease. Dr. Hoffman reported that histological observation of spores in cartilage confirms the whirling disease pathogen. He also stressed that there should be some sort of market so that properly processed infected fish can be used; these fish do have value. Dr. Hoffman does not support the wholesale destruction of fish, they should be utilized.

Dr. Hoffman feels that management with whirling disease is necessary because it can't be eradicated. He offered the following suggestions.

* All facilities with earthen ponds or raceways should be monitored.
* Convert earthen ponds and raceways to concrete.
* Treat water with ultra violet light or ozone.
* Consider adoption of indemnification procedures.
* Fish lightly infected with whirling disease should be used.

Roger Herman, USFWS, Leetown. Work has been carried out relative to the life cycle of the whirling disease pathogen. Work has been conducted with tubificid (Tubifex tubifex) worms. There seems to be an intermediate form of the whirling disease pathogen, the myxosporidian Triactinomyxon gyrosalmo. This form was found in tubifex worms following addition of whirling disease spores to negative tubifex worms and a period of 120 days at 12 C. It was also found that negative tubificid worms could be exposed to spores and following a 104 day incubation period at 12 C, the tubificid worms could be fed to salmonids which would developed
signs of whirling disease.

Charlie Smith, USFWS, Bożeman, Montana. Older fish are more resistant to whirling disease because of osteogenesis. The deformations observed in some infected fish result from formation of granulomatous tissue and associated pressure on nerves. It has been found that gill cartilage is also involved in whirling disease infections but sampling should not be restricted to the gill area.

M. cerebralis spores are most easily identified in fish tissue using Giemsa stain while H & E stain is best for the identification of the triactinomyxons.

Dr. Ron Hedrick, University of California, Davis. Dr. Hedrick has done experimental work with tubificid worms (not Tubifex) and has been unable to infect worms using whirling disease spores nor has transfer of whirling disease from these worms to trout been successful. There have been no successful experimental infections thus far. This work further suggests that hosts must include Tubifex tubifex. Dr. Hedrick is currently experimenting with DNA homologies regarding whirling disease.

John Modin, California. Mr. Modin recounted the history of whirling disease in northern California (Monterey and adjacent areas) and noted that the disease was likely moved through intrastate commerce before an awareness developed relative to the disease. It is known from areas where infected fish have been planted but there are also places where whirling disease is found where there is no history of planted fish. Also, there are locations where the disease cannot be found but where infected fish have been planted.

California has also experienced occurrences of the spores in the wild in parts of contiguous stream systems. There appears little explanation of why one location is positive for the pathogen and others nearby remain pathogen free. There appears to be some tie-in with low gradient, high alkalinity streams.

Mr. Modin also reported on the tremendous efforts that have been made in California to eradicate whirling disease with virtually no success. Fish cultural facilities have been depopulated, disinfected and even destroyed and the premises buried following chlorination or liming with the result that positive fish appeared again. In the case of a private facility so treated, the owner was compensated through California Fish and Game supplying rainbow trout from a clean source.

Mel Willis, California. Studied the effect of whirling disease on various sizes of fish (fry-fingerlings, subcatchables, and brood fish) held at a private facility consisting of earthen ponds connected by concrete raceways. The work was conducted over a six month period. The results showed;

* Fry and fingerlings were positive for whirling disease after 107 days.
* Initial incidence of spores was light and then increased to heavy infestation.
* 100% infection in fry and fingerlings.
* 92% light to moderate infection in subcatchables.
* 50% infection in brood fish at barely detectable levels.

Clearly, the small fish were affected to the greatest degree and level of infection decreased as the fish grew.

Mortality associated with the study showed 2% of fingerlings, 0.2% of subcatchables, and no mortality of brood fish. This was likely "normal" fish loss rather than attributable to whirling disease.

Diagnostic work using skull cartilage and gill arches showed that analysis of skull cartilage resulted in detection of whirling disease more often than analysis of gill arches. Analysis for whirling disease should not be based solely on examination of gill arches.

Bob Toth, California. Reported on the incidence of whirling disease at the Mt. Whitney Hatchery, the first occurrence of whirling disease at a California State fish cultural facility. Rainbow, brown, brook, and golden trout were infected. The water supply for the station is a stream (Oak Creek); the South Fork of Oak Creek was negative and the North Fork positive for whirling disease.

Clean-up at the station and the ancillary Blackrock Hatchery (grow-out facility for Mt. Whitney) was conducted in 1986, 1987, and 1988 with the result that spores remain. Through this exercise 2,000,000 fingerling and 100,000 catchable trout were buried. Additionally, 200,000 of the 300,000 catchable trout held at the Blackrock facility were caught from the ponds by fishermen; all fish were dressed before leaving the station. The remaining 100,000 catchables were buried.

There are large populations of tubificid worms in the stream and ponds but no Triactinomyxon gyrosalmo could be detected. There was no mortality even with the heavy infestations. Fish from seven waters planted from Mt. Whitney Hatchery revealed the following.

* Golden trout, 100% incidence of spores.
* Brown trout, 60% incidence of spores.
* Rainbow trout, 40-60% incidence of spores.
* Brook trout, 20% incidence of spores.

There has been virtually no mortality associated with any of this work.

Don Manzer, California. (The complete text of Mr. Manzer's presentation appears as Appendix 2.) As has been discussed, California has experienced and still is experiencing whirling disease. The State has an "advisory board" which advises California Department of Fish and Game on fish health matters.

California policy has required the destruction of fish infected with whirling disease and the closing, depopulation, and disinfection of facilities with infected fish. Over 22 years 185 tons of fish have been destroyed and $200,000 to $300,000 has been spent in efforts to eradicate the disease. Efforts to eradicate have been unsuccessful. The California Policy pertaining to whirling disease provides that infected fish cannot be stocked into waters where there is no whirling disease and can be stocked into waters where the disease is enzootic only when the waters show continued infectivity for three years without diminished intensity.

Joe O'Grodnick, Pennsylvania. The source of whirling disease in Pennsylvania is thought to be from frozen, processed fish from Denmark.

The disease was "transferred" to three hatcheries before detection. The hatcheries were quarantined such that infected fish were not planted in clean waters nor in the vicinity of commercial enterprises.

Following modernization of hatcheries (concrete ponds and raceways) the disease disappeared and appears to be manageable with proper care at fish cultural facilities. There was one incidence of possible transmission via birds but there was no confirmation.

Studies conducted following the stocking of infected fish showed that spores could not be found in wild systems characterized by high gradient and low alkalinity. This has remained the case following 10 years of stocking infected fish. Whirling disease spores have been found in brook trout stocked in low gradient, high alkalinity, farmland streams . . . . and in the effluent below hatcheries where spores have been found.

John Hnath, Michigan. Please refer to the earlier information presented for the State of Michigan. Mr. Hnath also spoke of the Great Lakes Fish Disease Control Program. The Great Lakes organization is international in scope because Canada is involved. The Great Lakes Fishery Commission is a technical advisory group relative to fish health. Whirling disease is in a restricted category and there is no importation of fish with whirling disease into the region. Facilities providing fish must be inspected and found pathogen free for two years before importations can be made.

Don Junell, Nevada. Nevada has had experience with whirling disease through two decades but has managed to keep from spreading the disease throughout the state. Infected fish are not stocked in interstate waters. The Lahontan National Fish Hatchery and the Verdi State Hatchery were confirmed positive for whirling disease in 1969; the fish at the federal hatchery were destroyed but the State opted not to destroy fish at the Verdi station . . . the fish were stocked and there was no documentation of fish loss or ill effects in the wild.

The Verdi station has had a chronic history of whirling disease and it seems apparent the positive fish were coming into the facility. The station has since been taken out of production. Ultra Violet light was used at the Verdi station to treat the water supply. Power failures and waterfowl (mergansers) muddying ponds made the use of UV light ineffective and it was discontinued because of cost and no guarantee of clean water.

Nevada does not stock infected or suspect fish in interstate waters or where positive fish have not been previously stocked.

Chris Horsch, U. S. Fish and Wildlife Service, Coleman NFH. Whirling disease was confirmed in the 1984 brood year of steelhead with the suspected source the creek outside the hatchery. There was no incidence of whirling disease in the 1985 brood year fish. Several things were apparent from analyses conducted during the whirling disease episode at Coleman.

* Several myxosporidians, including Myxobolus cerebralis were identified from a general analysis of all fish in the system.
* Spread of whirling disease is traceable to movement of fish within the drainage system associated with the Coleman station.
* Following the initial infection, incidence appears to be declining and fish can no longer be infected.

Mr. Horsch felt that if whirling disease of salmonids had not been described years earlier, the parasite would be considered a novelty much as other myxosporidians in other species are considered. He is not concerned about the seriousness of the disease.

Tony Amandi, Oregon. Whirling disease was discovered in Oregon in 1987 and the disease was anticipated from strays of anadromous fishes from California waters. Whirling disease has also been found in northeastern Oregon where fish above and below a private hatchery were positive for the disease. Returning adult salmonids have been found positive for spores of whirling disease which indicates the disease has been around for some time. The disease has been confirmed in rainbow (including steelhead) and brook trout, and in chinook salmon in hatchery fish and in wild fish returning to streams to spawn.

Management decisions Oregon has made relative to whirling disease include;

* Cease transfers of fish from infected sites.
* Catch out of infected fish from positive stations. Heads removed before fish leave station, treated with lime, and buried.
* Disinfected eggs are sold.
* Oregon Commission has downlisted whirling disease to a category that does not require destruction of infected fish.
* Infected fishes are stocked in areas where whirling disease appears endemic.
* Assistance is provided private growers in preventing whirling disease infection.

Pete Walker, Colorado. Clinical signs of whirling disease were found in November, 1987. The disease has (to the time of the conference) been found at 9 sites, all on the eastern slope. The disease was first found in the Arkansas River system (four sites), then in the South Platte (two sites), the Rio Grande (one site) and the Cimarron River system (one site). There has been no mortality detected to date and the problem appears to be biopolitical in nature . . . . neighboring states and commercial growers are concerned that the disease may spread.

Two State hatcheries on the Arkansas system have been confirmed positive for whirling disease. These stations account for 30% of trout

production in Colorado. Stocking guidelines for infected stations allow for stocking where warm water would act as a barrier or outside a 10 mile radius of negative sites. No positive fish will be stocked in areas where there are self-sustaining stocks of wild trout. All state hatcheries have been inspected and all private and federal stations soon will be.

Steve Henry, New Mexico. New Mexico considers itself as not having whirling disease although the disease has been confirmed from three locations where illegal plants of fish (from Colorado) were made. It is felt that all of these fish can be recovered and the pathogen eliminated. New Mexico now has a permit system in effect regarding all importations of fish and has a moratorium on shipments of fish from out of State.

Rick Cordes, South Dakota. South Dakota feels fortunate in not having the disease because regulations governing movements of fish have been lax. Whirling disease is currently listed as emergency prohibitive in South Dakota, however, classification is currently under review because eradication efforts appear to be futile.

Jim Gleim, Nebraska. Nebraska has not experienced whirling disease and wishes to keep the disease out. No fish or fish eggs from infected populations would be allowed into Nebraska. If whirling disease were to enter a State hatchery, the facility would be quarantined until disposition of fish to market could be accomplished. Mr. Gleim felt that destruction of fish appeared unnecessary.

Doug Mitchum, Wyoming. There has been no whirling disease found in Wyoming (to time of Conference) and the State wants to remain free of the pathogen. All stations in the State were routinely inspected for whirling disease for a period of 10 years with negative results and inspections were reduced. With the incidence of the disease in Colorado, the inspections will resume with all facilities (public and private) in State being inspected.
Since the conference whirling disease has been discovered in Wyoming in fish stocked from producers in Colorado.

Import permits for all fish brought into the State will be required and permission from the State must be granted before any fish can be purchased for import for private plants.

Gary Camenisch, Missouri. Missouri does not have whirling disease and wishes not to have it. About 1.2 million pounds of trout are reared in State facilities each year and there is some private production of trout. Regulations relative to whirling disease were established in the 1960's when outbreaks occurred in Michigan. Missouri has no plans to downlist whirling disease or relax requirements that eggs be imported only from sources free of whirling disease. Missouri relies on compliance with this regulation . . . . there is some question about enforcement of the regulation.

Roger Sorensen, Arizona. Arizona does not now have whirling disease and desires to maintain pathogen free situation. Fish health program began in the 1970`s; prior to that time import regulations were quite lax. The State hatchery system is being upgraded to include concrete ponds and raceways. Whirling disease is currently classed as an emergency prohibitive pathogen in Arizona. Fish coming into the State must have disease free certification and a stocking permit.

Disease regulations are reviewed each 5 years in Arizona and a review is scheduled for July, 1988 at which time changes will be incorporated to allow importation of eggs from infected parents if the eggs are disinfected and incubated in spore free water. Arizona will not accept infected fish.

Kent Hauck, Idaho. Idaho conducted limited testing for whirling disease prior to 1987. All imports of fish from Oregon were terminated in 1987 after discovery of positive fish from that State. Analysis of the Salmon River system indicates that whirling disease is endemic there because 3 and 4 year old returning salmon have been confirmed positive for spores of whirling disease. Idaho does not permit stocking of fish where the disease does not exist but does allow stocking in the Salmon river drainage. No losses of fish attributable to whirling disease have been experienced.

Steve Roberts, Washington. Washington has no confirmed incidence of whirling disease though it is likely that the disease may be in the Grande Ronde River system, a tributary from Oregon in areas where whirling disease has been confirmed. There are more than 100 fish culture facilities (State, federal and private) in Washington. Washington currently considers whirling disease as an emergency disease but that will likely change to a lesser category that doesn't require destruction of fish.

Ron Goede, Utah. Utah does not have whirling disease and wishes to insure that the situation remains that way. There are strong regulations regarding importation of fish into the State and also with respect to movement of fish within the State. The regulations place responsibility on both the buyer and the vendor. It is felt that many, if not most, State regulations control only import. In this case, if a serious pathogen should be moved into the State there is no mechanism in place to detect and contain it and it is likely that the pathogen would be widely spread before detection.

It was stressed that the effects of whirling disease on free-ranging stocks has never been adequately demonstrated. Current opinion is based merely on the fact that no impact has been observed. Possible subclinical effects such as increase in disease susceptibility, reduced fecundity, increased susceptibility to predators, impaired growth and performance, and so on have never been investigated. Utah is concerned about the role of M. cerebralis as a chronic stressor which once introduced to a wild system cannot be removed.

Jim Peterson, Montana. Montana does not currently have whirling disease and feels that there are many more options to fish production and management without whirling disease than there are with the disease. Any fish with whirling disease are less desirable than those without it and it is felt that any sportsman would rather catch a healthy fish than one with some sort of disease, whether serious or benign.

Montana currently considers whirling disease as an emergency disease. Montana's fish health program is evolving and regulations specify that no fish will be imported from a positive facility or from a drainage positive for whirling disease. Suggested legal requirements are to require an import permit for fish, disinfection of all shipments of eggs into the state, and development of intrastate controls on fish movement.

John Nickum, U.S. Fish and Wildlife Service, Washington, D.C. It is likely that whirling disease will be downlisted on federal regulations to a category that doesn't require destruction of fish.

Bob Wiley, Fish Disease Subcommittee, CRFWC. The Colorado River Fish and Wildlife Council is an organization composed of the seven states in the Basin and has no regulatory authority. The organization's Fish Disease Control Policy serves as a guideline for effective fish health management in the Basin. By agreement, programs in the respective States can be more restrictive but not more lenient. The Fish Disease Subcommittee has proposed downlisting of whirling disease from Emergency Prohibitive to Prohibitive in 1987 and may recommend Notifiable category.

Jim Warren, Pacific Northwest Fish Health Protection Committee. Whirling disease has been recommended for inclusion in a category that does not require destruction. The policy recommended by this group is not to stock fish into areas where the disease does not occur. Dealing with whirling disease in anadromous salmonid stocks is difficult because the fish migrate great distances and can stray.

John Thoesen, New England Salmonid Health Committee. Whirling disease is likely to remain an emergency pathogen in New England and it is likely that if infected fish were discovered they would be destroyed. Regulations call for no import of products from infected facilities and importation of eggs only if certified as disease free.

Experiences and perspectives of private growers. The following information is presented as summaries of thoughts expressed by the presenters and any inaccuracies that may appear are entirely the responsibility of the Fish Disease Subcommittee.

People involved in commercial aquaculture stressed the importance of being treated fairly with regard to the occurrence of whirling disease; that is, treated in the same way as agency facilities. Infected fish retain value and various uses for these fish need to be identified. Currently, "regulations" have the effect of causing private growers extreme business losses. Commercial interests desire to have

input into policy making and decisions that affect their operation. The Fish Disease Subcommittee will seek input from these people in discussions of fish health management within the Colorado River Basin.

Commercial growers have a great concern for fish diseases and whirling disease in particular because their livelihood depends on inter- and intrastate shipment of fish. They expect fair treatment with regard to fish health inspections and disease control. Many commercial growers expressed concern that whirling disease is not the serious threat that it has been held to be. Since whirling disease has been considered a very serious disease, the result of fish health regulations has had a great effect on some growers (public and private); fish have been destroyed, facilities have been required to close, and large sums of money have been spent in attempts to control or manage the disease.

Growers felt that there should be some form of indemnification available to them for fish destroyed as result of whirling disease or other disease regulations. No clear recommendations were offered concerning funding for indemnification. They would also like to have some voice in developing policies that will affect them. The Fish Disease Subcommittee is considering how best to involve this interest in Colorado River Fish and Wildlife Council fish health management issues.

Perhaps the most important consideration beyond maintenance of fish health is identification of various means to utilize the diseased fish. Consensus at the meeting indicated that fish affected by whirling disease are commercially usable if proper care is taken to insure that spores are not introduced elsewhere.

Individual presentations by private growers.

Harold Hagen, Hagen Western Fisheries, Colorado. Dr. Hagen reported that he has never seen a clinically diseased fish infected with whirling disease. His experience with whirling disease at one of his facilities has been a "horror" story. The situation is that no fish from his positive facility can be moved west of the Continental Divide and he must let customers know that the fish test positive for whirling disease. He said that the controversy relative to whirling disease is ridiculous and the disease should be recognized for what it is; not a very serious pathogen.

Harold Hagen, Jr., Hagen Western Fisheries, Colorado. Operates a facility that has been quarantined because of whirling disease. There has been a 1% loss of fish but that appears to be due to crowding of fish as a result of the quarantine. Money should be put into research rather than regulations and enforcement particularly related to whirling disease because whirling disease isn't a very serious pathogen. He has not observed loss of fish due to whirling disease and feels that viral infections are much more important and serious than whirling disease. He is losing his respect for the fish health profession.

He feels that his business is being ruined by the regulations associated with whirling disease and believes that the pathogen ought to be removed from the list of diseases of concern.

Dick Smith, Lost River Trout Farm, Idaho. His station was diagnosed positive for whirling disease in 1987 and is the only known private facility in Idaho positive for the disease. The history of inspections at his facility has been twice yearly inspections since 1982.

Mr. Smith voluntarily destroyed about 400,000 of his fish with heaviest infestations and has spent $30,000 in renovations to his facility including enclosing the spring water source and piping water to the station. Since detection of the disease his business has crashed; there is no longer a market for his fish. He remains willing to destroy fish if others are also willing and emphasized equitable treatment for all affected by whirling disease. He fears that downlisting of the disease will cause further spread.

Roger Ritzert, Castalia Farms, Ohio. Roger earlier reported about the experience with whirling disease at Castalia Farms. He also indicated that the private sector in the Great Lakes region have been able to participate in the regulation making process and has a vote on the Great Lakes Fish Health Committee.

Anne Putnam, Rainbow Springs Trout Farm, Colorado. Rainbow Springs Trout facility is near the Arizona-New Mexico border and is isolated from the rest of Colorado. Station remains free of whirling disease. She had several recommendations relative to fish health management.

* There is a need to work together in addressing all fish health needs.
* Support service (fish health inspections, for example) costs should be shared among all interests (agencies, growers, and customers).
* Standardize the paperwork associated with movement of fish in interstate commerce; in that way, everyone has a better understanding of what is required and expected.
* Communication needs to be more rapid than the mails.
* Make lists of certified eggs and fish available to both fish farmers and customers.
* The public should be educated about fish diseases and fish health so that misunderstandings about diseases are avoided.

Ken Cline, Cline Trout Farms, Colorado. Cline's have been in the trout farming business for 42 years and this conference represented the first opportunity to participate in any discussions about diseases or fish health. Fish are shipped from this facility through seven States, so the variation in regulations and import requirements are very obvious.

Private growers should be allowed to stock infected fish in the same waters where positive fish are stocked from State facilities. Mr. Cline stressed that all interests should cooperate in working towards good and equitable fish health regulations and that the issue should not be decided by the courts.

REGULATIONS

Agencies across the country do not allow the importation of fish with whirling disease although there is some importation of properly disinfected eggs from whirling disease positive sources. Opinions relative to regulation of whirling disease focus on a single issue; those that do not have whirling disease will do all possible to insure that it does not spread and those that have the disease would like to have some reasonable way to manage with the disease, yet not facilitate its spread.

Much of the discussion about regulation concerned interstate movement of fish. Equally important but seldom discussed is intrastate movement. Whirling disease has apparently spread in California (for example) through movement of infected fishes within the state. Larry Krisl (USFWS Enforcement Agent) pointed out that movement of fish in interstate commerce is governed by the Lacey Act and violations of the provisions of that act carry fines ranging from $100,000 for civil violations to $200,000 plus 5 years in jail for willful violations. The penalties for felony violations range from $250,000 for individuals to $500,000 for corporations.

Whirling disease has been considered (and treated) as a Class A (very serious) disease requiring drastic (destruction of fish, depopulation and disinfection of facilities) control measures. In areas where the disease has been identified a general downlisting of the disease to a classification requiring less drastic control procedures are favored. Where whirling disease does not occur, strong regulation and treatments are favored. General consensus suggested that whirling disease could be downlisted to a classification that requires inspection but does not carry requirement for depopulation and disinfection of stations and destruction of infected fish.

Commercial growers suggested that effective regulations are those that generate voluntary compliance. Voluntary compliance will occur if;

* Compliance is possible and economically feasible,
* Compliance is easily accomplished with a minimum of bureaucracy,
* Regulations are understandable,
* Commercial interests perceive that they have had opportunity to assist in formulation of regulations affecting their business.
* Law enforcement people are educated in requirements of regulations,
* There is some communication in developing regulations,

* Trusting relationships are established,
* Meaningful fines are established,
* Difference between the letter and spirit of law is recognized.

These general rules ought to apply to many things that are done in fishery and fish health management. Regulations should have the flexibility to provide for implementation of carefully considered management options.

CONFERENCE STATEMENT

The Fish Disease Subcommittee, Colorado River Fish and Wildlife Council having reviewed information presented at the Whirling Disease Conference has determined that the status of the disease should be reevaluated and reassigned. The considerable expertise at the conference represented historic and current perspective pertaining to whirling disease (Myxobolus cerebralis). The Fish Disease Subcommittee recommends that whirling disease be included in the Notifiable Pathogen category of the Fish Disease Control Policy, Colorado River Fish and Wildlife Council.

The Notifiable Pathogen category is defined in the Policy as follows.

> Notifiable Pathogen: A pathogen of special concern capable of causing fish losses and which is treatable or manageable through existing technology or effective management of fish culture facilities.

The Notifiable category requires inspection of facilities but does not demand attendant destruction of infected fishes or depopulation and disinfection of facilities.

Rationale for recommendation. The whirling disease pathogen is recommended for downlisting (Emergency Prohibitive to Notifiable) because;

1. Best evidence based on case studies indicates that fish losses and deformities attributable to whirling disease are minimal in fish culture activities.

2. Appropriate hatchery management techniques (as highlighted at this conference) are available to minimize the effect of whirling disease at fish cultural facilities.

3. Affected fishes retain value for use as food fish and sport fish given careful management strategies.

4. Inspection continues to be an important part of the whirling disease fish health management strategy providing for continued monitoring of occurrences and facilitating management of incidents.

Information Concerning the Whirling Disease Pathogen

Current knowledge includes:

* There is no known therapeutic.
* Skeletal deformities may be caused.
* Mortality caused appears insignificant.
* Pathogen is transmissable through spores retained in fresh or frozen processed fish and developing in alternate annelid hosts.
* Pathogen affects young fishes, though they can recover.
* Pathogen is environmentally persistent through spores.
* Pathogen has a complex life cycle with alternate hosts.

Research needs; Information concerning the following is either lacking or requires further definition.

* Effect of pathogen on wild stocks (growth, longevity, and other biological impacts).
* Cross infectivity with non-salmonid species.
* Understanding of the ecology of the pathogen in wild systems, particularly concerning the establishment of the pathogen in the wild.
* Clear definition for the life cycle of the pathogen with special reference to life stages most susceptible to control.
* Safe, practical use for infected fishes.
* Development of additional fish cultural management strategies.
* The role of M. cerebralis as a chronic stressor predisposing populations to other infectious diseases and increasing susceptibility to pollutants, toxins, etc.
* Control - clean-up technology relative to best water supply management.
* Efficacy of egg disinfection including best chemicals, time and dosage.
* Develop refined techniques for differentiating M. cerebralis from other forms of Myxobolus.
* Determine the significance of the bird vector in transmission of the pathogen.
* Further define procedures used in certification of "pathogen free" fish stocks.
* Develop methods to insure sanitation of vehicles transporting fish.

SUGGESTED MANAGEMENT GUIDELINES RELATIVE TO WHIRLING DISEASE

These guidelines are offered towards the effective maintenance of fish health in the Colorado River Basin in fish culture systems and in wild populations. The guidelines are equally applicable elsewhere.

1. Fish with confirmed presence of whirling disease should be liberated into contiguous salmonid waters only in locations where it has been confirmed that the pathogen is established or where the probability of establishing or spreading the disease is unlikely.

2. When fish with the confirmed presence of whirling disease are to be shipped in inter- or intrastate commerce, careful consideration should be given for protection of areas where the pathogen does not now occur.

3. Fish eggs, water and ancillary equipment should be properly disinfected. Where support materials (shipping cases and so on) must be discarded, they should be discarded such that there is no threat of contamination of clean water supplies.

4. Comply with state and federal regulation requirements in the distribution of fish and fish products.

5. Fish culture facilities positive for whirling disease should be modified or operated so as to minimize or ultimately eliminate the occurrence of spores in fish.

6. Within these guidelines, the policies and regulations should include consideration of the economic impact of whirling disease on commercial, federal, and State aquaculture programs.

The recommendations presented will allow development of effective management strategies. Such strategies should be consistent so that programs are comparable and understandable.

Consideration of a plan for Indemnification.

Regulations governing fish health apply to all producers of fish including State, federal, and private. There is a cost associated with replacing fish destroyed through compliance with regulations governing fish health. The fish health community including growers, fish health specialists, fishery managers and fishery administrators should consider how best to set up a plan that would provide for replacement of fish or fish eggs destroyed through compliance with fish health regulations.

LITERATURE CITED

Wolf, K. and M. Markiw. 1985. Salmonid whirling disease. Fish Disease Leaflet 69, U. S. Fish and Wildlife Service, National Fisheries Center-Leetown, National Fish Health Research Laboratory, Kearneysville, WV

APPENDICES

Appendix 1. List of conference attendees.
Appendix 2. Text submitted by Don Manzer (California Fish and Game, Fish Disease Laboratory),

APPENDIX 1

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APPENDIX 2

The fish disease control provisions of this new legislation are only an element of a broader mandate to the Department to generally encourage and assist in the development of aquaculture in the State. Although the fish disease language regulates activities associated with private aquaculture, it is apparent within its context that it contains a doctrine of fairness expected of the State in the treatment of State facilities. The law also privides for a Fish Disease Advisory Committee which has the authority to advise the Director of Fish and Game on matters relating to diseases in aquaculture and on changes and interpretations relevant to the aquaculture code. This committee is in large part comprised of private aquaculturists.

Shortly after the aquaculture code was enacted, its disease control provisions were implemented in the first new major California discovery of whirling disease in about 20 years. This involved in rapid sequence two private fish growers on the same major drainage. In short order the fish and Game Director was notified, the Disease Advisory Commitee met, owners were instructed to bury all fish on the facilities and begin disinfection. All appeals for more moderate actions were rejected because the intent of the code was clear. Eradicate whirling disease wherever it might occur at any cost. Hatcheries were shut down and literally buried under tons of earth. Not much later the Department suffered its first episode in a State facility. Our hatchery managers made the same observations -- "no mortalities or disfigurement" and asked the same question - "why" - when instructed to bury fish. Unfortunately, rapid compliance with our policy of "immediate destruction", prevented any prolonged observation of infected fish, or documentation of mortality or other clinical factors. There was insistence on immediate destruction of valuable broodstock groups regardless of reasonably safe opportunities to salvage some spawning potential prior to such action. This situation made more apparent the fallacy of strict adherence to inflexible policy when prudent judgement might lead to more moderate actions.

The situations at Coleman National Fish Hatchery and the most recent private trout grower facilities involvement created an environment for more questioning attitudes and insightful evaluation. By this time it seemed more reasonable to transfer and release infected fish to waters known to be enzootic rather than wastefully destroy them. In order to do this, it was necessary to request a reclassification of whirling disease in our regulations. This was quickly accomplished by Commission action supported by the Fish Disease Advisory Committee. Now whirling disease is classified with Ceratomyxa, IHN, BKD, PKD, and several diseases of non-salmonid fishes.

Our policy now is to prohibit any movement of infected or potentially infected fish to waters that are not known to be enzootic for whirling disease. Waters are not considered enzootic by virtue of having received plants of infected fish. Such waters must show a three year history of continued infectivity without evidence of diminishing intensity. These constraints have seriously impacted the trout management program in the southern eastern Sierra where we have demonstrated that infected fish were planted from the Mt. Whitney/Black Rock complex. We have also denied commercially grown infected fish transfers to Southern California waters which were presumably planted with infected fish in recent years. The wisdom for several of these prohibitions is founded only on the reputed grave and insidious nature of whirling disease: because the threat to other salmonid resources or risk of establishing the disease in new waters were certainly minuscule.

Managing, administering or regulating fishery activities on a daily basis under the burden of whirling disease is reason enough to frequently question the intellect of what we do. We have wrought personal anguish and financial tragedy for what might have appeared to some as no more than an interesting biological anamoly. Forcing otherwise viable business enterprises to fold up, burying tons and tons of fish at the license buyers expense and seriously restricting catchable trout programs and anadromous fishery resource management is all serious stuff. Are we sure that the value of what we are saving or what we are hoping to prevent is worth the cost of doing so? We're not certain, but from what we have seen in California over the last 22 years, I have serious doubts!

California's whirling disease policy was developed instantaneously with the need to react to our first discovery. The national concensus of the day seemed to say that this was a mortal and debilitating disorder which should be treated with immediate destruction and eradication. That was our perspective in 1966 and we acted accordingly.

Seventeen years later, in 1983 one year after our second major episode with whirling disease in private aquaculture facilities and after burying 100,000 pounds of fish, we had a new perspective. Three aquaculture businesses were dead and one seriously crippled. Many doubted that we had seen any fish die as a result of infection by M. cerebralis. Our perspective was modified.

One year later, 1984, we had suffered our first known outbreak in a State hatchery complex involving two separate facilities. One remains in intensive care and the other severely crippled as is the fishery management program. Even in populations approaching 100% prevalence of intense infec-

tions, we saw no fish losses nor significant gross deformity in these hatchery populations. Then we had a new perspective.

Now, 22 years of experience later, we have another major aquaculture operation severely restricted and an anadromous hatchery involvement added to our list. We have destroyed over 165 tons of fish in California plus the entire 1985 brood year population of steelhead at Coleman National Fish Hatchery. The State's out of pocket expense has been in excess of $280,000 for disposal and disinfection costs alone. The dollar values of all other losses to private business and fisheries management have not been calculated, but they are obviously immense. Out-of-state marketability of eggs from endemic areas is now always of question regardless of any real risk of transmissibility.

Fish disease control perspectives seem to me to fit well within many other areas of environmental concern and many similar issues subject to related questions. The biological issue is the one foremost in our minds as fisheries pathologists or fish health specialists. We are concerned with the biological impact of specific disease on fish under culture and the native or wild fisheries resources that some of us are responsible for.

There is also another issue which I'll call ethical for lack of a better word. In my mind this is characterized by something like "purity", and "healthfullness".

We feel a need to protect against foreign parasite introductions not only because they might be bad but also just because they can't be good. They are akin to pollution.

A third and very valid issue can't be avoided and that is politics. Like it or not, there are other people involved in these matters besides pathologists. Commercial and recreational fishermen, aquaculturists, their customers and all the politicians who represent these people. Their views may often be adverse to ours but no less important to them.

California's perspective now: after bringing to focus all of the experiences that we have related and the concerns for all of the issues at stake in the whirling disease dilemma, our perspective is still clouded by the historical stigma of "one of the most threatening of salmonid diseases".

If we had the freedom of conscience and the assurance of the majority of other fish disease policy makers that whirling disease is no more serious a threat than furunculosis or vibrio, we could adequately manage the problem like we do with those diseases and still avoid any real threat of extending its actual range of concern.

We respect the ethical position which states that "we don't have it and we don't want it". In fact, I'm sure that with enough introspection, we'd find some of that in our own philosophy. But I suggest that we don't even apply this ethic consistently. We require certification for whirling disease but not for any of the other myxosporeans except ceratomyxa. I wonder how widespread they might be and if the same ethical tenet should apply to them also.

Those of us who have been surveying for whirling disease are finding at least one other myxosporean in the brain. Our only concern for this seems to be for the diagnostic confusion it presents. Why shouldn't it be listed in fish disease policies?

In trying to evaluate our perspective in order to establish policy, we must be guided by what we have experienced, what we have learned, and the legitimate interests of others. We ask ourselves some questions about what we have done so that we will know whether we should continue to act in the same manner.

Some of the questions we ask are:

1. When are we over protecting so that the costs of protection exceed the value of that protected.
2. When does the severity of the protective action go beyond what is reasonable to everyone else who doesn't share our ethic.
3. When will the magnitude of our cries for alarm exceed the willingness of administrators to accept our rationale?
4. At what point might we lose all credibility with our administrators and others that we serve when we continue to demand such severe control actions.

This last question causes me some real anxiety. Prior to our more serious encounters with whirling disease during the last 8 years, the Fish Pathology Section in California had almost unquestioning support from our administrators. Because of the many doubts about the wisdom of actions dealing with those problems, I am concerned that we may not have full support in future situations involving controversial opinions--even if we have no question about the severity of the matter.

We obviously need more research on many aspects of the whirling disease probelms, but I believe that the information presented at this meeting will support a new understanding of this disease. I believe that the pathological nature of whirling disease has been seriously exaggerated and that its importance in biopolitics has been elevated only by mystique. We in California hope that at this meeting our collective

wisdom will put M. cerebralis in its place so that we can have a more clear and uniform nationwide perspective.

Yesterday John Hnath asked the question "was it worth it?" That question applied to California's actions would have to be answered with a qualified "No".

If the question is paraphrased to ask "will it be worth the cost to continue with our current restrictive policy indefinitely"--I would answer with a more emphatic "No", IF that cost means further restriction of fishery management programs, denying legitimate commercial opportunities or even worse, destroying more aquaculture businesses.

Proposed changes will depend in part upon the Conference Statement that results from our meeting here.

COLORADO RIVER FISH AND WILDLIFE COUNCIL

FISH DISEASE SUBCOMMITTEE

Suggested Management Guidelines Relative to Whirling Disease

The following management guidelines were developed, in part, from information discussed at the April 12 - 14, 1988 Emergency Conference on Whirling Disease and in response to assignment from the Colorado River Fish and Wildlife Council.
The recommendations provide a framework within which effective management strategies can be implemented.

1. Fish with confirmed presence of whirling disease should be liberated only in waters where there is confirmed presence of the pathogen or where the probability of spreading the disease is unlikely.

Rationale. It is important that infected fish (those carrying spores) not be released where the spores may become established in the wild. There is evidence that judicious use and movement of infected fish can control the spread of the pathogen. Whirling disease has been contained in reasonably localized areas in Nevada by careful use of the infected fish. There have been similar experiences in California.

2. When fish with the confirmed presence of whirling disease are to be shipped in inter- or intrastate commerce, careful consideration should be given for protection of areas where the pathogen does not now occur.

Rationale. See No. 1 above.

3. Fish eggs, water, and ancillary equipment should be properly disinfected. Where support materials (shipping crates and so on) must be discarded, it should be discarded such that there is no threat of contamination of clean water supplies.

Rationale. The spores of whirling disease are environmentally persistent and can be transmitted via contaminated shipping containers, water, and so on. It is thus very important that all equipment be thoroughly disinfected and disposed of properly.

4. Comply with State and federal regulation requirements in the distribution of fish and fish products.

APPENDIX 3

Rationale. Regulations were developed to safeguard the environment, resource, and suppliers of fish.

5. Fish culture facilities positive for whirling disease should be modified or operated so as to minimize or ultimately eliminate the occurrence of spores in fish.

Rationale. Work across North America has shown that whirling disease can be controlled by installation of concrete rearing facilities, covering spring sources, and otherwise making sure that facility water supplies remain free of pathogens. Facilities positive for whirling disease should not simply continue in operation without efforts to control the disease. Failing to do so virtually guarantees the spread of the pathogen wherever infected fish may be liberated.

6. Within these guidelines, the policies and regulations should include consideration of the economic impact of whirling disease on commercial, federal, and state aquaculture programs.

Rationale. The policies and regulations developed for whirling disease ought to affect all fish producers equally. There is cost involved in "restocking" a depopulated fish culture facility whether commercial, federal, or State. The costs of restocking State or federal facilities are no less important than those associated with commercial interests except that dollar costs of repopulating State and federal facilities are borne by public funds and may, therefore, not be as visible as costs to a commercial operator.

7. An indemnification plan should be established to provide assistance to commercial aquaculture enterprises required to destroy or otherwise dispose of diseased fish because of regulations.

Rationale. Commercial growers are subject to various regulations in raising and transporting fish. Costs of restocking such facilities are borne by the grower whereas costs of restocking State or Commercial facilities is borne by the public (largely the fishing license buyer). Indemnification funds could be established through contributions to a trust account from commercial growers and public funds.

STOCKING OF FISH INFECTED BY WHIRLING DISEASE

Concern was expressed during discussions at the April, 1988 meeting of the Technical Committee and at the July, 1988 meeting of the Council relative to the distribution of fishes infected with whirling disease. It was felt that allowing stocking of diseased fish into watersheds where whirling disease exists would encourage further stocking (illegal) into uncontaminated waters.

COLORADO RIVER FISH AND WILDLIFE COUNCIL

FISH DISEASE SUBCOMMITTEE

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Rationale. See No. 1 above.

3. Fish eggs, water, and ancillary equipment should be properly disinfected. Where support materials (shipping crates and so on) must be discarded, it should be discarded such that there is no threat of contamination of clean water supplies.

Rationale. The spores of whirling disease are environmentally persistent and can be transmitted via contaminated shipping containers, water, and so on. It is thus very important that all equipment be thoroughly disinfected and disposed of properly.

4. Comply with State and federal regulation requirements in the distribution of fish and fish products.

Bogdanova, E.A. 1970. On the occurrence of whirling disease of salmonids in nature in USSR. Second International Congress of Parasitology, Abstract #719, September 6-12, Washington, D.C. Journal of Parasitology. 56 (Section II, Part II): 399.

719. On the occurrence of whirling disease of salmonids in nature in USSR.

E. A. BOGDANOVA, State Institut of Lake and River Fisheries, Leningrad, USSR.

For a long period—about 50 years—*Myxosoma cerebralis* (Hofer) Plhen was recorded as the agent of whirling disease only in rainbow trout at the pond fish farms of the West and East Europe (including the USSR), North America, and Asia. It was not found in other representatives of Salmonidae at that period. This is mainly due to the fact that rainbow trout is one of the main species reared at the salmonid pond farms in the majority of European countries whereas in the USSR many salmonids—*Salmo salar, Oncorhynchus keta, O. gorbuscha, O. masu, O. nerka, S. ischchan*—are reared alongside rainbow trout.

The agent of whirling disease at the different stages of development has been recorded both in the above species and in salmonids from some rivers of northern Europe (*S. salar, S. trutta*) and the Far East (*Oncorhynchus masu, O. keta, O. gorbuscha, Salvelinus malma, S. leucomaenis*).

These data enable us to conclude that this species of Myxosporidia is peculiar to Salmonidae and occurs within their natural range.

In the Soviet Union regions of distribution of whirling disease of salmonids in nature are: Kola Peninsula, Far East, the Carpathians, and the Caucasus.

The results of studies have shown that the percentage of infection in natural water bodies is not the same for the salmon species differing in duration of river and sea period of life. It attains 60–70–100% in nonanadromous forms and is only 5–15% in forms descending to sea for feeding in 4–5 months after hatching.

Thus, we may point to the fact that all representatives of Salmonidae participate in distribution of the parasite in nature, but nonanadromous forms—*Salmo trutta, Salvelinus malma, S. leucomaenis*—are most important among them.

It was mentioned above that the percentage of invasion in natural water bodies attains sometimes

100%, however the intensity of infection is as a rule rather low and therefore no symptoms of disease are observed there.

The disease symptoms are recorded only under conditions of artificial rearing: In June of 1964 whirling disease was recorded in *Salmo ischchan* infsp. *gegarkuni* in the basin of Issyk Kul Lake. In September of 1967 in young salmon—*S. salar*—at Kola Peninsula. In May of 1960 external symptoms of disease were observed in young *Oncorhynchus keta* and *O. masu* at Sakhalin. Slides of cartilaginous tissue of cranium were studied histologically and plasmodia were found.

Cases of whirling disease affecting young salmon at the hatcheries situated within the region of its natural occurrence are the result of accumulation of parasitic spores in nursery basins; they accumulate there for a long period (4–5 years) for quicklime used usually for disinfection does not kill spores of the parasite.

Havelka, J. and F. Volf. 1970. Whirling disease of salmonids caused by *Myxosoma cerebralis* in Czechoslovakia. Second International Congress of Parasitology, Abstract #253, September 6-12, Washington, D.C. Journal of Parasitology. 56 (Section II, Part I): 137-138.

253. Whirling disease of salmonids caused by *Myxosoma cerebralis* in Czechoslovakia.

J. HAVELKA and F. VOLF, Fisheries Research Institute, Laboratory Praha. (English translation edited by G. L. Hoffman.)

This disease appeared in Czechoslovakia for the first time in the year 1954 in one trout hatchery and in 1956 in three trout hatcheries. In all cases it was identified microscopically as *Myxosoma cerebralis* in the skeleton of diseased fish. Externally the disease appeared with typical symptoms: whirling, black tail, gaped jaws, misshapen heads and trunks, mostly with spinal curvature (scoliosis, kyphosis) and slow growth. In one case, most expressive and frequent symptoms of diseased fry in rainbow trout (*Salmo gairdneri* Richardson) the fins were quite disintegrated, especially the caudal, dorsal, anal and pectoral fins; ventral fins remained intact. The losses by rainbow trout, brook trout (*Salvelinus fontinalis* Mitchill) and brown trout (*Salmo trutta morpha fario* Linne) reached 80–90%.

The most remarkable is that in one registered case, *Myxosoma cerebralis* was identified for the first time in the world in the year 1956 in the grayling (*Thymallus thymallus* Linne). The disease broke out suddenly to a great extent even without appearing in the brown trout and rainbow trout that were reared together with the grayling. The breeding graylings were caught in the running waters and held over the winter time in the raceway (211 m^2). The breeding rainbow trout were reared in the ponds and the brown trout were always caught before the spawning time in the running waters. In the year 1949 were imported to the fishery 200,000 eggs of rainbow trout from Denmark and in the year 1956 100,000 eggs of sea trout (*Salmo trutta trutta* Linne). The fry of grayling died in great numbers; black tail and whirling were the most obvious symptoms. The spinal curvation was quite rare. The fact, that among 3 kinds of fish, living together, grayling only was attacked by the whirling disease at that time demonstrates the possibility of some specificity of *Myxosoma cerebralis*.

According to records of attacked trout hatcheries, the symptoms of whirling disease were noticed soon after the second world war in the years 1948 and 1949 after importing eggs and yearling trout from Denmark. In the following years the symptoms vanished and reappeared again in the years 1954–1956, when it was ascertained and verified as above mentioned. We are convinced that whirling disease of trout was

brought to this country in rainbow trout from Denmark.

We exclude that the disease was brought in to our fishery from nature with water. Czechoslovakia is situated in a higher position, and has no inflow from neighbouring countries, and all waters rising in Czechoslovakia flow into the Baltic and Black Sea. The inland spreading of this disease by water doesn't come into consideration, because the attacked hatcheries are distant from each other and are not connected together. Even it is excluded that the disease is brought in by marine food fish which was not used by us at this time.

During the last 10 years we can state that whirling disease in our fish culture has not recurred. Its control was achieved by the following measures: limiting import of eggs and stocking fish of salmonids from abroad, and the effort of every trout hatchery to produce the stock fish from its own brood fish. In the trout hatcheries where whirling disease was ascertained, the ponds were drained, cleaned, cleared of plants and mud; strong repeated disinfection was carried out on the bottom and banks of ponds and inlet and outlet with quick lime or calcium cyanamide. The hatch house and all implements which came in contact with the diseased fish or contaminated surroundings were disinfected. It was forbidden to sell the fry, yearlings and breeding fish from affected hatcheries. The fry was fed in pure water at least 4 weeks, and after which they were planted in disinfected ponds at smaller stocking rates. The fry were not planted in the ponds where older fish were raised.

Heckmann, R.A. 1993. Whirling disease, *Myxobolus cerebralis*, in Utah salmonids. Annual Meeting of the Rocky Mountain Conference of Parasitologists. Abstract #9, May 20-22, Bozeman, MT.

WHIRLING DISEASE, MYXOBOLUS CEREBRALIS, IN UTAH SALMONIDS. R.A. Heckmann, Dept. of Zoology, Brigham Young University, Provo, Utah, 84604.

Abstract: During 1991 Whirling Disease of salmonids caused by Myxobolus cerebralis was reported for the first time in Utah. It was first reported in the Loa - Bicknell (Wayne County) area of Utah in a private aquaculture facility. Since that report the disease has spread to several other regions (Tooele, Cache County) and has been of major concern to the State Fisheries Biologists. Myxobolus cerebralis is not life threatening to the host but causes lesions and disintegration of the developing bone with subsequent misshapen heads, spines and prominent head depressions. This disease has not been reported in Montana but is found in most western states. State and regional regulations pertaining to Whirling Disease will be revived. In some states, "Zone regulations" have been instituted to permit transfer of infected fish only to areas where Whirling Disease exists. Infected fish could be deliberately stocked by malefactors in uncontaminated areas to expand the range of possible sales.

Heckmann, R.A. 1994. Politics and fish parasites; the Utah story. Annual Meeting of the Rocky Mountain Conference of Parasitologists. Abstract #7, May 19-21, Colorado Springs, CO.

POLITICS AND FISH PARASITES; THE UTAH STORY. Richard Heckmann*,Department of Zoology, Brigham Young University, Provo, Utah, 84602.

House Bill 262 was passed by the Utah Legislature this year whereby private aquaculture became part of the jurisdiction of the Department of Agriculture. Before it was under the Division of Wildlife Resources. The history behind this bill involves two fish parasites, ***Myxobolus cerebralis and Bothriocephalus acheilognathi,*** both recently introduced into Utah. Whirling Disease, caused by ***M. cerebralis,*** was first reported during 1991 in an isolated part of south central Utah near private fish production facilities. Since that time Whirling Disease has spread to Northern Utah and this year it was detected in wild trout populations. Whirling Disease has been the center of controversy between the private trout growers and the Chief Personnel in the Division of Wildlife Resources. The Asian fish tapeworm has a longer history in the state but has been isolated in one river in Southern Utah. During 1993 a shipment of grass carp, ***Ctenopharyngodon idella,*** were planted in ponds in Utah Valley without proper quarantine and inspection. Now ***B. acheilognathi*** threatens endangered fish in Utah Lake. Due to the connection of the Utah Governors family to private trout farmers and the dismissal of the state fisheries director, this problem has been considered Utah's version of Whitewater for the Governor.

Hermanns V.W. & W. Korting. 1985. Improved method for the detection of non-sporulated *Myxosoma cerebralis*, Hofer 1903, causative agent of whirling disease (WD) of salmonids. Berl. Munch. Tierarztl. Wschr. 98: 126-129. (GERMAN)

W. HERMANNS and W. KÖRTING: Improved method for the detection of non-sporulated *Myxosoma cerebralis*, Hofer 1903, causative agent of whirling disease (WD) of salmonids.

Summary

A method is described which supports the early diagnosis of whirling disease in salmonids. The entire head of trout is embedded in water miscible plastic without prior decalcification. The quality of sections allows the detection of vegetative sporoplasms and thus the diagnosis of WD before the onset of spore formation. Further a clear differentiation between parasite induced tissue alterations and normal bone formation of the skull can be made.

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Anschrift des Verf.: Dr. U. HERMANNS, Institut für Pathologie, Tierärztliche Hochschule Hannover, Bischofsholer Damm 15, D–3000 Hannover 1

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273. Whirling disease in the state of Michigan.

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Information received in August of 1968 made the State of Michigan aware of an *exchange* of fish between a Michigan trout farmer and an Ohio trout farm known to be currently affected by whirling disease (*Myxosoma cerebralis*), although it was not known whether the disease had developed before or after the transfer of fish. Immediate investigation showed that three stations in Michigan were contaminated with the protozoan.

Following detection of the disease in Michigan, an administrative order was issued by the Director of the Department of Natural Resources quarantining the infected establishments. The state and federal hatcheries within Michigan were also quarantined pending inspection, although there was no reason to suspect infections in these facilities.

Inspection of the records of the primary sources of infection disclosed distribution of fish to 159 smaller hatcheries, fee fishing ponds, and private recreational ponds. Sample inspection of these indicated spread of the disease and the quarantine was subsequently extended to all licensed trout farms and fee fishing ponds in Michigan.

Further inspection of private, state and federal hatcheries established that the protozoan was confined to hatcheries and ponds which had received fish from the primary sources. The extensive quarantine was relieved in September of 1968. Only establishments known to have received

fish from the primary sources remained quarantined. Concurrently, a temporary permit system was established to prevent further spread of the disease through importation or interstate transport.

Injunctions on the primary sources prohibited them from spreading the disease through movement of fish. Permission was granted to sell fish, providing the heads and entrails were removed and buried on the grounds.

Meanwhile, through voluntary agreements, 159 establishments originally receiving fish from the primary sources were treated with Fintrol 5 to kill the fish. These were then buried on the premises or in sanitary land fills.

After this the ponds were checked for whirling disease by using susceptible live fish. Rainbow fry were placed in free-floating test cages and distributed among the suspect establishments. Test fish were collected and examined for spores approximately three months later. To date, all these fish examined have been found to be free of spores.

The validity of the test cage procedure is being further checked by release of susceptible aged rainbow fry in a few ponds where the disease had once been shown to be present. These fish will be recaptured and examined after a 3–4 month period.

The primary sources remain contaminated with living diseased fish, pending the outcome of litigation between the State and the owners. Spores of whirling disease have been observed in wild brook and brown trout from a natural stream draining a primary source. Close watch is being kept on this area and current plans of the Department of Natural Resources are to survey the stream to determine the extent of the disease in the natural environment. If feasible, the fish population in affected portions of the stream will be eradicated and barriers erected to isolate these areas from other wild fish.

Current legislation is now in effect to prevent the importation of further diseased fish into the State.

UNITED STATES DEPARTMENT OF THE INTERIOR, STEWART L. UDALL, SECRETARY
Fish and Wildlife Service
Bureau of Sport Fisheries and Wildlife

Fishery Leaflet 508

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WHIRLING DISEASE OF TROUT

By
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INTRODUCTION

Whirling disease of trout is a serious hatchery disease in Europe and has recently spread to Russia, Italy, and the United States.

IDENTIFICATION

This disease derives its name from the rapid, tail-chasing type of whirling which is often seen when the fish is frightened or trying to feed. This whirling differs from the horizontal spiraling of the fish along its long axis which is characteristic of infectious pancreatic necrosis (Fishery Leaflet 453). The whirling symptom is associated with lesions and disintegration of the cartilaginous skeletal support of the organs of equilibrium caused by invasion of a parasite protozoan. The damage to the head skeleton is evident in older trout as a depression in the head or as misshapen jaws. Sometimes the spinal column is affected resulting in spinal curvature. Pressure on the nerves which control the caudal pigment cells results in "blacktail" in many fish. Symptoms may appear as early as two weeks after the fry start feeding and in heavily infected groups of fish there may be many mortalities. The whirling and black tail are less striking as survivors grow older and these symptoms tend to disappear in one to two years. Sunken heads and spinal curvatures do not disappear, however.

CAUSE OF THE DISEASE

Myxosoma cerebralis (Lentospora c.) a myxosporidian protozoan which was discovered in Europe in 1903 is the causative organisms. The development of the parasite takes four months at which time the spores can be demonstrated in wet mounts made from scrapings of cranial skeleton and auditory capsule (organ of equilibrium). The spores are about 10 microns in diameter and possess two polar capsules. Prior to this, verification can be made only by histological methods.

SOURCE AND RESERVOIR OF INFECTION

As far as is known this parasite attacks only salmonid fishes. Infected yearlings in the water supply are the usual source. The spores are very resistant and probably survive drying and/or freezing. It is difficult to eradicate the disease from earthen ponds.

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MODE OF TRANSMISSION

Although there is some evidence that spores are released through the intestine, it is probable that most fry become infected from accidentally ingesting spores that have been released from dead trout four months of age or older which are crushed or decomposed. The ingested parasite leaves its spore capsule in the intestine and migrates through the intestinal wall to the head cartilage where development continues.

INCUBATION PERIOD

The typical symptoms usually appear at one to two months after exposure to the disease. However, mortalities of fry may occur before this.

PERIOD OF COMMUNICABILITY

Spores remain in infected fish at least three years and probably longer.

SUSCEPTIBILITY AND RESISTANCE

Rainbow and eastern brook trout are susceptible to the disease. Very young fry are most susceptible; occasionally year-old fish may become infected but older fish do not become diseased. Brown trout become infected but do not develop disease conditions and, therefore, may act as carriers. Salmon and grayling have been found infected in Europe.

RANGE

This disease was known only in Europe until recent years. Apparently it originated in Central Europe and spread to other European countries, including Russia. It has been reported in Italy and the United States in the last few years.

METHODS OF CONTROL

A. Preventive measures

Destroy all fish from ponds containing fish known to be infected. Incineration or deep burial is recommended.

Fish must be reared in spring or well water for the period of maximal susceptibility (8 mo.). No adequate filtering device is available for stream water. Earthen ponds must not be used during this important period.

All concrete ponds must be cleaned thoroughly and disinfected with calcium cyanamide (0.08 lb/sq. ft. on wet concrete). Quicklime and sodium hypochlorite are chemicals of second choice.

Earthen ponds are usually responsible for the perpetuation of the disease. Drain the ponds and immediately apply calcium cyanamide as above. Allow to stand a month or more and clean out muck as thoroughly as possible--bury deeply or plow under in farm field. Fill pond with water, drain and repeat treatment. Pond may be used six weeks later. It may be necessary to repeat this treatment the following year.

The hatchery may be restocked the following season with fish from known uninfected source The young fish should be kept as long as possible in metal or concrete facilities. Keep ponds well cleaned and remove mortalities each day. Use earthen ponds only for eight-month or older fish until it is certain that the disease has been eliminated.

Brown trout and older rainbow and brook trout, although not obviously diseased, may serve as carriers.

B. Therapy

No proven chemotherapy is available at present although Scolari (1954) cites partial prophylaxis.

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Gives an account of first epizootics of this disease in Russia. Recommends that no fish be transferred from an affected hatchery. Describes the possibility of some spores being eliminated with the feces. She found the disease in rainbow trout primarily, but also brown trout and salmon.

Hoffman, G.L. 1970. International control of parasitic diseases of fishes. Second International Congress of Parasitology, Abstract #277, September 6-12, Washington, D.C. Journal of Parasitology. 56 (Section II, Part I): 151-152.

277. **International control of parasitic diseases of fishes.**

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The first United States livestock disease law was written in 1884. The first national fish disease law, a 1958 amendment (Part 13) to Title 50-Wildlife and Fisheries, attempts to exclude certain fish diseases from importation to the United States. Salmon and trout, live, fresh, or frozen, must be certified free of whirling disease (*Myxosoma cerebralis*) and viral hemorrhagic septicemia before they can be imported. The certification shall be made in the country of origin by a designated official acceptable to the Secretary of the Interior. This amendment was prompted, in part, by the appearance of whirling disease in northeastern U. S. in 1957. Up to that time the disease had been known only from Europe. It is probable that the disease came to this country in frozen table trout. The amendment was further necessary because viral hemorrhagic septicemia, another European disease, does not exist in the U. S. If other fish diseases threaten U. S. fish stocks, further amendment of the law will be necessary. This law is a part of Title 50 which also controls the importation of live fish, birds, and mammals into the U. S.

According to a recent survey by FAO (FI/EIFAC 68/SC II-17, May 7, 1968) 21 countries have adequate import laws to control fish diseases; they are: Argentina, Australia, Brazil, Bulgaria, Canada, France, Greece, Hungary, Ireland, Luxemburg, New Zealand, Nigeria, Panama, Phillipines, Sudan, Switzerland, Trinidad and Tobago, Uganda, United Kingdom, USA, and Zambia. In addition I have learned that Iran required disease-free certification of a bluegill sunfish importation and Costa Rica required certification of goldfish imports. The USSR requires disease-free certification for their collective fish farms and fish hatcheries. It is likely that other countries have control measures.

With the rapid increase of fish culture in many countries, and the transfer of table fish, live fish and fish eggs, disease prevention becomes a very important item. Adequate laws should also protect native fish stocks when imported and cultured fish are to be stocked in natural waters. In fish culture, inspection of fish stocks where the fish are raised is probably of more value than a single inspection of a shipment of fish at the time of ex-

port or import; some diseases, e.g., whirling disease, can be diagnosed more easily in small fingerling trout than in yearling or adult trout. Fish intended for stocking in natural waters should be inspected in the country of origin and preferably at the site where they were raised. Asymptomatic carriers of disease will continue to make this work difficult, therefore, more research on diagnostic methods is needed.

Hoffman, G.L. & C.E. Dunbar. 1961. Studies on *Myxosoma cerebralis* (Hofer) Plehn (Protozoa: Myxosporidea) the cause of whirling disease of trout. Annual Meeting of the American Society of Parasitologists, Abstract #53, August 27-31, Lafayette, IN, Journal of Parasitology. 47 (4, Section II): 29.

53. **Studies on *Myxosoma cerebralis* (Hofer) Plehn (Protozoa: Myxosporidea) the Cause of Whirling Disease of Trout.** G. L. HOFFMAN, C. E. DUNBAR, Department of the Interior, Bureau of Sport Fisheries, Kearneysville, West Virginia, and A. D. BRADFORD, Benner Spring Fish Research Station, Bellefonte, Pennsylvania.

This disease, apparently of central European origin, has more recently appeared in Russia, Italy, and the United States. Very young rainbow and eastern brook trout are most seriously affected. The trophozoites invade and erode cartilage causing malfunction of the organ of equilibrium which results in heetic, tail-chasing, whirling when disturbed. Other symptoms include crooked spinal columns, gaped mouths, sunken heads, and black tails. The multinucleate trophozoites grow and produce many pansporoblasts each of which produces several spores in about four months.

Whirling disease appeared at a Pennsylvania trout hatchery in 1956; one pond of fish was affected. The epizootic became widespread in 1957 and 1958 at which time a tentative diagnosis was made by Dr. S. F. Snieszko, USFWS. It was verified histologically by Dr. E. M. Wood, Seattle, Washington. In the spring of 1960 an attempt was made to clean and disinfect the hatchery using calcium cyanamide (1/10 lb. per sq. ft.) in the dirt ponds and chlorine gas (max. 300 ppm) in the spring water source. There has not been a recurrence of the disease in the 1961 fingerlings.

In 1960 Mr. Fred Howard discovered the disease at the National Fish Hatchery, Lamar, Pennsylvania, which is on the same watershed. There were relatively few fish affected and it is believed that only one series of ponds is contaminated.

During 1959–1961 we exposed early feeding rainbow and brook trout fry to the spores. Some of the fish developed whirling symptoms, but we could not verify the infections histologically.

Hoffman, G.L., C.E. Dunbar & A. Bradford. 1962. Whirling disease of trouts caused by *Myxosoma cerebralis* in the United States. Special Scientific Report. No. 427. 14 p.

WHIRLING DISEASE OF TROUTS CAUSED BY MYXOSOMA CEREBRALIS IN THE UNITED STATES

By

Glenn L. Hoffman, Clarence E. Dunbar[1/] and Arthur Bradford[2/]

ABSTRACT

This disease has recently appeared in North America for the first time. It appeared in a Pennsylvania trout hatchery and may have spread from there to the Lamar National Fish Hatchery which is on the same watershed, and from Lamar to the Kensington State Hatchery in Connecticut in transferred fish. The parasite develops in the cartilage, primarily of the head of very small trout. Symptoms of blacktail, whirling, spinal curvature and misshapen heads follow skeletal damage.

It was not possible to infect rainbow trout fry experimentally with the spores although the incidence was very high in one of the hatcheries. The development of the parasite was studied in infected fish brought from the Benner Spring Hatchery to Leetown. In histological sections the parasite can be seen as a small multinucleate trophosoite at 3 months after infection. The first spores can be seen at 4 months and persist for at least 3 years. Spores can be found in wet mounts prepared from head cartilage.

A program for control has been started, and the incidence appears to be declining in the Benner Spring Hatchery. The spring water reservoir was chlorinated, the ponds cleaned and potassium cyanamide applied. Acetarsone (Stovarsol) was fed to one lot of small trout at high concentrations with no noticed side effects, but control of whirling disease was not determined.

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This disease, apparently of central European origin, has more recently appeared in Russia, Italy, and the United States. Trout usually become infected during the first few weeks of feeding, mortalities ensue and most of the surviviors exhibit disease symptoms for 3 or more years. The spores gain entrance to the fish, presumably through accidental ingestion, and the sporoplasm of the spore emerges and migrates to the cartilage, mainly that of the head. The very small sporoplasm, now called a trophosoite, grows and its nuclei divide repeatedly to form a much larger organism which finally produces the spores. During the growth of the parasite much host cartilage is eroded and the skeleton weakened, resulting in the symptoms -- whirling, black tail, gaped jaws, misshapen heads and trunks. The whirling is caused by damage to the cartilaginous capsule of the organ of equilibrium; the blacktail is caused by damage to the skeleton in the region of the nerves which control the posterior pigment cells.

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To the best of our knowledge no other group of parasites has representatives which develop in cartilage or bone of the host, but among the Myxosporidia there are 6 other species which have been so recorded. They are: Myxobolus aegelefini from the head skeleton of Gadus, Pleuronectes, Molva, Merluccius (marine) (Kabata, 1957); Myxobolus dentium from the base of the teeth of Esox masquinongy (Fantham et al. 1939); Henneguya brachyura from the fin ray of Notropis (Ward, 1919); Henneguya sp. in the cartilage of gill of Pomoxis (Davis, 1923); and Myxosoma sp. in the head cartilage of Lepomis macrochirus (Hoffman, 1961, unpub. research). To date the pathogenicity of cartilage parasites has been studied only in M. cerebralis infections.

During the past 3 years, we have studied the symptoms, the cause, and have attempted to infect fish experimentally to study methods of control and treatment. Our results are here incorporated in a review of the subject.

HISTORICAL AND GEOGRAPHICAL

The parasite and disease were first described as Lentospora cerebralis by Hofer (1903) in Germany. His associate, Marianne Plehn (1904, 1924) described the disease, the parasite, and the histopathology in greater detail. Schäperclaus (1931, 1954) also described the disease in detail and outlined a method of control. Heuschman (1940, 1949), Tack (1951), and Luling (1952) in Germany have also studied whirling disease and its control. Vanco (1952) records it from France, Kocylowski (1953) from Poland, Scolari (1954) from Itally, Dyk (1954) and Volf (1957) from Czechoslovakia, Uspenskaya (1955, 1957) in trout and salmon from Russia, Schäperclaus (1959, pers. comm.) from Denmark, and Bogdanova (1960) in salmon from S.E. Russia. Hoffman and Dunbar (1961) briefly reported on it from the United States.

The chronology of the reports indicates that the disease originated in Central Eurpoe. In support of this hypothesis is the fact that the brown trout, a native of Europe, may become infected but is resistant to the disease, whereas the imported rainbow trout becomes seriously diseased.

OCCURRENCE IN THE UNITED STATES

Whirling disease appeared in brook trout at the Benner Spring Fish Research Station, Bellefonte, Pennsylvania, in 1956; one pond of fish was affected. European trout were circumstantially implicated in the appearance of whirling disease at Benner Spring; sample purchases of frozen imported table fish may have been spilled accidentally, or their viscera discarded in streams. The epizootic became severe in the hatchery in 1957 and 1958 at which time a tentative diagnosis was made by Dr. S. F. Snieszko. This was verified histologically by Dr. E. M. Wood, Fish Pathologist, Seattle, Washington. In the spring of 1960 an attempt was made to eradicate the parasite by using calcium cyanamide (1/10 lb. per sq. ft.) in the dirt ponds as recommended by Dr. Schäperclaus of Germany, and chlorine gas (max. 300 ppm) in the spring water source and small reservoir. A few trout were killed in the small reservoir, but it was not certainly known if this was the source of infection for the hatchery. There was no recurrence of the disease in 1961 fingerlings in the hatching building. We believe the disease is under control and can be eliminated completely in a year or so. Whirling disease did reappear later in some of the dirt ponds, however. As long as the spring water supply remains uninfested there probably will be no recurrence of the disease in the hatching building. We hope the spores presumably remaining in and around the ponds will eventually be eliminated.

The National Fish Hatchery, Lamar, Pa., is on the same watershed as Benner Spring and in 1960 Mr. Fred Howard discovered the disease there. The disease was verified by the Eastern Fish Disease Laboratory. There were only a few fish affected, and we believe that only one series of ponds was contaminated. The Lamar Hatchery plans to treat the ponds, when drained, with calcium cyanamide. All diseased fish were incinerated.

In December 1961, Mr. Lyle Pettijohn diagnosed Myxosoma cerebralis disease in fingerling rainbow trout from the Kensington State Fish Hatchery, Kensington, Connecticut. The hatchery personnel had noticed symptoms,

apparently of M. cerebralis disease, about a year earlier. The source of infection may have been in one lot of trout transferred from Lamar to Kensington in 1959.

To our knowledge whirling disease caused by M. cerebralis has not been verified anywhere else in North America. Dr. R. Bangham (pers. comm.) recalls identifying M. cerebralis at a northern Wisconsin hatchery about 1945 but no specimens are available for our verification. We have seen similar whirling at three other hatcheries but could find no M. cerebralis and assume that some physiological disturbance or other disease may also cause this symptom. No other characteristic symptoms were present in these fish.

If the spreading of this disease is not halted shortly, it can be expected to show up in trout and salmon hatcheries where infected fish are transferred and particularly those which have earthen ponds, trout in spring water reservoirs or stream water supply.

SYMPTOMS AND THE COURSE OF THE DISEASE

The symptoms are thoroughly discussed by Plehn (1904), Schäperclaus (1954) and Uspenskaya (1957) but will be reviewed here because of the lack of a previous English discussion. The symptoms we have seen are identical with those reported in Europe.

Trout may become infected up to one year of age (Schäperclaus, 1954) but usually become infected during the first few weeks of life--the earlier the infection, the more severe the disease because of the greater amount of cartilage present in younger fish. As we have pointed out, we were not able to prove that trout become infected by ingesting the spores, but this should be assumed until disproven. The disease takes the following course:

Period of "incubation". After exposure to the spores a lapse of 40-60 days ensues before the symptoms of whirling disease are evident (Schäperclaus, 1954). Our experimental fish in which we were never able to demonstrate the parasite exhibited symptoms in 12 to 16 days. We mention it here because there may be other conditions which simulate M. cerebralis whirling disease. We do not know for certain whether the disease causes mortalities during the "incubation" period. The parasites are so small during this period that histological verification is probably impossible or extremely difficult and mortalities might be attributed to other factors.

Initial symptoms. The most obvious symptoms, tail-chasing, whirling and black tail (fig. 1), become evident at about 40 to 60 days and may last about 1 year. The trophozoites have invaded and eroded the cartilage of the developing skeleton. Rather large "lesions" containing the parasites and debris can be seen in histological preparations. The cartilaginous capsule around the auditory-equilibrium organ behind the eye is usually invaded. Perhaps toxins released by the parasite (Plehn, 1904) or simply weakening of the capsule destroys the equilibrium of the fish to such an extent that each time it is disturbed or tries to feed it goes into a frantic tail-chasing whirl. This tail-chasing type of whirling differs from the horizontal spiraling of the fish along its long axis which is characteristic of a virus disease, infectious pancreatic necrosis (Snieszko and Wolf, 1958) and possibly hexamitiasis (octomitiasis) (Davis, 1953). Small fish, up to 3 months of age (about 2 inches long) may become so exhausted that they fall to the bottom and remain on their sides until they regain their strength. It is during this period that mortalities are likely to occur. Other debilitating factors such as other parasites, bacterial or viral diseases or malnutrition will probably increase mortalities. During the early part of this period the whirling symptom is at its worst, but it tends to subside gradually until it is only rarely seen in fish one year post-infection.

Very often the cartilage of the vertebral column, posterior to the 26th vertebra, is simultaneously affected (Plehn, 1904; Schäperclaus, 1954). The sympathetic nerves which control the caudal pigment cells have their origin at about the 26th vertebra. Apparently the weakened skeleton at this point causes pressure on the caudal nerves and pigment cell control is lost, hence the black tail. The black tail tends to dissappear earlier than the whirling symptoms. One lot of about 4,000 rainbows at Kensington became infected when brought to the hatchery at

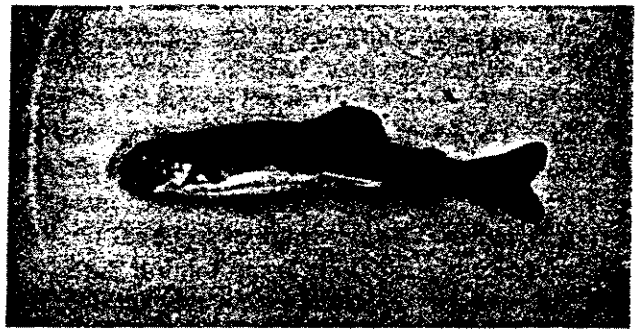

Figure 1:--Photograph of black-tail rainbow trout caused by Myxosoma cerebralis - about 3 months post-infection. Photograph by S. F. Snieszko.

Figure 2:--Photograph of eastern brook trout with spinal curvature and black tail due to Myxosoma cerebralis infection. Photograph by S. F. Snieszko.

6 months of age; an estimated 100 percent of these developed whirling symptoms, but no black tail.

Survivors' symptoms (figs. 2 and 3). Those infected fish which were not killed by the parasite during the early stages of the disease tend to recover although they may be misshapen, particularly in the head. The black tail and whirling usually disappear but the spinal curvature and misshapen head may reflect permanent damage. The two most common and obvious head symptoms are the sunken areas behind the eyes and the permanently open or twisted lower jaw. All of these symptoms are caused by the loss of cartilage during bone formation when the skeleton is weakened and support is lost. During the latter part of the first year much of the damaged area is filled in with an epitheloid granuloma type of tissue which tends to proliferate in many instances and cause secondary damage. Plehn (1904) p. 163 has an excellent illustration of such a tissue proliferating from a vertebra and causing pressure on a sympathetic nerve. Presumably these "recovered" fish can live for a long period of time so long as they are not crippled too badly to feed. In spite of apparent recovery, however, the spores remain in the tissues of the fish for at least 3 years.

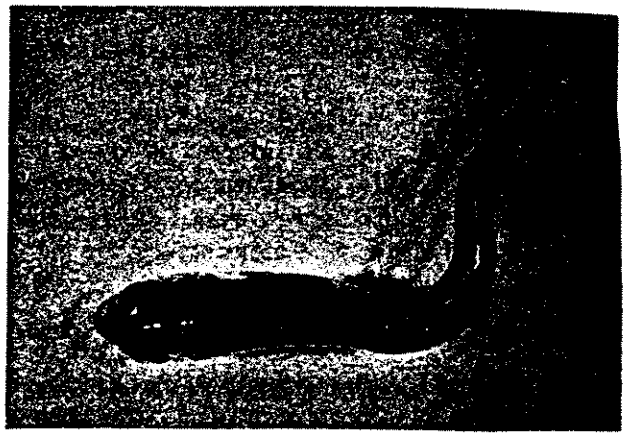

Figure 3:--Photograph of yearling rainbow trout with spinal curvature due to Myxosoma cerebralis infection.

TRANSMISSION AND HOST SPECIFICITY

It has long been assumed that, if ingested, the spores taken directly from infected trout will infect other trout (Plehn, 1904; Schäperclaus, 1931, 1954). As previously discussed, we were not able to do this experimentally at Leetown, and to our knowledge no one else has. Therefore, all knowledge concerning transmission and life cycle has come from studies on material collected during epizootics.

Trout fry can be infected by exposing them to water containing the silt, and presumably the spores, of ponds from a hatchery epizootic (Schäperclaus, 1931). One assumption is that the spores are ingested accidentally, but the possibility of an invertebrate transport host has not been ruled out. Another assumption is that spores are freed from infected fish when they decompose or are crushed. Uspenskaya (1957) has found isolated spores in various organs of the fish and suggests that they may be carried away from the site of infection by blood or lymph and be deposited in other organs. If this includes the intestine, they could be shed while the fish is still alive. However, it has not been determined whether fish can be infected by this method. Apparently the freed spores accumulate in the ponds (particularly earthen ponds) and the severity of the epizootic depends on the number so accumulated. Small trout up to four months are most severely affected. The disease can be controlled therefore, but perhaps not eliminated, by keeping the trout in spore-free water until they are four months or more of age. Older fish may become infected, but are usually not seriously affected because ossification of the skeleton prevents massive infection. Such fish, however, may act as "carriers".

On the basis of epidemilogical evidence the spores are apparently very resistant to drying, freezing, and survive a long period of time (Plehn, 1904, 1924; Schäperclaus, 1931, 1954). We have kept 2 vials of spores for 3 years, one at room temperature, the other at about 6° C. At the end of 22 months all spores appeared normal, but at 3 years the sporoplasm has completely disappeared from 85 percent and did not appear normal in the remaining 15 percent. We believe they were 100 percent non-viable.

It is likely that the spores can be carried from pond to pond or hatchery to hatchery on boots and other equipment. Schäperclaus (1931) found myxosporidean spores in the feces of kingfishers at an affected hatchery and believes that the disease can be spread in this manner.

Most myxosporidean species show varying degrees of specificity for certain species or closely related species of fish. They are also usually specific for a certain organ or tissue. Myxosoma cerebralis is no exception--it has been found in rainbow trout (Salmo gairdneri); eastern brook trout (Salvelinus fontinalis); brown trout (Salmo trutta) and recently in salmon (Salmo salar) (Uspenskaya, 1957), grayling (Volf, 1957), and in Salvelinus leucomaenis, S. malma, Oncorhynchus keta and O. masu (Bogdanova, 1960). Rainbow trout are most seriously affected by the disease, brook trout somewhat less severely, and brown trout show no symptoms at all but may act as "carriers". Likewise, any symptom-free but infected rainbow or brook trout may be serious "carriers". The initial infection in a fish is always in cartilage, but loss of cartilage and proliferation of tissue may leave the spores outside of the skeleton in little cyst-like structures.

LIFE CYCLE

The complete life cycle of Myxosoma cerebralis (Lentospora c.) has never been determined experimentally (fig. 4). We know of no one who has demonstrated the experimental life cycle of any Myxosoma or related Myxobolus or Henneguya species. These are all histozoic parasites. Plehn (1904, 1924), Schäperclaus (1954), and Uspenkaya (1957) assume that the spores are ingested, the sporoplasm leaves the spore, penetrates the intestinal mucosa and migrates to the cartilage. However, this has never been verified experimentally. Kudo (1930), p. 313, reviews experimental infections and cites two workers who successfully infected fish with three coelozoic myxosporideans--Myxidium, Chloromyxum, and Leptotheca; but none of the histozoic Myxosoma, Myxobolus, or Henneguya are mentioned.

The various tissue stages from hatchery epizootics have been described by Plehn (1904)

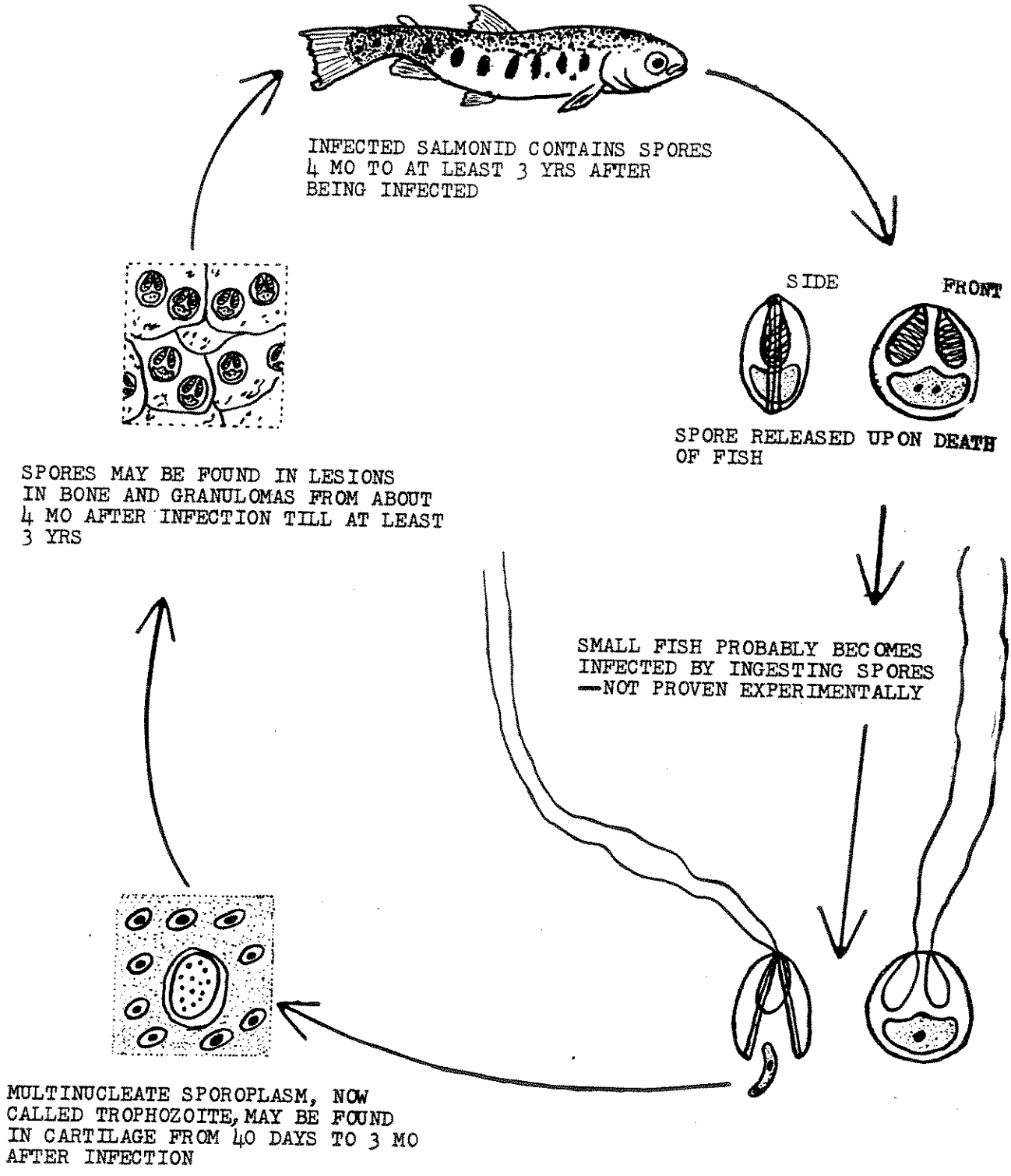

Figure 4:--Life cycle of Myxosoma cerebralis. (Note that experimental infection of fish has never been achieved).

and Schäperclaus (1931). In neither instance, however, was the material from experimental infections, i.e., the fish were not exposed to infection under controlled conditions. Schäperclaus (1931) held newly feeding fry in infested ponds for 11 days and then observed them in aquaria supplied with uninfested water. Symptoms of black-tail disease (tail-chasing whirl and black tail) developed at 35-46 days. The fish recovered in about 2 months but the infections were not verified histologically (Schäperclaus, 1961, pers. comm.). It is not known for certain exactly how they acquired the parasites.

The sporoplasm becomes the multinucleate amoeboid trophozoite that can be seen in the histological sections of cartilage from about 40 days post infection (Schäperclaus, 1954) to 3-4 months. The trophozoite grows and the nuclei divide and differentiate to produce units known as pansporoblasts, containing 12 nuclei each which eventually produces 2 spores at about 4-6 months. Spores are probably released after the death and disintegration of the fish. However, Uspenskaya (1957) has found spores in various organs other than skeleton and believes that some make their way to the outside through the intestine during the 4-9 month phase of the disease. Spores have been found in fish up to 3 years of age (Uspenskaya, 1957). It is assumed that newly feeding trout fry become infected by ingesting spores which have been released from the skeleton and associated lesions of older fish that have died or been crushed. However, no one has reported on establishing the disease experimentally by feeding fresh spores to fry or by holding the fry in water to which fresh spores have been added.

In an attempt to reproduce the life cycle and to test disinfectants, we set up 47 experiments in 1959. Spores were fed to 47 lots of 3-24 rainbow and brook trout each. The fish ranged from 2 weeks to 4 months of age. Individual lots of the spores were treated with one of the following: sodium hypochlorite, sodium hydroxide, formalin, phenol, calcium oxide, zephiran chloride (Roccal^{R}), calcium cyanamide, drying, heating, freezing, and others (94 fish) served as controls. The aquarium water was not changed until absolutely necessary in order to retain the spores. Compressed air was supplied and the aquaria cooled by immersion in running 54° F. spring water. Of the entire group of fish, 17, including two controls in uncontaminated, but otherwise similar aquaria, developed whirling symptoms at 12 to 19 days post exposure. Only one developed black tail. Whirling (tail chasing type) alone cannot be used as proof of M. cerebralis infection because we have seen whirling at two hatcheries where no spores could be found. Presumably other conditions, perhaps certain types of malnutrition, can cause whirling. Therefore, we attempted to verify our studies by identifying the developing stages of the parasite in histological sections but we were not able to demonstrate any parasites.

In 1960 seven lots of 10-75 (total 290) newly feeding rainbow fry were placed in aquaria at 54° F. Suspensions containing many spores were prepared by homogenizing infected yearling trout heads with the Waring blendor or macerating with mortar and pestle. This material was added to the aquaria just after the fish had begun to feed and the water was not changed in order to retain the spores. Another lot was also fed such a suspension for 6 days. All fish were kept in the "contaminated" water for 5 to 14 days and then transferred to running spring water. Whirling was seen in a very few at about 2 weeks but no infections have been verified and no symptoms persisted. From these experiments we assume that a transport host, pond envoronment, or different water condition is necessary for transmission of the disease. Another possibility for our negative results is that we may have rendered the spores non-viable during handling. We were not able, however, to ascertain any damaging factors and the spores appeared normal microscopically.

We have likewise been unable to infect very young bluegills with the spores of a different Myxosoma sp. that occurs in bluegill cartilage.

In an attempt to determine whether fry can become infected prior to feeding, 4 lbs. of rainbow sac-fry were kept at the affected Benner Spring Hatchery for 10 days and then brought to Leetown, before feeding, for rearing in uncon-

taminated water. They were observed for 5 months; except for one which developed whirling symptoms, growth and behavior were normal. The affected fish was sectioned at 3 months of age and the developing stages of M. cerebralis were readily recognized in the cartilage.

DEVELOPMENT STAGES IN TROUT

Prior to 3 months (fig. 5)

Presumably the sporoplasm has made its way through the intestinal wall and migrated via blood or lymph channels to the cartilage, mainly of the head. This seldom affects fish over 12 months of age (Plehn, 1904, 1924; Schäperclaus, 1931, 1954). Our attempts to infect fish experimentally failed, so we could not demonstrate this stage. Schäperclaus (1954) p. 379 (our fig. 5) has an excellent photomicrograph of the developing trophozoite which has "eroded" a cavity for itself in the cartilage. As near as we can determine this is from a 40-day-old fry, so the parasite must be 40 days or less of age.

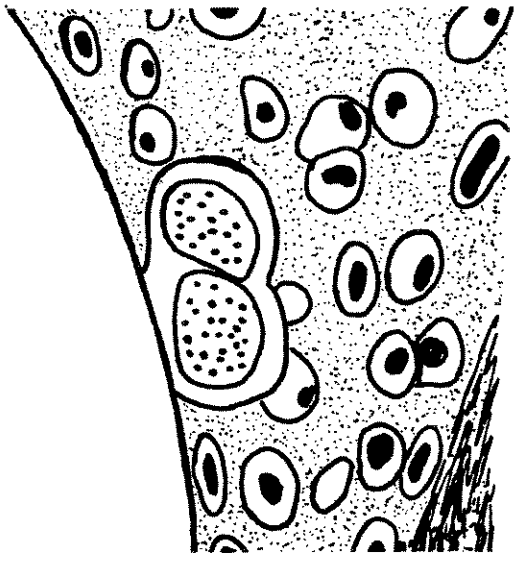

Figure 5:--Trophozoite of Myxosoma cerebralis in cartilage at about 40 days post-infection (drawn from photo of Schäperclaus, 1954).

Three months (figs. 6, 7, and 8)

Our only specimen of known age is this one. It was from one of many sac-fry brought from Benner Spring, Pa., during the epizootic, to Leetown for rearing in water free of M. cerebralis. Since it must have become infected at Benner Spring, we know the approximate age of the parasite. At this stage the parasites are multinucleate ameboid trophozoites and in cross sections range from 5 x 5 to 30 x 8μ in diameter the smaller ones are possibly cross sections of elongate trophozoites. There are at least 18 nuclei about 1-1/2 - 2μ in diameter in each trophozoite. These exist in lesions measuring about 300 x 100μ in the cartilage. Also in the lesions are freed cartilage cells and remnants of disintegrating cells and cartilage matrix. Apparently in normal osteogenesis in the trout studied, the cartilage of the skeleton in certain places is eroded from within by vascular action at the same time that bone is being laid down on the outside of the skeletal structures. Blood vessels penetrate the cartilage, and pockets of it are eroded, presumably by enzymes released from the capillaries. These pockets of cartilage erosion (fig. 9) resemble M. cerebralis lesions, but contain blood vessels, disintegrating cartilage cells and cartilage matrix and sometimes multinucleate host cells but no parasites. The multinucleate cells resemble phagocytic giant cells, but Ruth[3/] suggested that they might be tissue cells that have failed to divide.

Four months (figs. 10, 11, and 12)

The multinucleate trophozoite has grown considerably but is still in the cartilage. Some of the nuclei have divided repeatedly to form groups of nuclei (or cells?) which are now known as pansporoblasts. These distinct units produce the spores, usually two each. Kudo (1960) believes that myxosporideans are distinctly multicellular at this stage and, therefore, much different from other protozoa. Kudo (1930) and Noble (1944) have reviewed pansporoblast formation in other Myxosporidia. At this age some of the pansporoblasts have already produced spores. The trophosoite (entire parasite) has probably reached its maximum size at this stage; those measured were up to 1 mm in greatest diameter although we do not know for certain whether two or more trophozoites may be in such close association that their boundaries are not discernible.

3/ Ruth, Delbert. Anatomy Department, John Hopkins Medical School, Baltimore, Maryland, pers. comm., 1961.

Figure 6:--Photomicrograph of Myxosoma cerebralis in cartilage at 3 months post-infection (x 100). See fig. 7 for labelling.

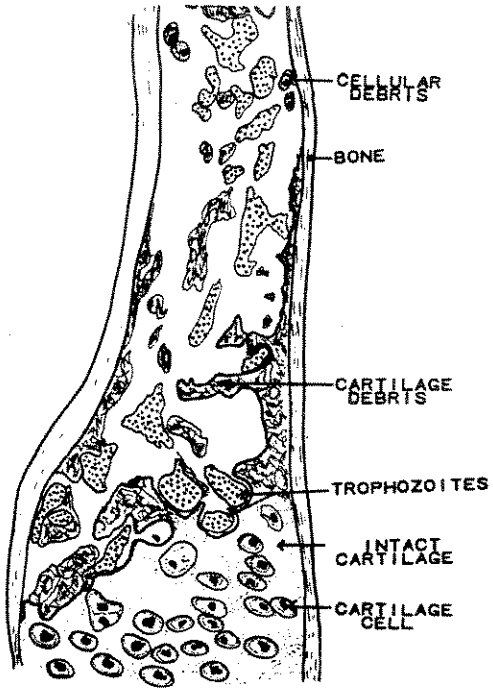

Figure 7:--Drawing of Myxosoma cerebralis in cartilage at 3 months post-infection. (Made from same slide as fig. 6) Drawn with aid of microprojection.

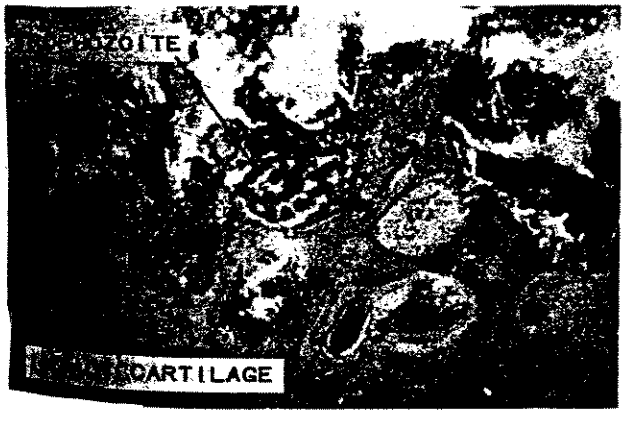

Figure 8:--Photomicrograph of Myxosoma cerebralis in cartilage at 3 months post-infection (x 430).

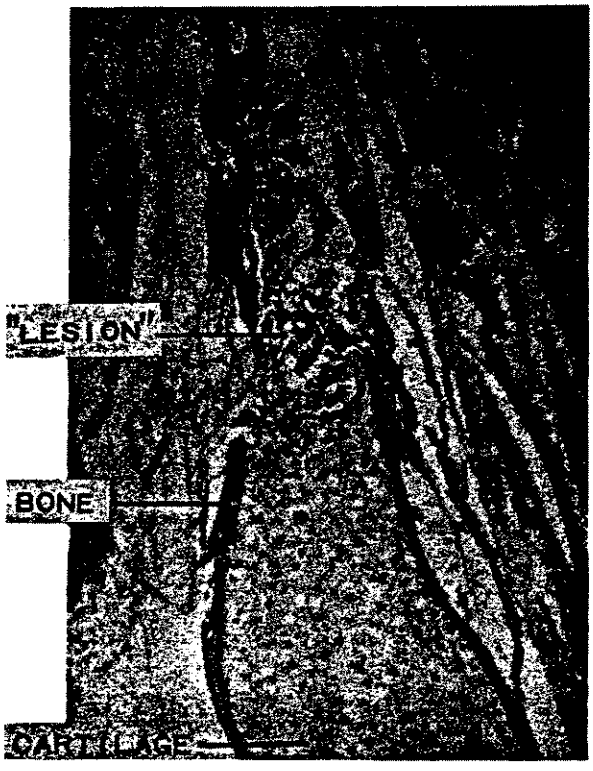

Figure 9:--Photomicrograph of normal cartilage resorption in trout about 3 months of age. These "lesions" grossly resemble Myxosoma cerebralis infections.

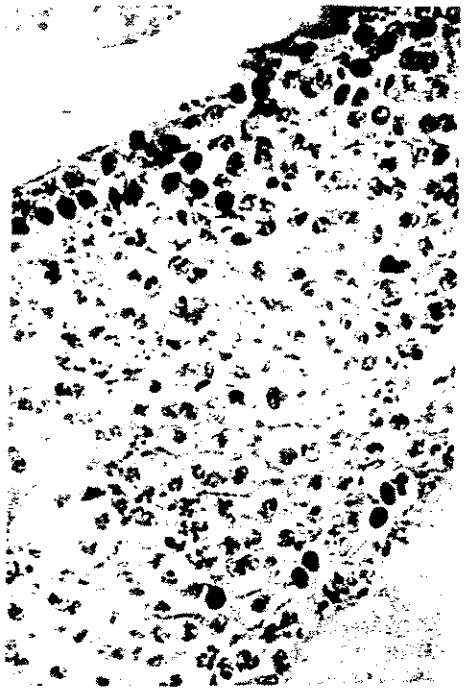

Figure 10:--Photomicrograph of Myxosoma cerebralis in cartilage at 4 months post-infection. Note that two spores develop in each pansporoblast. Stained with Giemsa's to show spores (x 430).

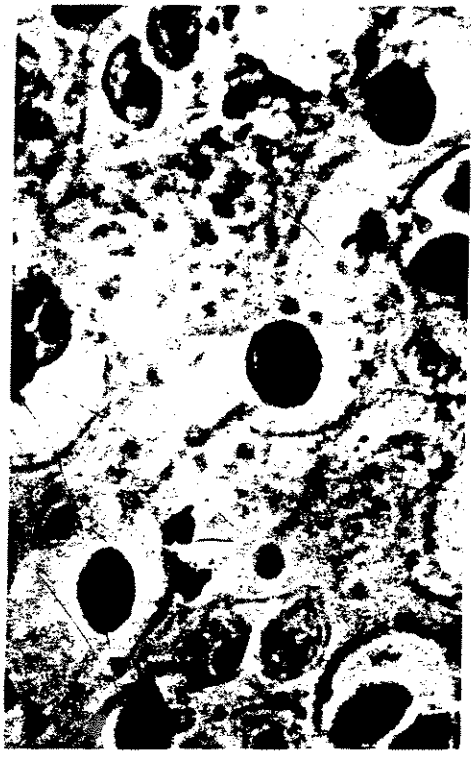

Figure 11:--Photomicrograph of Myxosoma cerebralis in cartilage at 4 months post-infection. Stained with Giemsa's to show spores (x 970).

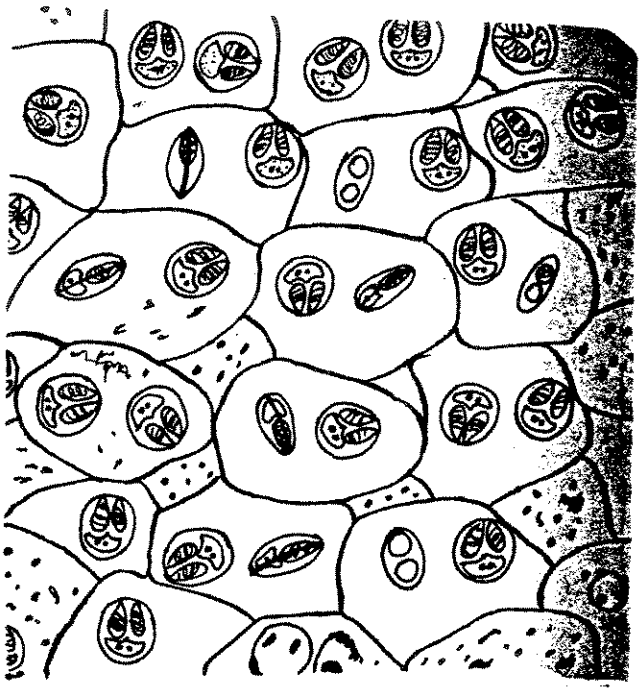

Figure 12:--Free-hand drawing of Myxosoma cerebralis pansporoblasts containing two spores each. Four months post-infection.

Eight months and older (figs. 13 and 14)

At this time all of the pansporoblasts have produced their spores, and these can be seen in the "lesion". Apparently the rest of the parasite disintegrates. The spores appear to remain in the lesion site permanently--Uspenskaya (1957) found them in 3-year-old fish. We have found many spores in 2-year-old fish and presume that they may remain much longer. It has been assumed that the spores remain in the site of the lesion, but she cites evidence that some of them are transported, presumably by blood, to other organs including the liver and lumen of the intestine. We believe that this is not the usual means of spore dissemination.

HISTOPATHOLOGY

There is no evidence of histopathology during the incubation period of 40 to 60 days. However, after this, as the trophozoite grows, cartilage is eroded, skeletal support is weakened, and simultaneously there is a proliferation of epithelioid-type granuloma about the parasite and its spores.

1. Forty days. Slight erosion of cartilage. Schäperclaus (1954) p. 379 (our fig. 5).

2. Sixty-five days. Still trophozoite only; no tissue proliferation. Schäperclaus (1931) p. 542, 554.

3. Three months (figs. 6, 7, and 8). Cartilage still being eroded by trophozoites in a lesion-like cavity in cartilage; cellular and cartilage debris present in lesion, bone being formed at periphery of skeleton. No tissue proliferation or inflammation.

4. Four months (figs. 10, 11, and 12). Some spores now present, but still no proliferation.

5. Eight - 12 months (fig. 13). Spores present. Trophozoites no longer present. Epithelioid proliferation of fish tissue now surrounds the mass of spores. This cyst-like structure is sometimes referred to as a granuloma which may cause pressure on vital organs (Plehn, 1904).

DIAGNOSIS

Before development of the spores (about four months) the disease can be tentatively diagnosed on the basis of the symptoms--tail-chasing, whirling, and black tail. Verification can be made only by finding the ameboid stages in the cartilage of the head in histological sections. Very early stages are more difficult to find but at three months they are often easily found and consist of trophozoites 5 to 30μ in size with many nuclei about 1-1/2 to 2μ in diameter.

From 4 months post infection to at least 2 years the spores can be found easily in wet mounts or histological section. Wet material may be prepared by dissecting out the auditory capsule and crushing it (Plehn, 1904) or splitting the head lengthwise and scraping the posterior part of the cranium with a scalpel to free the spores. A more reliable method for fish, of at least 2 years of age, is to cut up the head with scissors and macerate in a Waring blendor in about 50 ml of water--the spores can usually be found in a random drop.

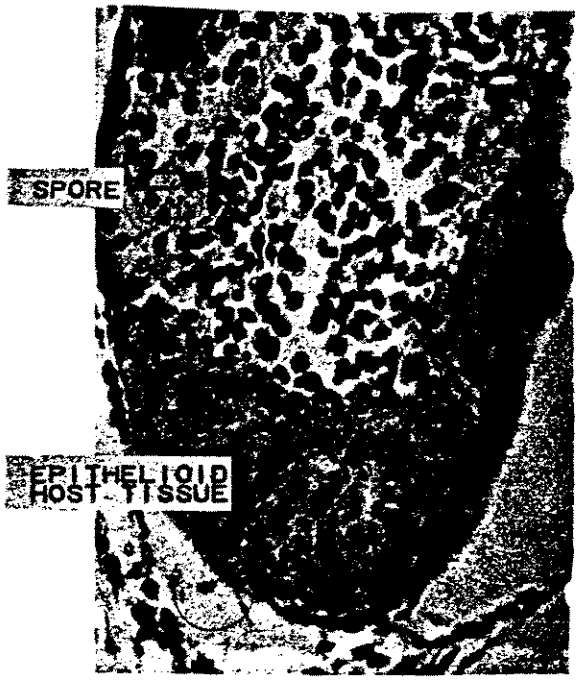

Figure 13:--Photomicrograph of Myxosoma cerebralis spores in an epithelioid granuloma at 12 months post-infection. Stained with Giemsa's to show spores (x 430).

Figure 14:--Photomicrograph of spores of Myxosoma cerebralis from rainbow trout at 12-15 months post-infection. Stained with Giemsa's (x 970). Photo by Dr. E. M. Wood.

In histological section the early stages (1-1/2 to 3 month post infection) can be recognized by their many small nuclei in the lesions in the head cartilage (figs. 5, 7, and 8). Lesion-like structures in normal bone formation (fig. 9) resemble whirling disease lesions. Sometimes these contain large multinucleate host cells (giant cells) which may be confused with parasites.

In histological section of the head the spores stain blue with Giemsa's (figs. 10, 11, 13, and 14), blue with carbol toluidin blue (Schäperclaus, 1931) and red with aniline water-saffranin (Plehn, 1904).

CONTROL AND TREATMENT

A modified version of Schäperclaus' (1954) and Tack's (1951) recommendations for control follows:

1. Destroy all fish from ponds containing fish known to be infected. Incineration or deep burial is recommended.

2. Water supply. That which supplies the hatching house fry and early fingerlings (up to 8 months at least) should be spore-free spring or well water. There should be no fish in the water system before it reaches the hatching house. Stream water may be used only if the stream contains no fish. No satisfactory filtering device for whirling disease has been described for hatcheries in the United States, but we have heard that sand-charcoal filters have been used successfully in France.

3. Disinfection of hatching house. Clean thoroughly (this is more important than chemical disinfection). We have found that overnight soaking with the following will dissolve the spores or cause them to extrude their polar filaments and probably render them uninfective: sodium hypochlorite (commercial or home laundry bleaches) as per instructions on container; zephiran chloride (Roccal^{R}) 800 ppm; sodium hydroxide 1/2 percent, calcium oxide (quicklime) 1/2 percent. It is imperative to have spore-free facilities for fry because the younger the fish the more seriously they are affected by whirling disease.

4. Disinfection of concrete ponds. Drain ponds and immediately apply calcium cyanamide at about 0.04 lb./sq. ft. (1780 lb./acre). Allow to stand 3-4 weeks, clean thoroughly, wet down and repeat treatment. Some of the chemicals listed in 3 above would probably be satisfactory for small ponds. It would take about 10 times as much chemical if used in water-filled ponds. Quicklime and hydroxides are strongly caustic chemical agents and personnel using them should wear protective clothing and use goggles and respirators.

5. Disinfection of earthen ponds. This is the most difficult aspect because organic matter usually interferes with disinfectants. Drain the ponds and immediately apply calcium cyanamide as in 4 above. Allow to stand a month or more and clean out silt as thoroughly as possible. Haul out to a farm field for plowing under or bury deeply. Fill pond with water, drain and immediately apply calcium cyanamide as before. Flush out and refill ponds 2 weeks or more later and stock with fish; Tack (1951) allowed 6 weeks before adding fingerlings.

Schäperclaus (1954) recommends quicklime as the chemical of second choice to be used at the same rate as calcium cyanamide. Snow and Jones (1959) have used quicklime successfully in treating bluegill ponds for fish diseases. We believe that the hot reaction of quicklime which produces a high transient pH is effective in killing spores--a pond so treated is safe to use 10 days after treatment with no flushing necessary. Sodium hydroxide is probably more effective than lime but may be more expensive.

6. Restocking hatchery. Eggs or fry should be obtained from a known uncontaminated source. Fry should be kept in hatching house as long as possible (3-8 months) because it is usually easier to control the disease here. It is advisable to maintain 2 series of ponds until it is certain that the disease is eradicated.

The first series of ponds should be concrete raceways supplied with spore-free water. Use great care not to contaminate with spores or infected fish. Keep fish here only 3 months.

It takes 4 months for the spores to develop, therefore, these ponds could not be contaminated by fish as long as the hatchery house, ponds, and water supply are spore-free. Pick off mortalities twice daily; incinerate or bury deeply.

The second series of ponds may be concrete or earthen. For best results the fish should be 8-12 months or more of age when stocked here. The older the fish when infected, the less serious the disease and it is doubtful that 13-month-old fish can be infected--certainly not heavily.

Following this routine it is expected that some disease will show up in the last series of ponds. If whirlers and mortalities are incinerated, however, one can expect the disease to disappear in 2-3 years. It may be necessary to treat earthen ponds annually for 2-3 years.

7. No fish from an affected hatchery should be transferred to an unaffected hatchery. However, the disease probably cannot be transmitted by eggs (Schäperclaus, 1931). It must be kept in mind that infected brown trout and lightly infected rainbow and brook trout may serve as carriers although they show no symptoms.

8. Fish from an affected hatchery should not be stocked in fishing waters unless there is no other hatchery on that watershed and no natural trout reproduction or stocked fingerlings. It is better to destroy the infected fish than to take a chance on spreading the disease which has apparently spread from Central Europe to Russia, Italy, and now North America in the last decade. Disposal or transport by fishermen of spore-bearing fish is an ever-present threat to spread of the disease.

Suppression of the disease, but not elimination, with Acetarsone (Stovarsol) at 10 mg per kilogram of fish daily on 3 consecutive days with weekly intervals between each course was reported by Scolari (1954). He recommends that it be used for 6 months for (partial) prophylaxis. Apparently the drug has no serious side effects on the fish. We fed it to several thousand rainbow trout for eleven months at Benner Spring at concentrations as high as 100 times that recommended by Scolari (1954). Although these Acetarsone experiments have not been completed as of this writing, the preliminary results are not promising. We thus believe it should be tested further before recommending it for prophylaxis of whirling disease.

SUMMARY

Whirling disease (black-tail), caused by the myxosporidean, Myxosoma cerebralis, is reported from the United States. It has made its appearence at 2 trout hatcheries located on the same watershed in Pensylvania and at another one in Connecticut. Young rainbow and Eastern brook trout were severly affected. European brown trout were not severely diseased, but probably served as "carriers".

Many attempts to transmit the disease in the laboratory failed.

The developmental stages of the parasite, histopathology, diagnosis, and control are discussed. The severity of the disease in the hatcheries has been reduced, but not eliminated, by removing fish from the water source and treating the ponds with calcium cyanamide.

Preliminary experiments with Acetarsone (Stovarsol), although inconclusive, indicate that the drug is not vary toxic to trout.

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Bird transmission of whirling disease

Seventy rainbow trout fry were added to aquaria containing mud and great blue heron feces containing whirling disease spores and "aged" about 4 months. One lot of feces was refrigerated, one was frozen, and one was dried at room temperature. There were no symptoms in any of the fish and no spores could be found in 17 necropsied from each aquarium. Seventy more swim-up rainbow trout fry were added to each tank but they were also negative after 7 months.

G. L. Hoffman and R. E. Putz

Hoffman, G.L. and R.E. Putz. 1970. Problems of research on *Myxosoma cerebralis*. Second International Congress of Parasitology, Abstract #278, September 6-12, Washington, D.C. Journal of Parasitology. 56 (Section II, Part I): 152.

278. **Problems of research on *Myxosoma cerebralis*.**

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Whirling disease caused by the cartilagophagous *Myxosoma cerebralis* was first recognized in the United States in 1958. Previously it had been known only from continental Europe. It is probable that it came to this continent in frozen table trout. Subsequently the disease spread throughout northeastern USA and later appeared in California and Nevada. Transfer of the disease in Europe and the United States has undoubtedly occurred with the shipping of live trout and eggs as well as frozen table trout.

Research on *M. cerebralis* has been economically oriented because of the great damage done to fish stocks and the danger of its continued spread.

It is very desirable that the disease be maintained in the laboratory so that research on the life cycle, disinfection, chemotherapy, etc. can be accomplished. Although the organism is very difficult to control once it has become established in fish culture it is, so far, almost impossible to infect fish under laboratory conditions. This is also the case for all other histozoic Myxosporidea. Uspenskaya (1965) succeeded in infecting small trout by pipetting 4-month-old spores into the stomachs of young rainbow trout. We were unsuccessful with this method. Over an 8 year period—and more than 26 attempts, we have been able to infect trout fry in only seven aquaria. Fish became infected only in those aquaria containing about 3 inches of mud and spores "aged" at least 3.5 months.

It has long been assumed that the fish become infected by accidentally ingesting the spores, but we believe that we have infected fish in the prefeeding stage, indicating that the organism might gain entrance to the fish by another route, perhaps the gills. These fry were held in contaminated water for three days and removed to spore-free water so there is a slight possibility of simple contamination.

Calcium cyanamide and calcium oxide have been used for years in Europe as pond disinfectants for *M. cerebralis*, but no controlled experiments have ever been reported. Once a reliable method of experimental infection is established it should be relatively easy to test disinfectants. We have attempted to test disinfectants by exposing the spores in test tubes and later using microscopic examination to determine destruction of the spores. These experiments provide us with indications of effectiveness but should be tested in experimental ponds. Very high concentrations of calcium oxide (calcium hydroxide), chlorine, and Roccal (Alkylmethylbenzylammonium chlorides) do destroy the contents of the spores.

Fish were infected in aquaria containing "aged" spores which had been frozen at –20 C for 2 months. Spores were microscopically visibly altered at as little as 60 C for 10 minutes. This information was necessary to establish recommendations on the shipping of table trout. It is assumed that hot-smoking kills the spores, but cold-smoking may not. Because salmon is sometimes shipped salted, the effect of such concentrations of salt on the spores should be determined.

M. cerebralis will infect at least *Salmo gairdneri*, *S. salar*, *S. clarki*, *S. trutta*, *Salvelinus fontinalis*, *S. leucomaenis*, *S. malma*, *S. namaycush*, *Oncorhynchus keta*, *O. masu*, *O. kisutch*, *O. tshawytscha* and *Thymallus thymallus*. *S. trutta* usually exhibits no symptoms; not all of the others have been adequately studied but the symptoms are very spectacular in *S. gairdneri*, *S. fontinalis*, and *T. thymallus*.

Lom, J. and G.L. Hoffman. 1970. Redescription of the spores of *Myxobolus cerebralis*. Second International Congress of Parasitology, Abstract #387, September 6-12, Washington, D.C. Journal of Parasitology.
56 (Section II, Part I): 213.

387. **Redescription of the spores of *Myxobolus cerebralis*.**

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Myxobolus cerebralis (syn. *Myxosoma cerebralis*), a causative agent of serious losses in cultured salmons and trouts is now being reported from additional localities in the U. S. and Canada. Supposedly, it is not a flare of latent autochthone infections, but the parasite is spreading across the North American continent from sources originally imported from Europe. To confirm or disprove the identity of these populations, a complex comparative analysis of *M. cerebralis* from European and American sources should be carried out, including a detailed morphological analysis.

The latter will depend—as in other myxosporidian species—largely on the structure of the spores. Curiously enough, no accurate description of spores of *M. cerebralis* exists in the literature, not even in the recent monographs. We have investigated the spores from trout fingerlings

(*Salmo gairdneri*) experimentally infected in the Eastern Fish Disease Laboratory. The spores are mostly broadly oval in outline, sometimes more elongated, rarely circular and in exceptional cases broader than longer. In side view, both shell valves are considerably vaulted, sometimes one more than the other. The average dimensions of the spore were 9.7 by 8.5 μ, average dimensions of polar capsules 4.2 by 3.1 microns; rarely, one polar capsule is slightly larger than the other. The polar filament makes usually 5 or 6 windings inside the capsule. Instead of regular intercapsular appendix, there is just a pointed thickening on the shell border. There are no shell markings in the frontal view of the spore, but the spore border is irregularly shaped in front view. India ink reveals a typical mucous envelope around the posterior end of the spore. The scanning electron microscope reveals some characters possibly special only for this species: a deep ridge separating the vaulted surface of the shell from the valve border, and a distinct opening of the canal for filament discharge as well as a pattern of the mucous envelope on the posterior half of the spore. To stress the potential use of scanning electron microscopy in myxosporidian taxonomy, micrographs of some other myxosporidian species will be demonstrated.

Meglitsch, P.A. 1970. Some outstanding features of *Myxosporida*. Second International Congress of Parasitology, Abstract #424, September 6-12, Washington, D.C. Journal of Parasitology. 56 (Section II, Part I): 235.

424. **Some outstanding features of *Myxosporida*.**

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Myxosporida are characteristic parasites of fishes, though some species have been recovered from other cold-blooded vertebrates. Almost 800 species have been named, but uncertainty about the stability and dependability of many of the characteristics used to distinguish species makes it impossible, at the present time, to present a realistic estimate of the number of valid species. They range from at least near-commensals to pathogens causing a high percentage of fatality.

They appear to have begun as coelozoic parasites of the biliary and urinary systems. Modern species of this type typically have small, amoeboid trophozoites producing one or a few sporonts, each giving rise to one or two spores. One line of evolutionary development led to the formation of very large, discoid trophozoites, forming thousands of spores. Another appears to have led toward histozoic habits, probably through migration into the tissues by way of the kidney tubules. In any case, the histozoic species tend to be walled off from the host tissues by a cyst membrane formed by host tissue. In a few species, no cyst membrane is formed. Some of the most pathogenic are among these.

The life cycle is not known adequately. Presumably spores are ingested with food or water, germinate in the gut, and release amoebulae that reach the final host organ by way of circulatory system. At least some species are known to appear first as small trophozoites within capillaries. However,

infection experiments have generally failed to produce consistent results.

The spores are characteristic and unique in several ways. The sporont appears first as a definite cell within the trophozoite; some have described a uninucleate beginning while others have described a binucleate initial stage. In any case, growth of the sporont, accompanied by nuclear divisions, produces a syncytial mass from which one or two spores develop, depending on the species. The number of nuclei produced includes one for each polar capsule to be produced, one for each valve of the spore membrane, and two for the sporoplasm. The next stage is drastically unlike that characteristic of Sporozoa as a whole. Discrete cells appear, one for each polar capsule, one for each shell valve, and one for the sporoplasm. The cells differentiate, the mass, and the valvular cells differentiating directly into the firm shell valves. As this occurs, the spore form characteristic of the species emerges. During the later stages of spore differentiation, the nuclei of valvular and capsulogenous cells become pycnotic, and are wholly lost in adult spores or nearly so. Haploid mitotic figures have been repeatedly observed in differentiating spores, and it is generally accepted that the life cycle is obligatorily uniparental as a result of autogamy of the two sporoplasmic nuclei.

Putz, R.E. 1969. Experimental transmission of *Myxosoma cerebralis* (whirling disease) and effect of freezing on the spores. Prog. Sport Fish. Res. pp. 55-57.

PARASITOLOGY

Experimental transmission of Myxosoma cerebralis (whirling disease) and effect of freezing on the spores

Before critical research can be done on treatment and control methods, whirling disease must be reliably reproduced under experimental conditions. Although young salmonids become

easily infected when placed in contaminated waters, it is very difficult to infect such fish at will in the laboratory. To find a reliable method of reproducing the disease, spores in infected fish tissues were introduced to 24 aquaria, 16 of which contained 3 to 5 inches of mud taken from warmwater fish ponds. Some of the aquaria were 150-liter stainless steel tanks, some were 75-liter glass aquaria, and some were 340-liter fiberglass tanks. Spring water (12° C.) flowing at about 600 ml/min was supplied during "aging" of the spores. After

the fish were added, the flow was increased to about 1800 ml/min. All aquaria had standpipe drains to facilitate the retention of spores. Fifty to 100 rainbow trout fry, usually 3-4 weeks old, but 10 weeks old in 2 aquaria, were stocked at regular intervals from 0 to 6 months after the spores were added. The fish were observed and were autopsied and examined for spores 4 to 6 months after stocking.

Fish became infected in 7 of the 24 aquaria. Only those aquaria containing mud and spores "aged" 3.5 to 6 months contained infected fish (Table 3). Symptoms were first noticed 2.5 to 3.5 months after the fry were placed in the aquaria. In 5 aquaria the symptoms were typical and spores were numerous in the fish. However, in 2 aquaria no symptoms were noticed but examination of cartilage showed spores in small numbers.

of the spores. In some of the experiments infected trout heads were frozen at -20° C. for 2 months, and then cut up and introduced to aquaria. Freezing did not kill the spores (Table 4).

Experiments 67-6(3) is being maintained to see how long a contaminated facility remains so. Spores were placed in the aquarium on August 9, 1967. After fry were added on November 28, 1967, the mud surface was stirred gently several times at weekly intervals. Symptoms were seen February 16, 1968, and immature spores were present. These fish were removed and new fry added on February 23; the second lot became infected and were removed May 21. This procedure was repeated 9 more times, with the fish becoming infected. The first 3 batches of fish were left in the aquarium about 3 months (long enough for spore

Table 3.--Effect of "aging" on Myxosoma cerebralis spores

Experiment No.	Facility	3-5" of mud added or not	Spores "aged" in aquaria, months	Age of fry at start, weeks	Presence of spores in fish
66-1B	150-liter steel tank	no	4	3	0
66-1A	" "	yes	4	3	+
68-14	" "	no	2	3	0
67-6(1)	340-liter fiberglass	yes	3	10	+
67-6(3)	" "	yes	3.5	3	+
67-6(4)	" "	yes	3.5	3	+
68-11	" "	yes	6	3	0
68-13	" "	no	2.5	3	0
68-14	150-liter steel tank	no	2.5	3	0
69-5A	75-liter aquarium	no	0	10	0
69-6	" "	no	3.5	3	0
69-7	" "	no	3.5	3	0
69-8	" "	no	3.5	3	0

From these experiments we assume that some mud is necessary to produce infection. The spores became infective 3 to 6 months after placing them in the aquaria, but we do not know the minimum and maximum infective ages

production), but the last 8 were removed before production. Dead fish were removed promptly during this experiment. The results demonstrate that the spores remain viable for

Table 4.--Effect of freezing and "aging" on Myxosoma cerebralis spores

Experiment No.	Facility	3-5" of mud added or not	Spores frozen -20°C, days	Spores "aged" in aquaria, months	Age of fry at start, weeks	Presence of spores in fish
66-16A	150-liter steel tank	yes	270	4.5	4	0
67-1B	" "	yes	18	4	10	+
67-6(2)	340-liter fiberglass	yes	3	0	10	0
67-6(5)	" "	yes	3	3.5	10	0
67-6(6)	" "	yes	60	2.5	10	0
68-5	" "	yes	60	6	3	+
68-6	" "	yes	60	6	3	0
68-7	" "	yes	60	6	3	+
68-8	" "	yes	330	6	3	0
68-9	" "	yes	330	6	3	0
68-10	" "	yes	330	6	3	0
69-1	" "	no	480	4.5	3	0
69-2	" "	no	960	5.5	3	0

22 months or the live infected fish shed infective units, or both.

The actual mode of transmission of M. cerebralis has never been experimentally determined; therefore a filtration experiment was initiated to pinpoint the size range of the infective unit. Water and sediment from an infected tank, where M. cerebralis has been maintained since the first quarter of 1968, were siphoned into a 20-liter container and from this filtered through filters of 14 μ to 1.2 μ potasity. This was done each day for 5 consecutive days. After running the water and sediment through each size filter, the filters with residue were placed in glass aquaria with susceptible trout. Results are shown in Table 5. Presence of spores of M. cerebralis in infected trout was confirmed by histopathological examination. This experiment is being run again with smaller trout and more consecutive days of filtering. The spores are actually 7.5 to 9.5 μ in diameter. The infective units, whether present as free spores or carried by larger organisms, were retained by the 12 μ filter. It may be that free spores were adsorbed to the filter pad or trapped in debris.

R. E. Putz

Putz, R.E. & I. McElwain. 1969. Serodiagnosis of whirling disease. Prog. Sport Fish. Res. p. 57.

Serodiagnosis of whirling disease

At present whirling disease of salmonids cannot be detected in asymptomatic carrier fish without sacrificing the fish, and it is sometimes very difficult to find spores in adult trout. Therefore, an experiment was initiated to try to diagnose the disease using a modified indirect fluorescent antibody technique. Rainbow trout globulin (to be used as an antigen) was prepared by fractionating the trout serum, and was injected into rabbits. The system was then tested.

A positive control system, using the indirect fluorescent antibody technique developed for salmonicida was run, as well as a negative control system using trout serum from non-myxosoma-infected trout. So far the experiments have not given satisfactory results because those not exposed to fluorescent antibody fluoresced as strongly as controls. Untreated spores of M. cerebralis fluoresced in ultraviolet light.

R. E. Putz and Ivan McElwain, trainee

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Walker, P.G. and R.B. Nehring. 1995. An investigation to determine the cause(s) of the disappearance of young wild rainbow trout in the Upper Colorado River, in Middle Park, Colorado. Colorado Division of Wildlife. 134 pp.

Due to the length of this document, only the Conclusions from the Executive Summary (p. xvi) is printed here. Please note the document is available from the authors, whose addresses are given below.

This report and recent observations in Utah are the first documentation of overt whirling disease in free-ranging fish populations. Moreover it is one of the first reports of overt whirling disease under any circumstances in brown trout. The evidence and inference developed in this report contravenes the oft-repeated assertion that the myxosporean parasite [*Myxobolus cerebralis*] has little discernable effect on stream-dwelling wild salmonids. Whirling disease, therefore, remains implicated as a factor, but not necessarily the sole cause contributing to the serious decline in recruitment of young wild rainbow trout in the upper Colorado River. There are, however, other factors (most notably gas supersaturation and gas bubble disease) which cannot be ruled out either. It is possible that the year class losses of rainbow trout in the Colorado River are the result of an interplay of both these important stressors (perhaps compounded in some cases by ectoparasites or bacteria and fungal diseases as well). These hypotheses can be tested both in the laboratory and by further investigation in the natural environment. Additional laboratory experimentation can determine whether or not the Colorado River strains of trout are genetically more vulnerable to whirling disease infection than other strains previously tested. Experimentation can also determine if the Colorado strain of [*Myxobolus cerebralis*] is more pathogenic then strains encountered in other parts of the world.

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LETTER TO THE EDITOR . . .

Salmonid Whirling Disease: Status in the United States, 1985

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In a recent description of the life cycle of *Myxosoma cerebralis*, the etiologic agent of whirling disease in salmonids, Wolf and Markiw (1984, Science 225: 1449–1452) demonstrated that a tubificid oligochaete was the obligate alternate host and that an organism produced in the worm and having the unique morphology of a triactinomyxon was the long-sought infectious stage for fish. That report presented the first scheme of a myxosporean life cycle that was supported by experimental data instead of authoritative opinion. Since then, inquiries from administrators, managers, and biologists have suggested a need to review the new findings as they relate to fisheries resources in the United States where this exotic disease was once considered rare, but where it now must be recognized as firmly established. My purpose here is to consider some aspects of whirling disease in the light of the new knowledge that tubificids are required in the life cycle, and of how that information relates to existing measures of control and to additional measures that should be considered.

Whirling disease is a chronic, noncontagious infection of salmonid fishes that is causally related to a protozoan parasite known as *M. cerebralis*. Because salmonids are propagated widely in the United States in areas where whirling disease is now established, the infection is found commonly in the highly susceptible young—fingerling to subadult—of rainbow trout (*Salmo gairdneri*) and brook trout (*Salvelinus fontinalis*). Clinical cases show external signs that are first evident as dark pigmentation of the caudal peduncle and caudal fin—the so-called black tail sign. Next to appear is the abnormal tail-chasing behavior from which the disease derives its name. In time, structural deformation of the head or body develops. However, fish with infections of low intensity show none of these signs and often appear and act normally.

Histologically, focal to regional areas of lysis and damage occur in cartilaginous tissue, and spores of *M. cerebralis* are present in or near such lesions. The metabolic activity of the parasite is credited with causing the lesions, which eventually result in structural deformation during bone deposition.

The presence of *M. cerebralis* spores is universally considered to be pathognomonic for whirling disease.

Whirling disease was introduced accidentally into the United States, presumably from its origin in Europe, during the early 1950's—to Nevada in the West and to Pennsylvania in the East. Within watersheds, the disease can spread naturally, but it has been disseminated also by man—sometimes unwittingly, but sometimes covertly. Consequently, whirling disease is now known to have been spread to 10 other states: California, Connecticut, Massachusetts, Michigan, New Hampshire, New Jersey, New York, Ohio, Virginia, and West Virginia. New Hampshire was added to the list in 1980 and New York in 1984.

No state has yet been successful in eradicating *M. cerebralis* from its waters. Unquestionably, the application of appropriate control measures can lower the prevalence of whirling disease, as well as

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reduce the more insidious problem of subclinical infection. However, once the organism is present in natural environments, eradication—a complete stamping out—will require extraordinary effort.

Although whirling disease has been known and studied in Europe since the early 1900's, the life cycle has remained an enigma. The breakthrough was made in the United States during the early 1980's as the culmination of a sequence of experimental research findings. First, Markiw and Wolf (1983, J. Protozool. 30: 561–564) found that a tubificid oligochaete was required for *M. cerebralis* to yield the stage that was infective for fish. Next, the long-sought infectious stage of the organism was reported and the life cycle was defined (Wolf and Markiw, 1984, op. cit.).

In brief, mature spores of *M. cerebralis* reach the environment when infected fish die and decompose or are consumed by scavengers. Alternatively, the spores are released in feces in viable condition when infected fish are cannibalized or eaten by a predator (Taylor and Lott, 1978, J. Protozool. 25: 105–106).

The freshly released small disk-like spores of the myxosporean *M. cerebralis* are not infectious for trout. Instead, the spores infect the tubificid and in the gut of the worm undergo a slow but dramatic morphologic change to a much larger three-tailed and grapple-shaped organism known as an actinosporean and more specifically as a member of the group with the genus name *Triactinomyxon*. Triactinomyxons have been recognized as parasites of annelid worms for more than 90 yr, but have never been shown to initiate autoinfection in their host. Instead, the triactinomyxon—for which the name *T. gyrosalmo* was proposed (Wolf and Markiw, 1984, op. cit.)—produces whirling disease when infected worms are eaten by a susceptible trout or when fish encounter the organism when it is waterborne. The grapple-like appendages are thought to enable waterborne organisms to lodge in fish gills and there to effect transfer of the parasite's internal bodies or sporozoites to the respiratory capillaries of the fish.

Tubificids are normal inhabitants of aquatic environments and are particularly abundant in rich organic soils. Dense populations are commonly considered to be an indication of organic enrichment or (in the general sense) pollution. Tubificids are typically abundant and occur in dense red patches in settling basins and streams that carry effluent (bearing residues of food and feces) from trout hatcheries. More important, the worms find ideal habitat in earthen ponds and raceways where cleaning is both difficult and infrequent. Once introduced, the whirling disease organism readily becomes established in such habitats. In contrast, concrete or plastic-lined hatchery facilities that are kept clean and have an uncontaminated water supply can be kept free of the disease.

Knowledge of the life cycle logically leads one to consider measures that could be applied to prevent whirling disease. Such measures include eradication of the essential tubificid or the waterborne triactinomyxon. In theory, tubificids can be killed by thorough drying or chemical treatment of the aquatic soil. Although the life cycle was not then known, Hoffman and Hoffman (1972, J. Wildl. Dis. 8: 49–53) conducted laboratory tests of several compounds and provided presumptive evidence that chemical disinfection—that is, killing the spores of *M. cerebralis*—was possible. In actual practice at hatcheries, however, chemical disinfection has been only partly successful and has not resulted in eradication of the disease. The biology of the tubificid, its burrowing behavior, and the nature of aquatic soils virtually ensure survival of some worms beyond the reach of chemicals that can be applied and maintained economically in adequate concentration and to sufficient soil depth. Considering that rainfall is appreciable at

most locations where trout are reared, the drying of pond soils is not a realistic eradication measure.

Selectively lethal chemicals have a potential for achieving eradication, although no such compounds are yet known. Application would probably have to be prolonged to kill deeply buried worms or the progeny worms inside cocoons. Moreover, the safety of other aquatic biota must be considered. The overall requirements for registration of such compounds by regulatory agencies of the United States government are formidable.

Decontamination of water supplies to remove or kill the triactinomyxons is an alternative approach to eradication. Cleanup can be effected by filtration, chlorination, ozonation, or ultraviolet irradiation—alone or in combination.

Although the actual infectious stage was not known, Hoffman (1975, J. Wildl. Dis. 11: 505–507) showed in laboratory tests that filtration and ultraviolet irradiation (UV) reduced waterborne infectivity, and that a combination of 25-μm filtration and irradiation at 27,650 microwatts/sec/cm^2 was most effective. Quantification of ozone and chlorine needed for disinfection of water supplies has not been determined.

Although two methods of decontamination have been partly successful, all of the various systems require an energy source, and thus are vulnerable to interruption. Accordingly, back-up fail-safe systems are required. Filtration is additionally vulnerable to heavy incursion of silt and debris that can occlude the devices and negate the effects of UV irradiation.

Drugs and chemicals are used routinely to treat and, in some instances, to help prevent certain bacterial and parasitic conditions in fishes under husbandry. Drugs are often highly successful in controlling infection or reducing mortality, but their use neither eliminates the pathogen nor prevents its entry into some fish; in fact, some fish are almost certain to become infected and to harbor the pathogen.

Year-long continuous feeding of six drugs to young trout held in water known to be contaminated with the infectious stage of the whirling disease organism showed that although clinical signs did not appear, infection was not prevented (Taylor et al., 1973, J. Wildl. Dis. 9: 302–305). One antibiotic, one nitrofuran, one sulfonamide, and three antiprotozoal compounds were tested. Best results were achieved with the nitrofuran, which partly inhibited spore formation but did not prevent infection.

In many regions of the United States whirling disease has yet to be found; the stocking of such regions with trout that are minimally or even potentially infected with the whirling disease organism is wholly unacceptable.

Significant but nonetheless incomplete control of certain insect pests of agriculture and forestry has been achieved by introducing predator insects or bacterial or viral pathogens. Prospects for biological control of whirling disease are dim, inasmuch as the host fish themselves are prime predators of the tubificids and demonstrably contract the infection by ingestion. The tubificids undoubtedly have their own array of pathogens, but the identity of virulent bacteria or viruses is not yet known.

Now that the actual infective stage, the triactinomyxon, has been identified and produced experimentally, the application of immunologic methods holds promise for future control of whirling disease. As a prime example of progress in parasitology, researchers are looking toward vaccination of man against malaria as a new weapon to eradicate the disease. Although not yet developed, malarial vaccine is to be targeted against the infective stage produced by the mosquito.

The antigenic components of the triactinomyxon must be determined and

methods must be developed by genetic engineering to mass produce the antigen economically. Appropriate methods of administration would have to be determined, as well as safety and efficacy. Eventually such a product would require licensing by the U.S. Department of Agriculture.

The task of developing a practical vaccine against whirling disease is, as seen from the present vantage point, formidable but not impossible. Whether such a product would evoke complete and population-wide protection cannot now be determined. Beneficial but less-than-absolute protection will affect the disposition of fish stocks involved and the waters to be stocked.

In Europe the rainbow trout is propagated mainly as a table or food fish and in some places water supplies needed for the necessary large-scale production are ineradicably contaminated with the infective stage of whirling disease. Trout producers in such situations incubate eggs and rear the young—usually to fingerling stage or larger—in pathogen-free spring or well water. That practice exploits the fact that, although even yearling trout might become infected and produce spores of *M. cerebralis*, older fish seldom develop clinical signs of the disease. The rearing of trout to at least fingerling size in noninfective water takes them past the highly vulnerable fry stage. When carrying capacity of available pathogen-free water is exceeded and fish must be removed and exposed to the pathogen, their response can be tolerated. Fish become infected, but severe signs such as deformities are avoided, and the product can be successfully marketed because it is normal in appearance.

An alternative method has been applied in the United States. Brown trout (*Salmo trutta*) and coho salmon (*Oncorhynchus kistuch*) have high, but not complete, resistance to whirling disease. The brown trout is generally considered to be the original host of *M. cerebralis*, and hence its resistance is a feature of selection and adaptation.

In places where the only water supplies are contaminated, production of the highly susceptible rainbow and brook trout has been discontinued. Instead, the resistant brown trout and coho salmon (O'Grodnick, 1979, Trans. Am. Fish. Soc. 108: 187–190) are propagated. Even these resistant species are reared for 6 mo or longer in noncontaminated water—just as is done in the European method.

Statistically valid samples (5% prevalence) of trout and salmon so produced have been examined and found free of spores of *M. cerebralis*. However, resource managers of areas or regions where whirling disease is absent nevertheless look at exposed populations hypercritically. Quite understandably, fish of such exposed stocks should not be stocked in environments that are free of the infection.

Attempts to arrive at the overall significance of whirling disease lead to mixed results. From an ethical or conservative viewpoint—that of responsible resource protection—whirling disease in the United States is an exotic infection. Accordingly, if it cannot be eradicated, every effort should be made to confine it to areas or regions where it now exists; it should not be introduced to environments, regions, or areas where it is not now present. The responsible viewpoint is rational, safe, and readily defended, for it is axiomatic that existing fish resources should be protected. Also, it is more effective and less costly to exclude a pathogen than to eradicate it once it is established.

"What harm does whirling disease do in the wild—or even in the hatchery?" "Is it measurably harmful or just theoretically harmful?" "Is *Myxosoma cerebralis* really that bad?" "How can you justify ranking whirling disease with viral hemorrhagic septicemia?" It is true that whirling dis-

ease infections of high intensity in a hatchery kill some of the young, cripple survivors, and result in reduced growth. Yet, some hatcheries with enzootic whirling disease have continued to operate (and presumably profitably) for years. In the absence of the disease, the profit margin could probably be increased—but by how much? The answer is not known, and data are not available.

The effect of whirling disease in wild populations is totally unknown, but one can speculate that, because population densities are lower than in hatcheries, the risk of intense infection, clinical disease, and serious impact is reduced.

Questioning the significance of whirling disease is not just an academic exercise. North America has had the problem for nearly 30 yr and the formerly rigid attitudes seem to be softening. In relation to commercial operations (not equivalent to natural fishery resources) in the United Kingdom, official attitudes changed when it became evident that the disease was widely disseminated. As a consequence in early 1984, whirling disease was taken off the list of notifiable diseases.

Judging by the small number of diagnostic cases submitted to our laboratory and by the paucity of reports of new occurrences, whirling disease seemingly has not spread much during the past several years. That judgment should not lead to a complacent attitude, because one must ask, "Is anyone looking?" and the answer must be "Probably not—unless there are signs of the disease or other reasons to scrutinize fish."

Whirling disease exists in streams of several of the states, but just how extensively is not known. Determination of the extent of the infection depends on the methods used for detection. The most widely used method of detecting spores of *M. cerebralis* is the plankton centrifuge method that was popularized by O'Grodnick (1975, J. Wildl. Dis. 11: 54–57) and modified for greater sensitivity by Markiw and Wolf (1980, Can. J. Fish. Aquat. Sci. 37: 2225–2227). Unquestionably, the most reliable method of determining the presence or absence of whirling disease infection is that of exposing susceptible young trout as sentinel animals.

The significance of whirling disease in various kinds of natural environments with different kinds of resident, or short-term, salmonids needs to be determined. Eradication procedures will undoubtedly be considered under some circumstances. Where that approach is to be applied, it should be directed against both host animals of the parasite's life cycle—the salmonid fish and the tubificid worm.

Until reliable eradication procedures are developed, it is highly probable that man will have to tolerate the presence of whirling disease in wild waters of the United States and in fish hatcheries where it is already present.

Wolf, K. and M.E. Markiw. 1985. Salmonid whirling disease. U.S. Fish and Wildlife Service. Fish Disease Leaflet 69. National Fisheries Center, Leetown. National Fish Health Research Laboratory. 12 p.

SALMONID WHIRLING DISEASE[1]

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FISH DISEASE LEAFLET 69

UNITED STATES DEPARTMENT OF THE INTERIOR
Fish and Wildlife Service
Division of Fishery Research
Washington, D.C. 20240

1985

[1]Revision of *Fish Disease Leaflet* 47 (1976), "Whirling disease of trout," by G. L. Hoffman.

INTRODUCTION

Salmonid whirling disease—sometimes referred to as "blacktail"—is a chronic, noncontagious parasitic infection of trout and, to a lesser extent, other members of the family Salmonidae. The parasite has specific tropism for cartilage. If the infection is heavy and the fish is young, mortality sometimes results. More commonly, the parasitosis evokes abnormal swimming behavior and the transient melanism of the caudal fin and peduncle that is the blacktail sign (Fig. 1).

When the parasite is given sufficient time for development and the infection is sufficiently intense, the head and axial skeleton become disfigured in some victims (Fig. 2). However, the behavior and appearance of lightly infected fish are commonly normal, or nearly so (Fig. 3).

The parasite belongs to the group of protozoans known as myxosporeans. Although it was first encountered in Europe at the turn of the century, its life cycle was not discovered until the early 1980's. During the intervening years, methods of diagnosis, detection, and identification were developed and improved.

Newly discovered features of the parasite's life cycle plus suggested procedures for detection, identification, and control are discussed here.

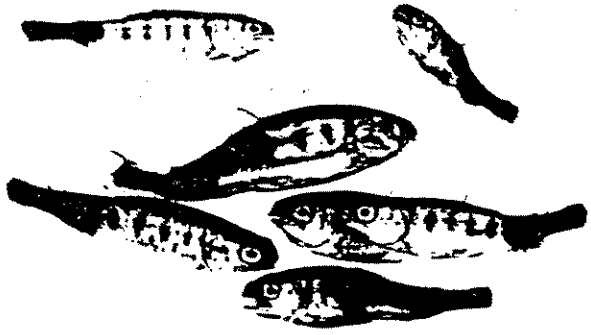

Fig. 1. Fingerling rainbow trout showing the blacktail sign of whirling disease. Inability of the fish to control skin pigmentation is a result of neural impairment.

Fig. 2. Extreme distortion of the skeleton caused by whirling disease. These fish were so disabled that they could no longer compete for food, even in a hatchery.

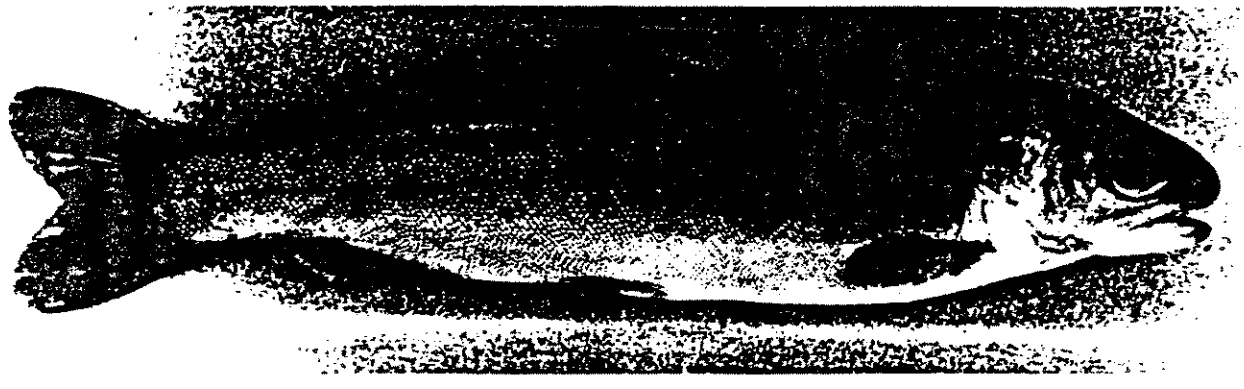

Fig. 3. An otherwise normal-looking fingerling trout showing a slightly sunken dorsal head surface. Outbreaks of whirling disease often involve damage no worse than this. However, such fish usually show whirling behavior.

DIAGNOSIS

Whirling disease can be diagnosed clinically on the basis of changes in fish behavior and appearance. When alarmed or feeding, some infected individuals show an abnormal tail-chasing (whirling) behavior while swimming. The caudal peduncle and tail sometimes become unusually dark or even black (Fig. 1), but that sign is not persistent nor is it specific; moreover, the blacktail sign fades when fish are anesthetized. Deformities of the head or axial skeleton eventually become apparent (Figs. 2, 3). Individually, these signs are less than conclusive, but if all are evident within a population, they collectively constitute a reasonably sound clinical diagnosis.

Internal organs are normal, but histologic sections through cartilage show focal to regional areas of lysis and damage. If the infection has existed for 3 or more months, small, biconvex, disk-like spores of the myxozoan *Myxosoma cerebralis* will have had time to form and mature, and occur in or around the cartilage lesions (Fig. 4). The presence of *M. cerebralis* spores is universally considered to be pathognomonic for whirling disease.

Although hematoxylin and eosin are routinely used in histology, those stains do not enhance the appearance of spores of *M. cerebralis*. In contrast, Giemsa, May-Grunwald-Giemsa, and Ziehl-Neelsen stains are decidedly better because the polar capsules react strongly and distinctly; when one of these stains is applied, the spores become prominent and are easily seen.

Fig. 4. Stained spores of *Myxosoma cerebralis*. The several spores are 8 to 10 μm in greatest dimension and show two prominent ovate polar capsules within their nearly circular profile.

IDENTIFICATION

As in many myxosporeans, spores of *M. cerebralis* are not particularly distinctive, and attempts at morphologic identification by inexperienced persons commonly result in uncertainty. Referral to a qualified person or laboratory is suggested. The mature spore is biconvex or lenticular and features a nearly circular outline that is 8 to 10 μm in greatest dimension. Two prominent ovate capsules containing coiled filaments of unknown function are at the anterior or polar end of the spore (Fig. 4). However, identification that is based solely on morphology may be erroneous because myxozoans are common parasites, and it is not unusual to find mixed infections of *M. cerebralis* and one or more other myxosporeans. Accordingly, the prudent diagnostician also takes into account the anatomical location of the spores, the geographical location and history of the hatchery, the species of fish involved, and the clinical signs.

The soundest identification is based on size and morphology of the spores, epizootiological data, and serology. In serology, a direct fluorescent antibody technique is used (Markiw and Wolf 1978). Rabbit antiserum against *M. cerebralis* is conjugated with fluorescein isothiocyanate. This preparation reacts with homologous spores and prespores (developmental forms that are smaller than spores and lack polar capsules) and the reactivity can be visualized by fluorescence microscopy, preferably with epiillumination (Fig. 5). Antiserum prepared at the National Fish Health Research Laboratory showed unacceptable cross-reactivity with other genera of myxosporeans when indirect fluorescent antibody tests were used. When the direct test was used, cross-reactivity was found only with another member of the genus *Myxosoma*.

The fluorescent antibody test works best with fresh spores. Spores that have been stored in formalin for a week or more show reduced specific fluorescence, and older specimens show little or none.

Fig. 5. As seen by fluorescence microscopy, spores and prespore stages (small bodies lacking internal features) of *Myxosoma cerebralis* react with specific antiserum that has been conjugated with fluorescein isothiocyanate. This direct fluorescent antibody test provides serological identification.

DETECTION

Spore detection in full-blown outbreaks of whirling disease—infections that have extended over several months—is relatively easy because mature spores are then abundant. Individual fish sometimes harbor hundreds of thousands of spores. Quantitatively, about two-thirds of all spores are in the head, and more than half of those are in the cartilage of the gill arches (Markiw and Wolf 1974a).

When fish are to be examined for the presence or absence of spores, the sequence of progressively more sensitive procedures that follows is suggested. The search is terminated when spores are found.

The most simple and rapid first step consists of removing and grinding gill arches, and suspending the resulting homogenate in several volumes of water or physiological saline. After the particulate matter has been allowed to settle for a few minutes, several drops of the supernatant are examined microscopically. An objective lens of about 40× is suggested, but experienced persons use 20× or even 10× objectives with confidence. If no spores are found after a search of 5 to 10 min, additional portions of the homogenate should be examined.

If no spores are found during this first simple procedure, one of several more-sensitive methods should be used. The so-called plankton centrifuge method (Prasher et al. 1971; O'Grodnick 1975) is now the most widely used procedure in laboratories where significant numbers of whirling disease examinations are made. (The plankton centrifuge used is Model 903 of G.M. Manufacturing and Instrument Corp., Manuet, New York 10954.) This method works well with either fresh or preserved specimens. It has the advantages of being relatively fast (2–3 h) and of

accommodating the processing of batches of specimens (such as statistical samples) when populations of fish are examined.

When fresh or frozen material is to be processed, heads of fish are removed and held in water at about 50°C for 5 to 10 min to loosen skin, eyes, and other soft tissues for easy removal. The defleshed bony and cartilaginous cranial elements and gill arches are then pooled and reduced by mincing, or by mechanical grinding if much bone is present. The resulting material is suspended in about 10 volumes of water and then reduced by several minutes of processing in a high-speed blender. The homogenized material is then passed through a continuous plankton centrifuge. Centrifuge harvests are collected and suspended in several volumes of water and examined microscopically.

A modification of the plankton centrifuge method resulted in a 10-fold increase in sensitivity (Markiw and Wolf 1980). However, the modification—30 min digestion of the harvest in 0.25% trypsin at pH 7.2 to 7.5—removes residual fish tissue only if the original material is fresh or frozen. Fixation of tissue in formalin denatures protein and prevents enzymatic digestion.

Laboratories that process whirling disease materials are advised to decontaminate equipment after each lot is processed. Half-strength household bleach (about 2.6% sodium or calcium hypochlorite) is suggested because this alkaline solution dissolves spores that might adhere to the instruments and equipment and thus yield a false positive when the next lot is processed.

The most sensitive method of spore detection involves digestion in pepsin and then in trypsin (Markiw and Wolf 1974a,b). The resulting material is then centrifuged on 55% solution of dextrose or sucrose, which retards sedimentation of debris but allows spores to pass through and be concentrated. The method works only with fresh or frozen material—not with tissues that have been fixed in formalin. Elapsed processing time is 6–8 h, but the spores are released efficiently, are concentrated in a small volume, and are virtually free of tissue residues. Accordingly, the method is also used in the preparation of antigens of *M. cerebralis*.

GEOGRAPHIC RANGE

Whirling disease occurs in much of Europe, and all indications are that it originated there. It occurs in the Soviet Union and, apparently by introduction, is now common in the British Isles. It was accidentally introduced into New Zealand and twice into the United States. Halliday (1976) compiled an extensive list of the then-current world distribution of the infection. However, he noted that the cited occurrence in several countries was subject to dispute. The matter could be resolved by making critical examinations in which sentinel populations of susceptible fish and contemporary procedures of spore detection and identification were used.

LIFE CYCLE

The life cycle of whirling disease consists of two phases (Fig. 6): the long-known phase of infection in fish involving the myxosporean *M. cerebralis*, and the newly discovered alternate phase that takes place in *Tubifex tubifex* (Fig. 7), a common oligochaete worm of freshwater environments (Wolf and Markiw 1984). A morphologically distinctive transformation takes place when the organism leaves the fish and begins its change in the worm. The resulting form produced in the worm is an actinosporean provisionally called *Triactinomyxon gyrosalmo*

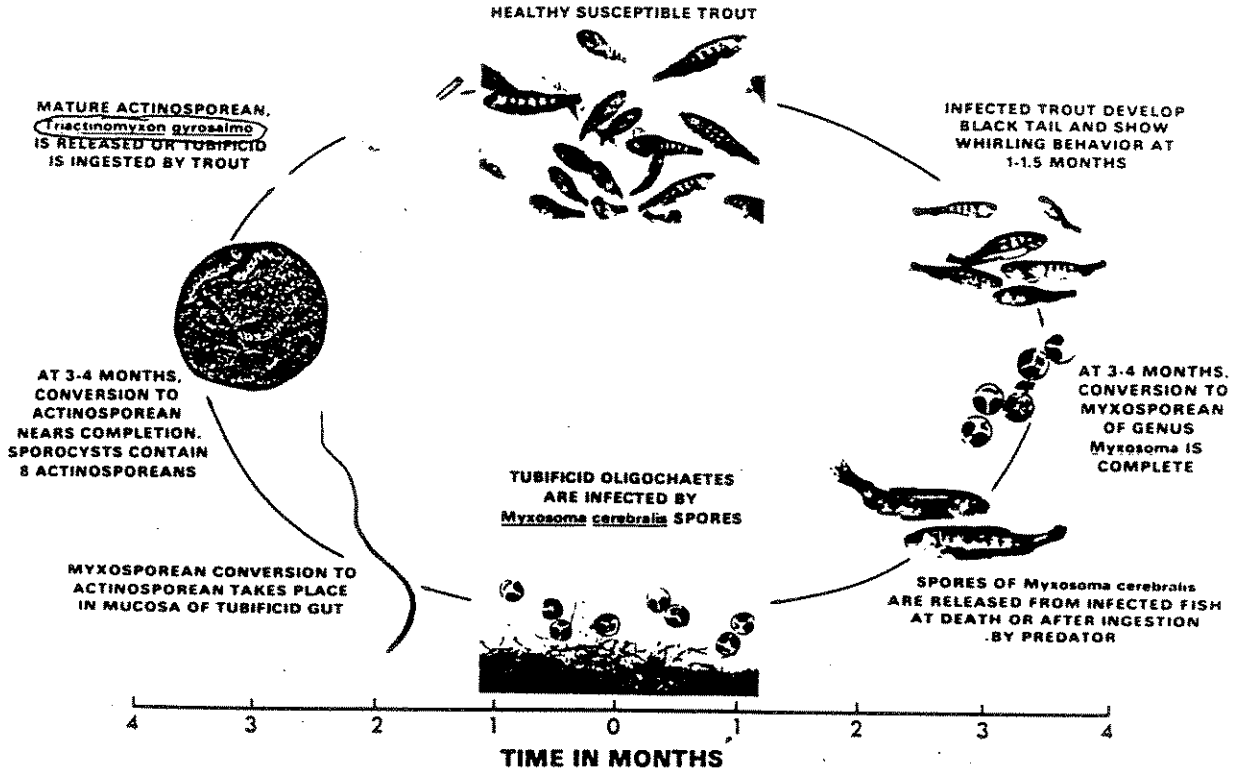

Fig. 6. A diagrammatic representation of the complete life cycle of the whirling disease organism.

Fig. 7. Tubificid oligochaetes are small reddish worms (2–8 cm long) that are common in organically rich aquatic environments such as earthen trout ponds and streams carrying hatchery effluents.

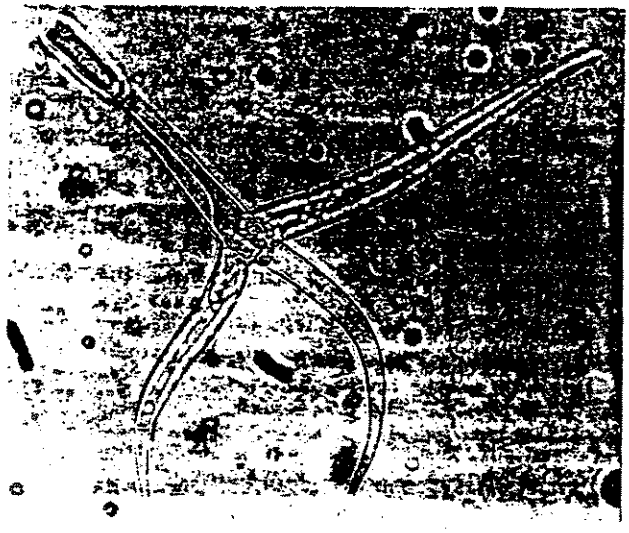

Fig. 8. The organism that initiates whirling disease—a three-parted actinosporean provisionally named *Triactinomyxon gyrosalmo*.

(Fig. 8). It is critical to an understanding of the whirling disease life cycle to note that the small disk-like myxosporean form in the fish cannot initiate infection in other fish; neither can the grapple-shaped *Triactinomyxon* form infect the worm. The life form produced in each kind of host can infect only the alternate host.

In brief, spores of *M. cerebralis* are released into the environment when the fish dies and decomposes or is consumed by scavengers. Spores are also released in the feces of predators that have eaten infected fish. Within a few days at most, the myxosporean-type spores are ingested by tubificid worms and the new phase develops in the gut. Transformation takes place slowly, but after several months abundant new forms of the actinosporean *Triactinomyxon* (Fig. 8) become mature. Fish develop whirling disease after ingesting infected worms or after encountering waterborne *Triactinomyxon*. The route or portal of entry of that infection is believed to be through the gills. The three grapple-like appendages of *Triactinomyxon* are believed to lodge between gill lamellae, and there transfer the numerous internal sporozoites (Fig. 9) to the vascular system of the fish. Once in the fish, spores of *M. cerebralis* mature after about 3 months.

Fig. 9. The epispore or anterior end of *Triactinomyxon* contains three prominent polar capsules (two shown) and 30 to 50 small sporozoites (2–3 μm in diameter).

TRANSMISSION

Whirling disease occurs when fish encounter the infective phase of the protozoan—the waterborne form that is provisionally termed *T. gyrosalmo* or, alternatively, when they ingest the tubificid oligochaete in which the *Triactinomyxon* is produced. Attempts to effect fish-to-fish transmission of whirling disease have been unsuccessful.

INCUBATION

Incubation time is directly related to temperature. Trout fry that are fed infected worms or are exposed to waterborne *T. gyrosalmo* show the blacktail sign after 35 to 45 days at 12°C. Whirling behavior first appears at about the same time or slightly later. Fully mature spores of *M. cerebralis* can first be found in infected fish after 2.6–3.5 months, but spore production continues for weeks and perhaps for months. Incubation time is shortened or length-

ened at temperatures above or below 12°C, to about 50 days at 17°C and 120 days at 7°C (Halliday 1973).

Incubation time in the worm is defined as the interval between first contact with *M. cerebralis* spores and the release of the first *Triactinomyxon*. At 12°C, that interval is about 3.5 months, or about equal to the incubation time required for the development of *M. cerebralis* spores (Wolf and Markiw 1984). Incubation time in the worm is precisely the length of time—3.5 months—that spores have long been known to need to produce infectivity.

HOST RANGE AND RESISTANCE

Whirling disease typically occurs in species of the family Salmonidae. Under husbandry conditions, the usual victims are trout, and to a lesser extent salmon; however, the infection also occurs in wild populations. Investigators generally agree that the rainbow trout (*Salmo gairdneri*) is the most susceptible species and that the brown trout (*S. trutta*) is highly resistant (Halliday 1976; Hoffman and Putz 1969; O'Grodnick 1979). On the basis of tests conducted during 3 years with seven species, O'Grodnick (1979) rated three species as intermediate in susceptibility: brook trout (*Salvelinus fontinalis*), chinook salmon (*Oncorhynchus tshawytscha*), and sockeye salmon (*O. nerka*). The coho salmon (*O. kisutch*) was usually refractory. Although O'Grodnick (1979) found the lake trout (*Salvelinus namaycush*) to be consistently refractory, Hoffman and Putz (1969) reported that exposed lake trout developed clinical signs of whirling disease and produced spores of *M. cerebralis*.

Graylings (*Thymallus*) and whitefishes (*Coregonus* and *Prosopium*), which are generally regarded as salmonids, have not yet been tested and their susceptibility or resistance to whirling disease remains undetermined. According to early accounts, whirling disease was found in nonsalmonids. However, Halliday (1976) believed that these reports might be erroneous. The matter deserves critical reexamination and the application of a serological method of spore identification.

The only tubificid that has been demonstrated to be susceptible to *M. cerebralis* is *Tubifex tubifex*. Members of the genera *Limnodrilus*, *Quistadrilus*, and *Ilyodrilus* were present with *T. tubifex*, but did not yield *Triactinomyxon*. Other genera of oligochaetes that have been tested—*Dero*, *Stylaria*, and *Aeolosoma*—did not produce infectivity for whirling disease (Markiw and Wolf 1983).

IMMUNITY

The immune response of fish to whirling disease has great potential in the development of nondestructive methods of detecting the causal organism. In addition, it provides for possible applications in vaccinating fish to induce immunity. All that can now be said for the immune response, however, is that some evidence has been found that rainbow trout produce antibody against *M. cerebralis* but that little use has been made of this finding. The situation is not clear because, even though antibody is produced, its specificity remains to be demonstrated—or conversely, the tests used need to include adequate precautions to exclude nonspecific reactions. Also, the presence of antibody does not necessarily mean that protection exists.

Relevant literature on the immune response of fish to whirling disease consists of three reports

describing searches for trout antibody against *M. cerebralis*. Griffin and Davis (1978) reviewed reports of two earlier unsuccessful efforts and described their own more productive search. Using an indirect fluorescent antibody technique and serum from rainbow trout known to have whirling disease, they found evidence of antibodies in 16 of 18 samples. However, when serum from specific-pathogen-free fish was tested, 3 of 18 also showed reactivity—presumably a false positive reaction.

The critical point in immunological studies thus far reported is that the myxosporean phase used as the source of parasite antigen is not the stage that is infectious for fish. The new knowledge that the stage termed *T. gyrosalmo* is the infectious stage for fish suggests that that form should be tested as antigen in serological procedures. Moreover, that stage should be the more logical antigen for use in immunization, because it is the form that the fish first encounters and probably has some antigenic components not present in the myxosporean form (*M. cerebralis*).

CONTROL

The broad term control includes the component aspects of avoidance, therapy, chemoprophylaxis, accommodation, decontamination, eradication, and prevention.

Avoidance requires a starting brood stock that is free of whirling disease plus a water supply that is free of the initiator *T. gyrosalmo* or its invertebrate host *Tubifex tubifex*. As an added precaution, fish stocks from geographic regions where whirling disease is enzootic should be avoided. Although all indications are that eggs from infected brood stock do not harbor spores of *M. cerebralis*, discretion suggests that such eggs should not be taken to hatcheries that are free of whirling disease.

Therapy, or an effective treatment of existing whirling disease—i.e., reduction of mortality or clinical signs or both—remains to be developed. Moreover, unlike some other parasites that can be eliminated with drug treatment, *M. cerebralis* is likely to persist in a population in spite of therapy. In that respect, whirling disease is like certain bacteremias such as furunculosis and renibacterial kidney disease. Therapy can effectively reduce losses, but inevitably results in an attendant development of pathogen carriers among the survivors. Although it is not considered therapy, continuous administration of drugs—chemoprophylaxis—has been tried for control, but it too has been found wanting. In no instance was infection prevented, but the most effective products did achieve a measure of reduction in the number of spores produced. Taylor et al. (1973), who fed six drugs to young trout throughout a 1-year test period, found that the greatest benefit was among fish that had been fed furazolidone; they had fewer spores than did control fish and those fed antiprotozoal drugs. However, food containing furazolidone was not palatable, and growth of fish so treated was only half that in the nontreated controls. O'Grodnick and Gustafson (1974, 1975), who conducted continuous feeding tests with 20 different drugs, concluded that, although they exhibited toxicity to trout, the best drugs—furoxone, benomyl, and fumagillin—all somewhat inhibited spore development. Whirling disease has not yet been prevented with drugs, and prospects for effective and practical chemoprophylaxis are poor.

Accommodation is the approach used to minimize mortality and clinical signs in facilities where the principal water supply carries infectivity that cannot be removed or where low levels of infection are acceptable. Accommodation is practiced in Europe, where trout are raised for the table and the stocking of fishing waters is not a major consideration. Economics also contributes to the practice of accommodation. Although the technology is available to decontaminate water supplies that bear infectivity, the

methods require capital investment for equipment and continuing cost of operation and maintenance. Accommodation is based on the fact that fish develop resistance to whirling disease with increasing size or age. In practice, eggs are incubated and the resulting fry and fingerlings are reared in pathogen-free water as long as possible. The objective is to rear fish to fingerling size or larger before crowding necessitates moving them to production facilities and contaminated water. Fingerlings reared in pathogen-free water for several months or more may become infected, but the effects of whirling disease are greatly reduced; the fish often behave normally and appear to be perfectly healthy.

Common methods of food preparation—cooking, canning, and pickling—inactivate spores of *M. cerebralis* in infected fish. Under properly controlled conditions that ensure 66°C for 40 min, hot smoking also kills spores (Wolf and Markiw 1982). Accordingly, such products pose no threat to the spread of the disease.

Where whirling disease cannot be avoided and where the potential for spread of the infection is not a consideration, the propagation of resistant species of salmonids is another approach to accommodation. Brown trout and coho salmon are notably resistant to the disease; however, these species can become infected and not show signs of the condition.

Most of the literature dealing with decontamination, eradication, and prevention was published before the infective stage, *T. gyrosalmo*, and its invertebrate host, *Tubifex tubifex*, were known. In addition, conclusions drawn from such early work were based on methods of spore detection that are much less sensitive than those available today. In the best method of assessment now used, sample populations of susceptible young "sentinel" fish are used to determine the results of decontamination or eradication procedures.

In theory, tubificid worms can be killed by thorough drying or by chemical treatment of their aquatic soil habitat. In practice, however, only partial kills have been effected. Climatic factors and worm biology contributed to the incomplete success. As examples, precipitation occurs at most locations where trout are raised and, although earthen holding facilities might be drained, the soil realistically cannot be thoroughly dried. Some worms usually survive as a result of their burrowing ability and reproduction by the formation of resistant cocoons, and the invariable presence of burrows of vertebrates and invertebrates in aquatic habitats. Chemicals such as calcium hydroxide or calcium cyanamide have been recommended for and used in decontamination or eradication.

It is worth noting, however, that such recommendations were made before the role of tubificids was recognized. In practice, whirling disease has not been eradicated by chemical treatment of earthen holding facilities. It has not been possible to maintain effective concentrations of chemical compounds long enough. Distribution of chemicals to uniformly adequate depth is probably a contributing factor. Success has been achieved when the water supply was free of infectivity and when earth was replaced with concrete in the construction of holding facilities.

Although chemicals that are selectively lethal for tubificids are not now known, such compounds could, if applied regularly and at adequate concentrations, result in eradication. However, possible effects on other aquatic biota would have to be considered.

Decontamination of waterborne infectivity has been effected; the best results have been obtained by combining filtration to remove or reduce suspended material with ultraviolet irradiation (Hoffman 1974, 1975).

ANNOTATED BIBLIOGRAPHY

Griffin, B. R., and E. M. Davis. 1978. *Myxosoma cerebralis*: detection of circulating antibodies in infected rainbow trout (*Salmo gairdneri*). J. Fish Res. Board Can. 35(9):1186–1190.

Indirect fluorescent antibody technique was used to detect circulating antibodies against *Myxosoma cerebralis*. Antibodies were found in 14 of 18 infected fish. Of 18 sera from specific-pathogen-free or control fish, 15 showed no evidence of antibodies. Correlation between the presence of spores and antibodies was significant ($P \leq 0.01$), but less than absolute.

Halliday, M. M. 1973. Studies on *Myxosoma cerebralis*, a parasite of salmonids. II. The development and pathology of *Myxosoma cerebralis* in experimentally infected rainbow trout (*Salmo gairdneri*) fry reared at different water temperatures. Nord. Vet. Med. 25:349–358.

Experiments with fish held at 7, 12, or 17°C showed that maturation of *Myxosoma cerebralis* was temperature dependent. The highest temperature accelerated the process and the lowest one slowed it.

Halliday, M. M. 1976. The biology of *Myxosoma cerebralis*: the causative organism of whirling disease of salmonids. J. Fish Biol. 9(4):339–357.

Somewhat outdated, but nevertheless the most comprehensive review of the subject. More than 100 references are cited.

Hoffman, G. L. 1974. Disinfection of contaminated water by ultraviolet irradiation, with emphasis on whirling disease (*Myxosoma cerebralis*) and its effect on fish. Trans. Am. Fish. Soc. 103(3):541–550.

Whirling disease was avoided under experimental conditions in which waterborne infectivity was passed through a 25-μm filter and an ultraviolet irradiation unit that provided at least 35,000 microwatt seconds per square centimeter at a wavelength of 2,537 angstroms.

Hoffman, G. L. 1975. Whirling disease (*Myxosoma cerebralis*) control with ultraviolet irradiation and effect on fish. J. Wildl. Dis. 11(4):505–507.

Combined 25 μm filtration plus ultraviolet irradiation at 18,000 or 27,650 microwatt seconds reduced but did not eliminate infectivity. A dosage of at least 35,000 microwatt seconds is suggested.

Hoffman, G. L., and R. E. Putz. 1969. Host susceptibility and the effect of aging, freezing, heat, and chemicals on spores of *Myxosoma cerebralis*. Prog. Fish-Cult. 31(1):35–37.

Coho and chinook salmon and lake trout tested showed clinical whirling disease and produced *Myxosoma cerebralis* spores. The work confirmed an earlier finding that spores had to be "aged" to yield infectivity. Frozen spores survived only 18 days. Heat and drastic chemical treatment gave presumptive evidence of spore death.

Markiw, M. E., and K. Wolf. 1974a. *Myxosoma cerebralis*: isolation and concentration from fish skeletal elements—sequential enzymatic digestions and purification by differential centrifugation. J. Fish. Res. Board Can. 31(1):15–20.

Defleshed skeletal elements are reduced in size, and subjected to pepsin and then trypsin digestion the resulting material is centrifuged through a sugar solution that retains debris but allows spores to sediment. The method is sensitive and provides purified spores for use as antigen. The method does not work with fixed specimens. The report concludes with a list of stepwise procedures to be used for efficient detection and diagnosis.

Markiw, M. E., and K. Wolf. 1974b. *Myxosoma cerebralis*: comparative sensitivity of spore detection methods. J. Fish. Res. Board Can. 31(10):1597–1600.

A sequence of four methods was applied. The most sensitive method was that of pepsin then trypsin digestion plus differential centrifugation. One group of fish with a population incidence of 18% yielded about 150 spores per fish—a light infection—whereas more heavily infected populations yielded as many as 10,000 spores per fish.

Markiw, M. E., and K. Wolf. 1978. *Myxosoma cerebralis*: fluorescent antibody techniques for antigen recognition. J. Fish. Res. Board Can. 35(6):828–832.

Rabbit antiserum was prepared against homogenates of prespores and spores of *Myxosoma cerebralis*. When it was conjugated with fluorescein isothiocyanate and used in indirect fluorescent antibody tests on other myxosporeans, an unacceptable level of cross-reactivity occurred. In direct tests, cross-reactivity occurred only with an organism of the genus *Myxosoma*. The antiserum provides a serological method of identification. (In later research, Wolf and Markiw [1984] showed that the preparation gave a homologous reaction with *Triactinomyxon gyrosalmo*, and thus supported the claim that an actinosporean was the alternate life stage of *M. cerebralis*.)

Markiw, M. E., and K. Wolf. 1980. *Myxosoma cerebralis*: trypsinization of plankton centrifuge harvests increases optical clarity and spore concentration. Can. J. Fish. Aquat. Sci. 37(12):2225–2227.

Typical harvests of spores from plankton centrifuge processing were digested with 0.25% trypsin for 30 min at pH 7.2–7.5. More than 20% additional spores were released, resulting in a 10-fold increase in concentration. Reduction of tissue residues aided visualization. The method does not work with specimens that have been fixed.

Markiw, M. E., and K. Wolf. 1983. *Myxosoma cerebralis*: (Myxozoa: Myxosporea) etiologic agent of salmonid whirling disease requires tubificid worm (Annelida: Oligochaeta) in its life cycle. J. Protozool. 30(3):561–564.

The so-called "aging" process during which spores develop or become infectious was demonstrated not to occur endogenously. Instead, a tubificid oligochaete of the genus *Tubifex* was essential to production of infectivity. Trout developed whirling disease after having been fed tubificids from a fish hatchery where the disease was known to occur or tubificids that had been kept with *M. cerebralis* spores for about 4 months. Tubificids of the genera *Dero*, *Stylaria*, and *Aeolosoma* did not develop whirling disease infectivity.

O'Grodnick, J. J. 1975. Whirling disease (*Myxosoma cerebralis*) spore concentration using the continuous plankton centrifuge. J. Wildl. Dis. 11(1):54–57.

The plankton centrifuge method of spore release and concentration is described. (See also Prasher et al. 1971.)

O'Grodnick, J. J. 1979. Susceptibility of various salmonids to whirling disease (*Myxosoma cerebralis*). Trans. Am. Fish. Soc. 108(2):187–190.

During a period of 3 years, seven species were tested. The rainbow trout was found to be most susceptible, and brown trout and coho salmon the most resistant. Sockeye and chinook salmon and brook trout were judged to be intermediate in response. In contrast with findings of Hoffman and Putz (1969), lake trout were completely refractory.

O'Grodnick, J., and C. C. Gustafson. 1974. A study of the transmission, life history and control of whirling disease of trout. Pa. Fish Comm., Fed. Aid Fish Restor., Prog. Rep. F-35-R-6. 31 pp. Mimeographed.

The work summarized showed egg transmission to be unlikely; also that the infection was not prevented by chronic feeding of nine medicinal compounds. The sequence of histological changes in infected cartilage is il-

lustrated xerographically. Concluding sections on disinfection with calcium hydroxide and ultraviolet radiation indicated that infectivity was reduced but not eradicated.

O'Grodnick, J., and C. C. Gustafson. 1975. A study of the transmission, life history and control of whirling disease of trout. Pa. Fish Comm., Fed. Aid Fish Restor. Prog. Rep. F-35-R-7. 34 pp. Mimeographed.

Twelve drugs were tested in chronic feeding trials. The most effective ones reduced but did not prevent spore development. Sequential development of the parasite was followed by histology and illustrated xerographically. Soilborne infectivity was reduced by the application of calcium hydroxide. Waterborne infectivity was eliminated by filtration combined with ultraviolet irradiation.

Prasher, J. B., W. M. Tidd, and R. A. Tubb. 1971. Techniques for extracting and quantitatively studying the spore stage of the protozoan parasite *Myxosoma cerebralis*. Prog. Fish-Cult. 33(4):193-196.

First report of the use of a plankton centrifuge for concentrating *Myxosoma cerebralis* spores.

Taylor, R. E. L., S. J. Coli, and D. R. Junell. 1973. Control of whirling disease by continuous drug feeding. J. Wildl. Dis. 9(4):302-305.

Six drugs were fed continuously for 1 year to young susceptible trout that were held in water containing whirling disease infectivity. Spore development occurred in all lots but was least in fish fed furazolidone. That compound was unpalatable, however, and retarded fish growth by 50%.

Wolf, K., and M. E. Markiw. 1982. *Myxosoma cerebralis*: inactivation of spores by hot smoking of infected trout. Can. J. Fish. Aquat. Sci. 39(6):926-928.

Carcasses of 2-year-old infected rainbow and brook trout were held in 8% brine for 16 h and then smoked at 66°C for 40 min. The complete process inactivated spores of *Myxosoma cerebralis*, but brining alone did not.

Wolf, K., and M. E. Markiw. 1984. Biology contravenes taxonomy in the Myxozoa: New discoveries show alternation of invertebrate and vertebrate hosts. Science 225(4669):1449-1452.

Describes the discovery of the life cycle of *Myxosoma cerebralis*. After host fish death or ingestion by a predator, spores enter the environment and infect tubificid oligochaetes. A new phase begins and ends several months later with maturation of new forms known as actinosporeans. The transformed organism, provisionally named *Triactinomyxon gyrosalmo*, infects trout and initiates the alternate phase of whirling disease.

Note: Use of trade names does not imply U.S. Government endorsement of commercial products.

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Yasutake, W.T. 1970. *Myxosoma cerebralis* infection of trout in the western United States. Second International Congress of Parasitology, Abstract #691, September 6-12, Washington, D.C. Journal of Parasitology. 56 (Section II, Part I): 375-376.

691. ***Myxosoma cerebralis*** **infection of trout in the western United States.**

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Whirling disease of trout, caused by the highly infectious myxosporidium, *Myxosoma cerebralis*, has been prevalent in fish hatcheries in the eastern United States since 1956 (Hoffman, Dunbar, and Bradford, 1962). It was not until 1966 that the disease was diagnosed in fish in the western United States. In that year, histological confirmation was made of the disease from specimens sent to the Western Fish Disease Laboratory, Bureau of Sport Fisheries and Wildlife (Yasutake and Wolf, 1970).

Rainbow trout (*Salmo gairdneri*) suspected of having whirling disease were received from two different sources. The initial samples were from a private trout hatchery located on Garapata Creek just south of Carmel, California. The hatchery owner's description: "nervous twisting and spiraling particularly when startled," was typical of fish with *M. cerebralis* infection. This behavior was first noticed when the fish were approximately 6 months old and it continued through the summer and winter months.

Extensive myxosporidium infection was found upon histopathological examination of cranial cartilaginous tissue. That plus the absence of iodinophilous vacuoles made a definitive diagnosis possible.

The second group of rainbow trout (4½ months old) suspected of having whirling disease was received in June 1966 at the Western Fish Disease Laboratory from a state hatchery located on the Truckee River in Nevada. These fish also exhibited typical behavior. Histological examination, however, revealed numerous trophozoite-like cells in the cranial cartilaginous area and an absence of spores. Subsequent samples of 7-month-old fish taken from the same lot revealed many spores.

Three years after the initial occurrence of whirling disease in western United States hatcheries, a third incidence of the disease was diagnosed in rainbow and cutthroat (*Salmo clarki*) trout at a National Fish Hatchery located on the Carson River, Nevada (Waldsdorf, personal communication). It is believed that this is the first incidence of whirling disease of trout reported in cutthroat trout.

In order to control the spread of the disease, approximately 100,000 rainbow trout from the California hatchery were destroyed. The infected fish from the 1966 Nevada epizootic were planted in several streams before a definite diagnosis had been made. Although the precise sources of the infection of all three epizootics are not known, it is suspected that the 1969 case was the result of stocking infected fish in the watershed of the National Fish Hatchery.