FACTORS AFFECTING THE RESISTANCE OF JUVENILE RAINBOW TROUT TO WHIRLING DISEASE

by

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ABSTRACT

Whirling disease has been linked to significant losses of rainbow trout in wild trout populations. I examined the relationships among the whirling disease pathogen (*Myxobolus cerebralis*), its hosts, and the environment, with emphasis on factors affecting the resistance of rainbow trout to whirling disease. I first determined the effects of age on the development of whirling disease in rainbow trout. Whirling disease was substantially reduced when the fish were exposed to the parasite for the first time at 9 weeks posthatch (756 degree-days) or older. Second, I determined if the relationship of increasing age at exposure causing a reduction in disease severity was a factor of age or size of fish at exposure. I demonstrated that both the age of fish at first exposure and the size of fish at first exposure were important for the development of whirling disease. Third, I determined if rainbow trout can develop an acquired immune response to *M. cerebralis* and whether it provides the fish with protection against subsequent exposures. Rainbow trout did develop an acquired immune response to the parasite and an initial immunization exposure provided the fish with protection against subsequent exposures. However, the immunization, which provided the fish with protection, also induced disease in the fish, which outweighed the benefits of the protection. To reduce the effects of whirling disease on rainbow trout, they should be reared in *M. cerebralis*-free waters for 756 degree-days of development or until they are 40 mm in length, whether in the wild or in a hatchery situation.

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CHAPTER 1

INTRODUCTION

Whirling disease is caused by the myxozoan parasite *Myxobolus cerebralis*. The whirling disease parasite was first described as the cause of significant losses of farm-reared rainbow trout (*Oncorhynchus mykiss*) in Germany by Dr. Bruno Hofer of Munich University in 1898 (Hofer 1903). *Myxobolus cerebralis* is now found worldwide in central and western Europe, Australia and New Zealand, and the United States, and is thought to have spread from its origins in Eurasia by the movements of live or frozen fish (Halliday 1976).

Myxobolus cerebralis has a two-host life cycle. The parasite has two alternating spore stages, the myxosporean and the actinosporean spores. Each stage was originally thought to be in different taxonomic classes, but both have now been confirmed by molecular evidence to be alternating stages of the same organism (Andree et al. 1997). Wolf and Markiw (1984) were first to describe the two-host life cycle involving an oligochaete host and a salmonid fish host, and the alternating spore stages of the parasite. Wolf et al. (1986) later showed that the oligochaete host was *Tubifex tubifex*. The actinosporean stage of the parasite, known as the triactinomyxon spore, is released from *T. tubifex* into the water column. The triactinomyxon spores infect the salmonid host by releasing their sporoplasms into the fish epidermis. Penetrated sporoplasms begin to multiply and then migrate via peripheral nerves and the central nervous system, whereby they reach cartilage where they further multiply. Sporogenesis begins at about 50 days after infection, at 13 °C, and after another proliferation phase, large numbers of *M.*

cerebralis myxosporean spores are formed in the cartilage of the fish (El-Matbouli et al. 1995; El-Matbouli et al. 1999). *Myxobolus cerebralis* myxospores are released back into the aquatic environment from the fish only when the fish dies and degrades, or when the infected fish is consumed and the myxospores are excreted by predators (El-Matbouli and Hoffmann 1991a; Taylor and Lott 1978). The myxospores are then ingested by *T. tubifex*. The parasite develops intercellularly in the worm intestine through four phases: schizogeny, gametogony, gametogamy and sporogeny. After a period of three months, at 12 – 13 °C, the actinosporean triactinomyxon spore is released from the worm; this is the only stage of the parasite that is infectious to salmonid fish (El-Matbouli and Hoffmann 1998). The cycle is completed by the triactinomyxon infecting the salmonid host. Both a mechanostimulant, represented by the movement of the fins and swimming of the fish host, and a chemoreceptor, probably located only on the body surface of salmonid fish, are required for attachment and penetration of triactinomyxon spores and their sporoplasm germs (El-Matbouli et al. 1999). Sexual stages of the parasite are present only during the development of *M. cerebralis* myxospores into triactinomyxons in *T. tubifex*, and do not occur during the development of triactinomyxon sporoplasts into myxosporean spores in salmonids. Therefore, the oligochaete is the definitive host of *M. cerebralis* and the salmonid fish is the intermediate host (El-Matbouli et al. 1998).

The disease signs are highly dependent on the stage of development of the parasite within the host (Schaperclaus 1991). The characteristic tail chasing or whirling of diseased fish, from which the disease gets its name, is a result of spinal cord and brain stem abnormalities that are caused largely by the invasion of granulomatous inflammation of the vertebrae and skull (Rose et al. 2000). Skeletal deformities,

especially curvature of the trunk and tail, often appear at the same time as swimming disturbances and are caused by the lysis and damage of cartilaginous tissue (Wolf 1986; Schaperclaus 1991). Blacktail, a darkening of the caudal area, is caused by an impairment of the pigment-motor nerve fibers contained in the sympathetic nerves, and is associated with cartilage damage and an accompanying inflammatory response (Schaperclaus 1991). Other signs associated with cartilage damage include shortened opercula and head and jaw deformities. Clinical signs appear in susceptible fish 2 to 8 weeks after exposure to the parasite, depending on water temperature (Hoffman 1976; Markiw 1992c; Kent et al.2001). Histologically, damage ranges from minimal to extensive areas of lysis and damage in cartilaginous tissue. Myxosporean spores of *M. cerebralis* are present in or near such lesions along with granulomatous inflammation. The lesions eventually result in structural deformation during bone deposition (Wolf 1986; Hedrick et al. 1999b).

The physical impairments caused by the parasite, if not initially fatal, are thought to severely compromise survival and, in part, explain losses of up to 90% in certain year classes of rainbow trout populations (Nehring and Walker 1996; Vincent 1996). Whirling disease was initially thought to be only a problem for fish culturists. The presence of whirling disease was often associated with high mortality of trout fry in earthen ponds resulting in severe economic implications, and became known as one of the most dangerous pathogens of salmonids kept under hatchery conditions (Hofer 1903; Hewitt and Little 1972; Hoffman 1974). Prior to any reports on the effects of whirling disease on wild trout populations, the risk of intense infection, clinical disease, and serious effects on wild populations were speculated to be significantly reduced in the

wild because of low fish population densities in comparison to high densities used in culture (Wolf 1986). However, such speculation is now known to be incorrect. Young-of-the-year rainbow trout and brown trout (*Salmo trutta*) in the upper Colorado River, Colorado, were severely parasitised by *M. cerebralis* and in many cases suffered from clinical whirling disease (Nehring and Walker 1996). By 1997, population declines among wild rainbow trout linked to the effects of whirling disease had been documented in eight major trout streams in Colorado (Nehring et al. 1998). Whirling disease also significantly affected wild trout populations in Montana. Rainbow trout numbers in the Madison River declined almost 90% from historical averages by 1994, and these losses were attributed to the presence of whirling disease (Vincent 1996).

The significant negative ecological and economic consequences of the reported population declines prompted an intensive and coordinated research effort to understand and attempt to control the pathogen among wild fish. Previous research efforts were concentrated around the development of control measures that could be used in culture situations. The renewed research effort changed direction and focused on measures to control the parasite and the disease in the wild. The control measures adapted for culture situations included chemotherapy treatments, ultra-violet irradiation of the water supply, maintaining susceptible fish in parasite-free waters and the disinfection of rearing ponds (Hoffman 1974; Alderman 1986; Wolf 1986; El-Matbouli and Hoffmann 1991b; Schaperclaus 1991; Molnar 1993). These methods are however, not applicable for use in the wild to control the disease. Additionally, when considering the control of a pathogen and disease in wild fish populations other considerations have to be taken into account, which are not an issue in culture situations. For example, Vincent (1996) suggested that

Montana's wild trout management should not be compromised when developing management strategies based around the control of whirling disease.

The severity and not simply the presence or absence of infection determines the onset of disease (Markiw 1992a). Therefore, control measures do not have to be 100% effective at eradication to reduce the disease and its effects. The occurrence of disease results from a series of complex interactions of the host, pathogen and the environment (Snieszko 1973). Controlling and managing the effects of diseases on wild fish can only come from a better understanding of these complex interactions. Among the most important interactions involved is the acquisition of immunity after exposure to the pathogen (Hedrick 1998). The purpose of my dissertation research was to better understand the relationships among the whirling disease pathogen, its hosts, and the environment, with emphasis on factors affecting the resistance of rainbow trout to whirling disease. Understanding what affects the development of resistance to whirling disease in salmonids will help to determine the factors that can be manipulated to control the disease. This research will hopefully result in the development of management strategies that can be adapted for the control of whirling disease in wild salmonid populations as well as in salmonid culture situations.

Rainbow trout are the best model to use for studying this disease. Salmonid species are not affected by the whirling disease pathogen equally (O'Grodnick 1979; Hedrick et al. 1999a; Hedrick et al. 1999b; Thompson et al. 1999; Hedrick et al. 2001; Hiner and Moffit 2001; MacConnell and Vincent 2002). Rainbow trout are known to be the most susceptible of all salmonids to whirling disease, whereas other salmonid species display various lower levels of susceptibility. The reasons for the range in susceptibility

to the whirling disease pathogen among salmonids are unknown; however, these differences have been hypothesized to be related to immune mechanisms (Hedrick et al. 1999a; Hedrick et al. 1999b). The high susceptibility of rainbow trout to the whirling disease pathogen was one reason this species was chosen as the experimental unit for this work. They were also chosen because of the known effects on wild rainbow trout populations by *M. cerebralis*, and because of their ease of adaptability to experimental culture.

The objectives and hypotheses of my work were to:

1. Determine the effects of rainbow trout age at exposure to *M. cerebralis*, and the effects of varying dose rates of the parasite on the development of the disease.

   Hypothesis 1.1: The age of rainbow trout at time of first exposure to *M. cerebralis* does not affect the development of whirling disease.

   Hypothesis 1.2: The dose of *M. cerebralis* which rainbow trout are exposed to does not affect the development of whirling disease.

The development and severity of whirling disease pathology in salmonids has long been known to be dependent generally on the age or size of fish when first exposed to the triactinomyxon spores of *M. cerebralis* (Hoffman 1961; O'Grodnick 1979; Wolf 1986; Lom 1987; Markiw 1991; Markiw 1992a; Thompson et al. 1999) and on the density of triactinomyxons to which the fish are exposed (Hoffman 1974; Hoffman 1976; O'Grodnick 1979; Wolf 1986; Markiw 1992a; Markiw 1992b; Thompson et al. 1999). However, these early studies failed to demonstrate at what age rainbow trout become resistant to the development of whirling disease when exposed to different densities of triactinomyxons. The purpose of the first objective was to systematically determine the

age at which trout become resistant to the effects of the parasite when they are exposed to different dose levels of *M. cerebralis* triactinomyxons. Knowing when young trout first become resistant to the parasite will aid the management of wild trout fisheries and hatcheries. Knowing when they become resistant to specific dose rates will enable fisheries managers to develop management strategies to protect and enhance wild trout populations based around maintaining young trout in parasite-free or nearly parasite-free waters until they are resistant to the disease. Similarly, hatchery managers would be able to optimize operations and produce disease-free trout by knowing how long the trout need to be reared in parasite-free water.

2. Determine the effects of age versus size at time of exposure to the parasite on the development of whirling disease in rainbow trout.

   Hypothesis 2: Size of rainbow trout at first exposure to *M. cerebralis* does not affect the development of whirling disease.

The age of rainbow trout when they become resistant to the development of the disease was identified in objective 1. However, the ages and sizes of fish used in that experiment were significantly correlated; therefore, it could not be determined whether the development of resistance with increasing age was a factor of age or size of fish. The purpose of this objective was to expose rainbow trout of same size but different age, and same age but different size, to determine whether the age or size of the fish is more important in development of resistance to the disease. Findings would allow recommendation of an actual age or size of fish that should be maintained in parasite-free water before being stocked or entering into *M. cerebralis*-positive waters.

3. Determine if rainbow trout can be protected against subsequent exposures after an initial immunization exposure and whether they can develop an acquired immune response to *M. cerebralis*.

   Hypothesis 3.1: Prior exposure to *M. cerebralis* fails to give rainbow trout protection against a subsequent exposure.

   Hypothesis 3.2: Rainbow trout do not develop an acquired immune response to *M. cerebralis*.

The presence of an acquired immune response after first contact with triactinomyxons of *M. cerebralis* could allow for the development of management strategies to minimize the effects of whirling disease on wild trout populations, hatchery trout and stocked trout. For instance, if a light infection can provide fish with immunity or resistance against a subsequent higher parasite dose, a young wild trout in *M. cerebralis*-positive waters may gain a benefit from being infected with a low level of the parasite if it later enters waters where the risk of higher infectivity is great. Similarly, a low level of exposure in a hatchery may be beneficial to fish stocked into positive waters, and critical to their subsequent survival and performance. Management and control of the pathogen in hatcheries could involve vaccination, the deliberate induction of an acquired immune response to a dead or attenuated (non-pathogenic) form of the pathogen. Vaccination would provide protection for the fish only if an acquired immune response could be induced against the pathogen. Thus, demonstration that rainbow trout could develop an acquired immune response to *M. cerebralis* would be necessary prior to the development of a vaccine against the pathogen. Vaccination could facilitate

operation of rainbow trout hatcheries in whirling disease-positive areas, or where rainbow trout are cultured for stocking into whirling disease-positive waters.

My third objective was therefore to determine if rainbow trout develop an acquired immune response to *M. cerebralis* and if present, whether the immune response provided the fish with protection against subsequent exposures. I hypothesized that a low level exposure or immunization with *M. cerebralis* would induce an antigen-specific antibody response, which would provide the fish with protection against subsequent higher levels of exposure. I tested whether specific anti-*M. cerebralis* antibodies were produced in rainbow trout after immunization with *M. cerebralis* triactinomyxons and secondly, I tested whether this immune response provided the fish with protection against the development of whirling disease after subsequent exposures. The immune response, and its timing, in rainbow trout after immunization with different parasite doses and the subsequent exposure with a range of parasite doses were compared using serological determination of the fish immune response.

Each of the three objectives is addressed in a separate chapter. The final chapter, Conclusions and Management Implications, integrates the various sections and discusses possible avenues resulting from this work for management and control of whirling disease. The final chapter also suggests possible avenues for further research.

CHAPTER 2

OBJECTIVE 1: EFFECTS OF RAINBOW TROUT AGE AND PARASITE DOSE ON THE DEVELOPMENT OF WHIRLING DISEASE

Introduction

The development and severity of whirling disease pathology in salmonids has long been known to be dependent generally on the age or size of fish when first exposed to the triactinomyxon spores of *Myxobolus cerebralis* (Hoffman 1961; O'Grodnick 1979; Wolf 1986; Lom 1987; Markiw 1991;Markiw 1992a; Thompson et al. 1999). The intensity of whirling disease decreases with increasing size (O'Grodnick 1979), or age (Markiw 1992a), and trout exposed to the parasite at larger mean weights will have better survival (Thompson et al. 1999). Hoffman (1961), Wolf (1986), and Lom (1987) suggested that fish older than 6 months, or larger than the fingerling stage, would not become diseased. The youngest age at which trout can become infected with the parasite is 2 days posthatch (Markiw 1991). Although estimates have been made on when rainbow trout become resistant to the development of whirling disease, no studies have been conducted to systematically determine what that age is.

The development of the disease is also known to be dependent on the density of triactinomyxons to which the fish are exposed (Hoffman 1974; Hoffman 1976; O'Grodnick 1979; Wolf 1986; Markiw 1992a; Markiw 1992b; Thompson et al. 1999). Rainbow trout exposed at 2 months posthatch to either 1,000 or 10,000 triactinomyxons per fish developed clinical signs of whirling disease (Markiw 1992b). *Myxobolus*

cerebralis myxospores recovered from rainbow trout increase linearly with increasing exposure to triactinomyxon doses between 100 and 10,000 triactinomyxons per fish, and the myxospore burden plateaus at doses of 10,000 to 100,000 triactinomyxons per fish, for fish exposed at 2 months posthatch (Markiw 1992a). However, no systematic determination of the interaction between age of fish at exposure and dose of parasites to which the fish is exposed has been conducted. Uncertainty is therefore present as to the ages at which young trout become resistant to the development of whirling disease when exposed to different parasite doses. Consequently, effective management of wild salmonid fisheries in whirling disease-positive areas has been prevented because fisheries managers do not know when resistance to the disease develops in fish and thus, do not know how long trout must rear in *M. cerebralis*-free waters to reduce or eliminate the effects of the disease after entering *M. cerebralis*-positive waters.

Similarly, the control of whirling disease in hatcheries has relied on rearing fry and fingerlings in parasite-free water for as long as possible before transfer into earthen ponds or into waters known to contain the parasite, but required durations were unknown. Recommendations range from 4 to 8 months after hatch (5 to 13 cm in length) (Halliday 1976; Hoffman 1976; Schaperclaus 1991; Garden 1992), which were generally based on the untested premise that resistance to whirling disease increases as a function of ossification of the skeleton. More recently it has been suggested that not only does ossification of the skeleton increase with age and hence increase the resistance to the parasite, but also that younger fish are more vulnerable than older fish to nerve damage caused by the parasite (Rose et al. 2000). Narrowing the range of recommended rearing

times of rainbow trout in parasite-free water would make for better management of rainbow trout in whirling disease-positive areas, whether in the wild or in hatcheries.

The purpose of this objective was to fill some of these information deficiencies by testing the disease susceptibility of different rainbow trout age groups exposed to differing parasite doses in a replicated factorial experiment under standardized laboratory conditions. The specific objective was to determine the effects of age of rainbow trout at exposure to *M. cerebralis,* and the effects of varying dose rates of the parasite on the development of the disease. By assessing how pathogenesis differs in fish of different ages and under different parasite doses, a better understanding of this parasite and the disease it manifests will be achieved, and thereby management activities can be designed to enhance viability and productivity of trout populations in *M. cerebralis*-positive river systems and in culture situations. Knowing when rainbow trout become resistant to the effects of the disease when exposed to varying dose rates will enable fisheries managers to develop management strategies to protect and enhance wild trout populations based around maintaining young trout in parasite-free waters until they are resistant to the disease. Similarly, hatchery managers would be able to optimize operations and produce disease-free trout by knowing how long the trout need to be reared in parasite-free waters to ensure their resistance against the development of the disease.

Methods

Whirling disease severity was compared among rainbow trout exposed to the parasite for the first time at a range of ages. The fish were also exposed to a range of parasite doses at each age. The severity of whirling disease was compared among the groups exposed at different ages to different parasite dose levels to determine if a threshold age could be reached after which the fish no longer develop the disease after exposure to the parasite.

Traditional indicators of whirling disease severity measured included mortality, clinical signs, microscopic pathology and *M. cerebralis* myxosporean spore counts. Additionally, swimming performance was measured. Swimming performance tests are a convenient means to assess physical condition of fish (Wedemeyer et al. 1990) as well as being a good indicator of stress (Webb and Brett 1973). The advantage of using swimming performance over more traditional methods of condition indicators (for example, growth rate) is that its demands are immediate and any failings can be measured by short-term tests. Swimming performance was used in this instance to determine if a correlation existed between severity of disease and endurance. Such a relationship would facilitate prediction of the actual effects this disease has on young-of-the-year wild fish subjected to rigorous natural conditions, as impairment of swimming performance correlates to reduced survival (Thomas et al. 1964).

Experimental Procedures

Myxobolus cerebralis triactinomyxons were produced in the laboratory. Cultures of *Tubifex tubifex* worms were exposed to myxospores of *M. cerebralis,* and triactinomyxons were collected using a procedure similar to that described by Hedrick et al. (1999b).

Erwin strain rainbow trout were hatched from eggs supplied by the Ennis National Fish Hatchery, United States Fish and Wildlife Service, Ennis, Montana, and maintained at the Bozeman Fish Technology Center (BFTC), Bozeman, Montana, until scheduled for exposure. Exposures were conducted at the Wild Trout Research Laboratory (WTRL), Montana State University, Bozeman, Montana. Each lot was exposed to *M. cerebralis* triactinomyxons in aerated 5-liter exposure chambers for 2 hours. Following exposure, lots were maintained separately at 13 °C in 38-liter glass aquaria supplied with filtered and oxygenated water by a recirculating process system at the WTRL. The fish were fed a commercial trout diet at 2 to 3 percent body weight per day. Mortalities were counted and removed daily.

Fish were exposed to a range of parasite doses over a range of ages in a replicated factorial experiment. Fish were exposed at eleven different ages ranging from 1 to 24 weeks posthatch (1, 3, 5, 7, 9, 11, 13, 15, 17, 20 and 24 weeks posthatch). All groups were reared at 12 °C before exposure (Table 2.1). Fish were exposed at each of the eleven ages to four levels of parasite dose (0, 100, 1,000, and 10,000 triactinomyxons per fish). Three replicate lots of 55 fish were exposed to each dose level at each of the eleven ages for a total of 12 lots for each age.

Table 2.1. Mean weight and degree-days of development at exposure for fish exposed at different ages.

Age at exposure (weeks posthatch)	Degree-days at exposure	Mean weight at exposure (g)
1	84	0.05
3	252	0.10
5	420	0.23
7	588	0.43
9	756	0.86
11	924	1.30
13	1092	1.72
15	1260	2.59
17	1428	3.33
20	1680	4.22
24	2016	6.05

The swimming performance of individual fish was tested 17 weeks after exposure and at 33 weeks posthatch in a stamina tunnel (Figure 2.1). Swimming stamina was measured as the length of time a fish could maintain its position in the tunnel at a constant velocity (45 cm/s). Time to fatigue was recorded for three randomly selected fish from each replicate at each of the two times.

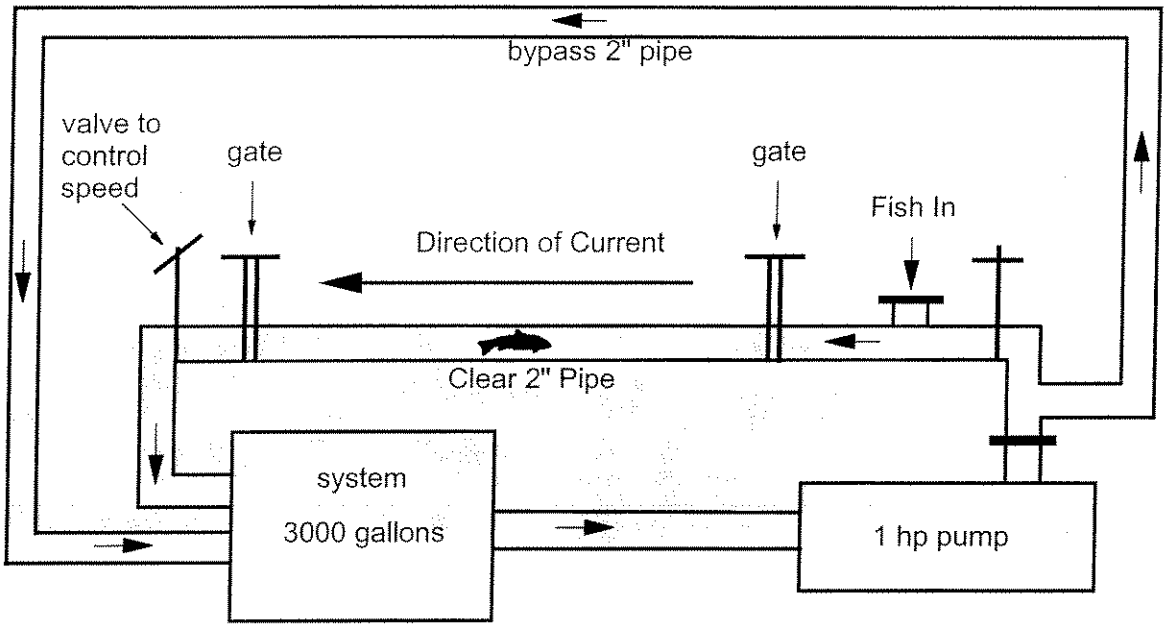


Figure 2.1—Stamina Tunnel. Arrows indicate direction of flow.

Swimming performance was not recorded at 17 weeks after exposure for any fish exposed at 1 week posthatch to either 1,000 or 10,000 triactinomyxons per fish. The numbers of fish remaining within these lots were low at this time (less than 20 per tank) with 15 weeks to go before the end of the experiment. Therefore, they were not sampled to allow them the chance to survive until the end of the experiment and be sacrificed at 33 weeks posthatch. The fish were not run through the tunnel and then returned to their tanks without sacrificing them to keep the experimental procedures equal for all treatment groups. Swimming performance at 33 weeks posthatch could not be measured for fish exposed at 1 week posthatch to 10,000 triactinomyxons per fish because only one fish from this exposure group was still alive at this time and had grown too large (73.45 g) for

the stamina tunnel. The fish exposed at 20 and at 24 weeks posthatch were also not subjected to the swimming performance test because of their size.

The fish used in the stamina test, and an additional six randomly selected fish from each replicate at 17 weeks after exposure and at 33 weeks posthatch, were euthanatized. The fish exposed at 20 and 24 weeks posthatch were euthanatized at 17 weeks after exposure. The heads of the euthanatized fish were removed, cut in half along the midsagittal plane, preserved in Davidson's fixative, and prepared for microscopic examination using standard histological techniques. From each fish, one head half and the tail were prepared for histology. Microscopic pathology was categorized according to the methods by Hedrick et al. (1999b), in which cartilaginous tissue was examined for the presence of the parasite and associated lesions. The abundance of parasites, cartilage damage, inflammation, extent of lesions, involvement of other tissues, and bone distortion were evaluated and categorized into one of six categories: no infection, minimal, mild, moderate, moderately/severe or severe (E. MacConnell, United States Fish and Wildlife Service, personal communication, Table 2.2). Two head sections and two tail sections were evaluated histologically for each fish collected.

Disease severity, as measured by microscopic pathology, was not determined at 17 weeks after exposure for fish exposed at 1 week posthatch to either 1,000 or 10,000 triactinomyxons per fish. Few fish had survived in these exposure groups (less than 20 per tank) to 17 weeks after exposure, with 15 weeks to go before the end of the experiment; they were not sampled in the hope that some would survive until the end of the experiment. Histology was not performed on fish exposed at 24 weeks posthatch because of time constraints.

Table 2.2. Definition of categories used for grading histological sections of *Myxobolus cerebralis*-exposed fish. Numbers commonly associated with the categories are 1 = minimal, 2 = mild, 3 = moderate, 4 = moderately severe, and 5 = severe.

Pathology Severity	Areas of Cartilage Damage	Inflammatory Response
None	None	None
Minimal	Few	None
Mild	Several	Focal
Moderate	Several in each section	Diffuse
Moderately severe	Several to numerous	Extensive
Severe	Throughout	Extensive

The other halves of the heads from the fish sacrificed at 33 weeks posthatch were used to obtain spore counts. Spore counts were made on samples collected at 17 weeks after exposure for fish exposed at 20 or 24 weeks posthatch. The standard plankton centrifuge method (O'Grodnick 1975) was used for spore extraction. After extraction, spores were resuspended in a known volume of deionized water, and 1-ml aliquots were placed on both sides of a standard 1-ml hemocytometer counting chamber. Total spores per original head were calculated as follows: (2 × total number of spores counted × 10^4 × volume of suspension) / (number of 1-mm^2 areas counted). Three counts of spores were made from each suspended sample; the mean of the three was used in analyses.

Cumulative mortality, swimming performance, and microscopic pathology were measured twice during the experiment, first at 17 weeks after exposure and second when the fish reached 33 weeks posthatch. Disease development, in all lots of fish, at an equal

time after exposure, and when the fish were all at the same age, could thereby be estimated. Clinical signs (i.e., blacktail, skeletal deformities, cranial deformities and whirling behavior) were only estimated at the end time, 33 weeks posthatch, as this was the only time all the fish in the tanks could be handled and visually inspected for signs of disease. Fish exposed at 20 and 24 weeks posthatch were examined for clinical signs at 17 weeks after exposure. Survival was not estimated for fish exposed at 20 and 24 weeks posthatch because their survival was influenced by uncontrollable factors unrelated to exposure to the whirling disease pathogen such as over crowding and low dissolved oxygen.

Bias was reduced throughout the experiment wherever possible. Fish were randomly assigned to lots and lots were assigned to tanks randomly. Samples were collected randomly from the tanks for swimming performance tests, histology, and spore counts. Histology and spore count samples were examined blindly and in random order. The exposure designation of each sample was not determined until all samples had been examined and recorded.

Statistical Analysis

The experiment was designed and analyzed as a two-way factorial. The two factors, or treatments, were age of fish at exposure (1, 3, 5, 7, 9, 11, 13, 15, 17, 20 or 24 weeks posthatch) and parasite dose (0, 100, 1,000, or 10,000 triactinomyxons per fish). The number of mortalities, percent of fish with clinical signs, spore counts, swimming performance and microscopic pathology were compared among the 44 treatment groups

(i.e., eleven ages and four parasite dose levels). All responses were treated parametrically with the exception of microscopic pathology.

Spore counts and swimming performance were analyzed by including random factors in the model for tank and fish, and the fish were treated as the experimental unit. Mortality and clinical signs were analyzed in the same way as spore counts and swimming performance, with the exceptions that tanks were the experimental units and no factor for fish was included in the model. A mixed linear model was used that combined both the fixed (age of fish at exposure and parasite dose) and random (tank and fish) effects. Type 3 *F*-statistics were used (Montgomery 1997). ANOVA tables are in Appendix A. The important assumptions supporting this analysis are that the data are normally distributed and that they are independent with constant variance. Visual inspection of residual plots of data for all responses confirmed that these assumptions were met. The units of measure (fish or tank) were not independent; however, this assumption could be dropped by modeling statistical correlation into the analysis, which assumes constant variance and constant covariance (PROC MIXED, compound symmetry covariance option; Littell et al. 1996). The model used for the analysis was the following:

$$y_{ijklm} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \gamma_{k(ij)} + \delta_{l(ijk)} + \varepsilon_{ijklm},$$

where

$i = 1,\ldots,a,$

$j = 1,\ldots,b,$

$k = 1,\ldots,c,$

$l = 1,\ldots,d,$

$m = 1,\ldots,e$,

and

μ = the overall mean,

α_i = the effect of the ith level of the fixed factor A (age at exposure),

β_j = the effect of the jth level of the fixed factor B (parasite dose),

$(\alpha\beta)_{ij}$ = the interaction effect between the ith level of factor A and the jth level of factor B,

$\gamma_{k(ij)}$ = the effect of the kth level of the random factor C (tank nested in factors A and B),

$\delta_{l(ijk)}$ = the effect of the lth level of the random factor D (fish nested in factors A-C; this effect is not included in the model when analyzing the mortality or clinical signs response), and

ε_{ijklm} = a random error caused by sampling.

Bonferroni's multiple comparison procedure was used to compare all pairwise differences of the least-square means. For each significance test, $\alpha = 0.05$. The chi-square test of homogeneity was used to determine whether age at exposure significantly affected microscopic pathology within each dose and to determine whether parasite dose significantly affected microscopic pathology within each age group (Daniel 1990). This non-parametric procedure was used because these data were categorical. All statistical analyses were conducted with the statistical software program SAS/STAT (SAS Institute 1996).

Results

Cumulative Mortality

Mortality at 17 weeks after exposure and at 33 weeks posthatch significantly increased with decreasing rainbow trout age at exposure ($F = 49.53$, $P < 0.0001$; $F = 27.55$, $P < 0.0001$, respectively for 17 weeks after exposure and 33 weeks posthatch, Figure 2.2 and Figure 2.3). Fish exposed at 1 week posthatch had significantly more mortality than all other age groups at all triactinomyxon doses. Fish exposed at 3 or 5 weeks posthatch had significantly less mortality than those exposed at 1 week posthatch and generally, significantly more mortality than those exposed at older ages.

Mortality at both time periods was also significantly affected by the parasite dose level the fish received ($F = 8.66$, $P < 0.0001$; $F = 6.28$, $P = 0.0007$, respectively for 17 weeks after exposure and 33 weeks posthatch, Figure 2.2 and Figure 2.3). Mortality increased significantly with increasing parasite dose in groups exposed at 1, 3, and 5 weeks posthatch. Fish exposed at 7 weeks posthatch or older to 100, 1,000, or 10,000 triactinomyxons per fish did not suffer significantly higher mortality than those of comparable ages exposed to 0 triactinomyxons per fish.

A significant interaction effect on mortality was present between the age of fish at exposure and parasite dose at 17 weeks after exposure ($F = 2.65$, $P = 0.0006$; Figure 2.2) and at 33 weeks posthatch ($F = 1.69$, $P = 0.0442$; Figure 2.3). Significant interactions occur when the difference in response between the levels of one factor is not the same at all levels of the other factor. Increasing the level of parasite dose significantly increased the number of mortalities only when the fish were exposed at 5 weeks posthatch or

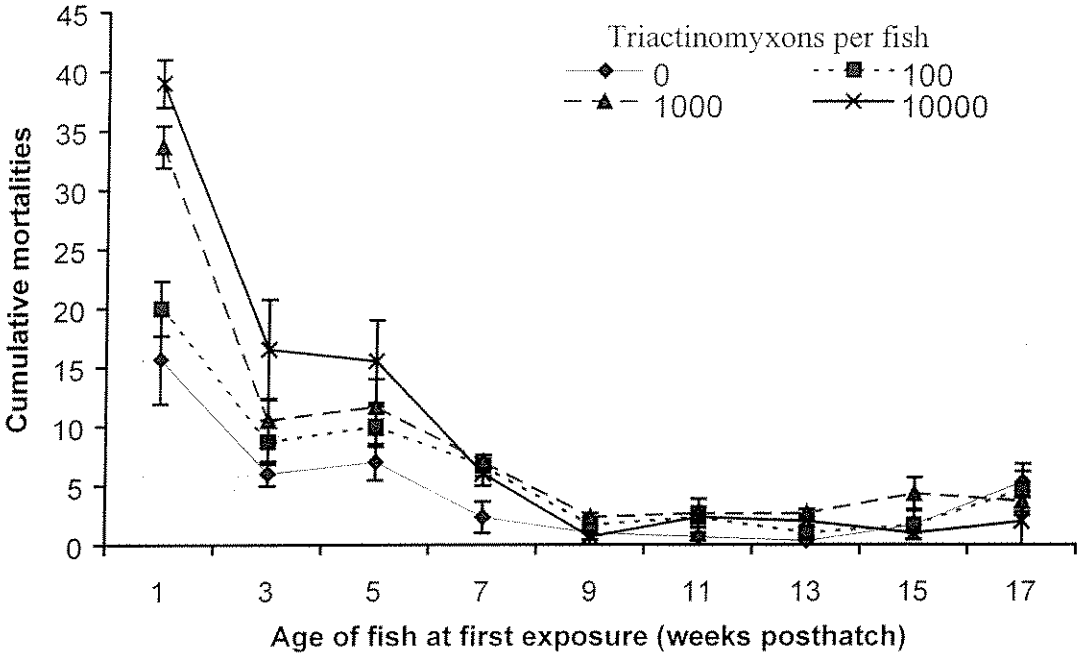


Figure 2.2—Mean (±SE) number of cumulative mortalities of rainbow trout 17 weeks after exposure at varying ages to 0, 100, 1,000 or 10,000 triactinomyxons of *Myxobolus cerebralis* per fish.

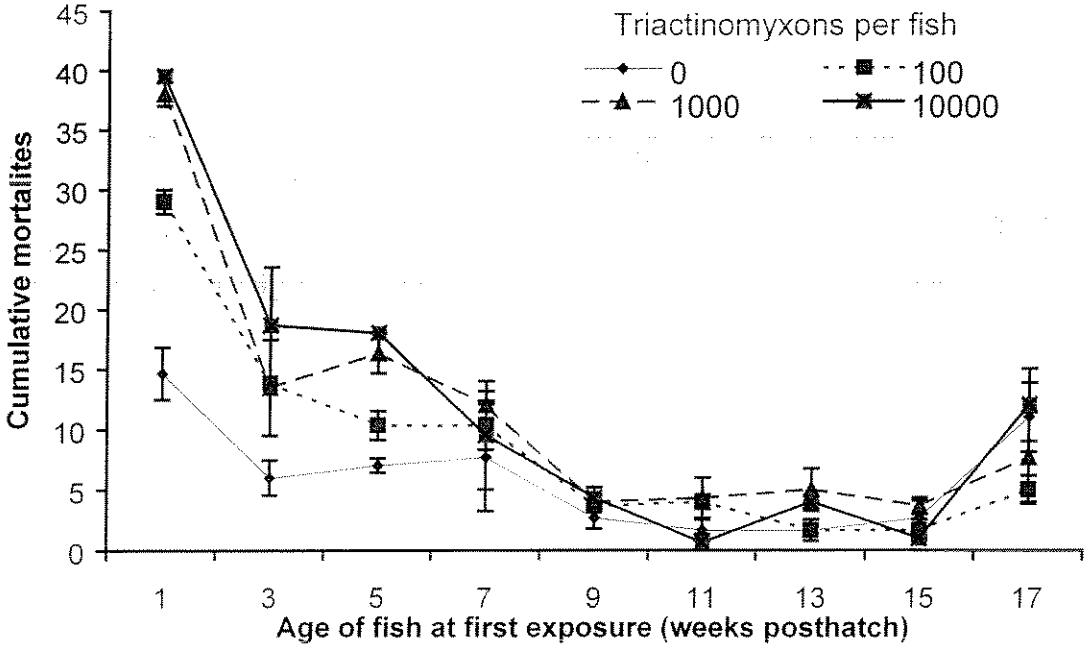


Figure 2.3—Mean (±SE) number of cumulative mortalities of rainbow trout at 33 weeks posthatch, exposed at varying ages to 0, 100, 1,000 or 10,000 triactinomyxons of *Myxobolus cerebralis* per fish.

younger. Parasite dose did not significantly affect the number of mortalities when the fish were exposed at 7 weeks posthatch or older.

Swimming Performance

Age of fish at exposure did not significantly affect the swimming performance of fish 17 weeks after exposure ($F = 1.65$, $P = 0.1194$) or at 33 weeks posthatch ($F = 1.30$, $P = 0.2523$), but level of parasite dose did at 17 weeks after exposure ($F = 6.08$, $P = 0.0029$; Figure 2.4) and at 33 weeks posthatch ($F = 4.57$, $P = 0.0045$; Figure 2.5). The swimming performance of fish 17 weeks after exposure significantly decreased with increasing parasite dose among fish exposed at 7 weeks posthatch or younger, but not among fish exposed at 9 weeks posthatch or older. Swimming performance did not decrease significantly with increasing parasite dose at 33 weeks posthatch for fish exposed at 7 weeks posthatch or older, but did decrease significantly with increasing parasite dose among fish exposed at 5 weeks posthatch or younger.

Microscopic Pathology

Microscopic pathology category decreased with increasing age at exposure when sampled 17 weeks after exposure and at 33 weeks posthatch. Frequency distributions of pathology category for all age groups were significantly different at $P < 0.0001$ (chi-square test of homogeneity) within the same parasite dose level. The pattern of changing frequency distributions occurred within all dose levels with the mode decreasing with increasing age (an example is shown graphically in Figure 2.6, all other distributions are tabulated in Tables 2.3 and 2.4). The frequency distributions of pathology

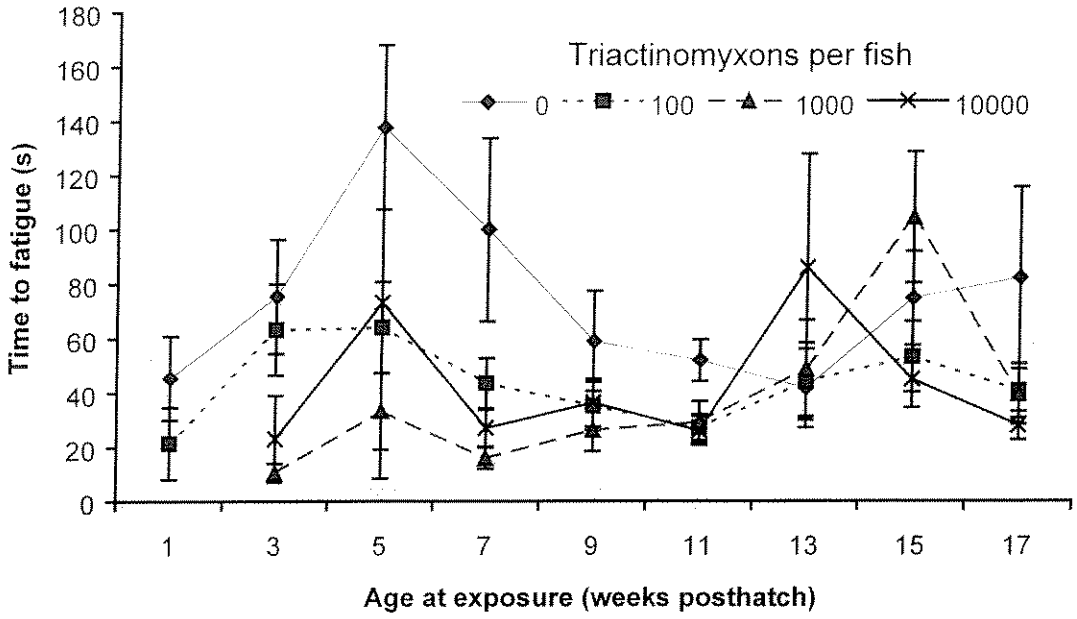


Figure 2.4—Mean (±SE) time to fatigue of rainbow trout at a water velocity of 45 cm/s 17 weeks after exposure at varying ages to 0, 100, 1,000 or 10,000 triactinomyxons of *Myxobolus cerebralis*.

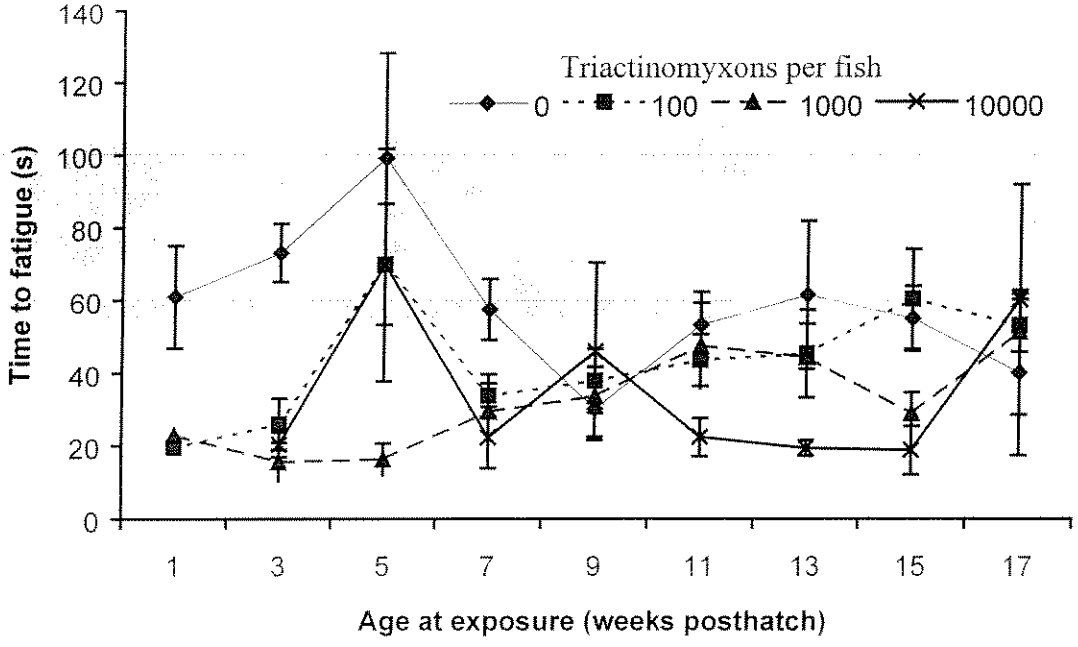


Figure 2.5—Mean (±SE) time to fatigue of rainbow trout at a water velocity of 45 cm/s at 33 weeks posthatch, exposed at varying ages to 0, 100, 1,000 or 10,000 triactinomyxons of *Myxobolus cerebralis*.

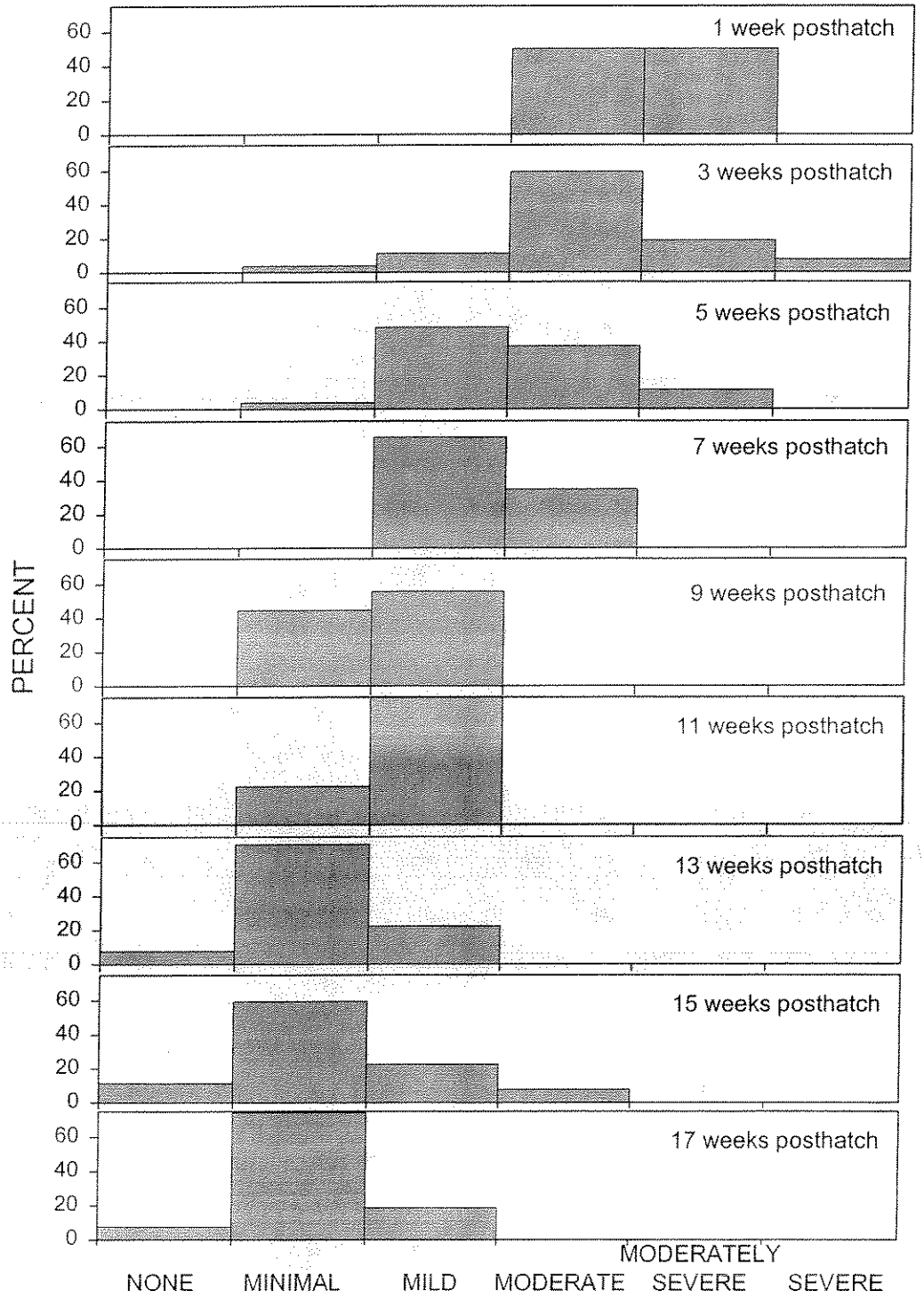


Figure 2.6—Frequency distributions of microscopic pathology category at 17 weeks post exposure to 100 triactinomyxons per fish.

Table 2.3. Frequency distributions of microscopic pathology category at 17 weeks post exposure for rainbow trout exposed to different levels of *Myxobolus cerebralis* dose at different ages.

Age (weeks posthatch)	Dose (triactinomyxons per fish)	Microscopic pathology category (%)					
		None	Minimal	Mild	Moderate	Moderately severe	Severe
1	0	100	0	0	0	0	0
	100	0	0	22	30	37	11
3	0	100	0	0	0	0	0
	100	0	7	26	52	15	0
	1,000	0	0	19	54	27	0
	10,000	0	0	7	60	27	7
5	0	100	0	0	0	0	0
	100	0	30	56	15	0	0
	1,000	0	0	0	78	22	0
	10,000	0	0	33	67	0	0
7	0	100	0	0	0	0	0
	100	0	22	59	19	0	0
	1,000	0	26	63	11	0	0
	10,000	0	0	60	40	0	0
9	0	100	0	0	0	0	0
	100	0	37	59	4	0	0
	1,000	0	41	59	0	0	0
	10,000	0	22	78	0	0	0
11	0	100	0	0	0	0	0
	100	0	63	37	0	0	0
	1,000	0	37	56	7	0	0
	10,000	0	6	89	6	0	0
13	0	100	0	0	0	0	0
	100	4	59	37	0	0	0
	1,000	0	11	44	41	4	0
	10,000	0	0	67	33	0	0
15	0	100	0	0	0	0	0
	100	0	48	41	11	0	0
	1,000	0	22	59	19	0	0
	10,000	0	22	44	33	0	0
17	0	100	0	0	0	0	0
	100	11	59	26	4	0	0
	1,000	4	37	44	15	0	0
	10,000	0	17	44	33	6	0
20	0	100	0	0	0	0	0
	100	60	40	0	0	0	0
	1,000	14	71	14	0	0	0
	10,000	0	25	67	8	0	0

Table 2.4. Frequency distributions of microscopic pathology category at 33 weeks posthatch for rainbow trout exposed to different levels of *Myxobolus cerebralis* dose at different ages.

Age (weeks posthatch)	Dose (triactinomyxons per fish)	Microscopic pathology category (%)					
		None	Minimal	Mild	Moderate	Moderately severe	Severe
1	0	100	0	0	0	0	0
	100	0	0	0	50	50	0
	1,000	0	0	0	67	33	0
	10,000	0	0	0	100	0	0
3	0	100	0	0	0	0	0
	100	0	4	11	59	19	7
	1,000	0	4	48	43	4	0
	10,000	0	26	53	21	0	0
5	0	100	0	0	0	0	0
	100	0	4	48	37	11	0
	1,000	0	0	44	52	4	0
	10,000	0	0	0	60	33	7
7	0	100	0	0	0	0	0
	100	0	0	65	35	0	0
	1,000	0	11	67	22	0	0
	10,000	0	0	29	71	0	0
9	0	100	0	0	0	0	0
	100	0	44	56	0	0	0
	1,000	0	52	41	7	0	0
	10,000	0	26	56	18	0	0
11	0	100	0	0	0	0	0
	100	0	22	78	0	0	0
	1,000	0	26	63	11	0	0
	10,000	0	15	78	7	0	0
13	0	100	0	0	0	0	0
	100	7	70	22	0	0	0
	1,000	4	52	26	18	0	0
	10,000	0	33	44	22	0	0
15	0	100	0	0	0	0	0
	100	11	59	22	7	0	0
	1,000	15	37	41	7	0	0
	10,000	0	44	44	12	0	0
17	0	100	0	0	0	0	0
	100	7	74	19	0	0	0
	1,000	0	48	48	4	0	0
	10,000	0	46	38	16	0	0

category also shifted with increasing parasite dose level within an age. The mode of the distribution increased with increasing parasite dose (an example is shown graphically in Figure 2.7, all other distributions are tabulated in Tables 2.3 and 2.4). Frequency distributions of pathology category for different dose levels within all age groups were significantly different at $P < 0.0001$ (chi-square test of homogeneity).

Spore Counts

Myxobolus cerebralis spores were not found in any of the control fish exposed to no triactinomyxons. The age of rainbow trout at exposure significantly affected the number of spores present in the heads ($F = 35.05$, $P < 0.0001$; Figure 2.8). Spore counts decreased significantly with increasing age at exposure. Fish exposed to 100 triactinomyxons per fish at 13 weeks posthatch or older had significantly lower spore counts than all other age groups exposed to the same parasite level. Fish exposed to 1,000 or 10,000 triactinomyxons per fish at 9 weeks posthatch or older had significantly lower spore counts than all other age groups exposed to the same parasite levels.

The number of *M. cerebralis* spores present in the fish heads was also significantly affected by the dose of triactinomyxons the fish received when exposed at certain ages ($F = 77.70$, $P < 0.0001$; Figure 2.8). Spore counts increased significantly with increasing parasite dose for fish exposed at 11 weeks posthatch or younger, but not for fish exposed at 13 weeks posthatch or older. A significant interaction was present between age of fish at exposure and parasite dose ($F = 8.23$, $P < 0.0001$), providing further evidence that the relationship between parasite dose and number of spores was not the same for fish exposed at all ages.

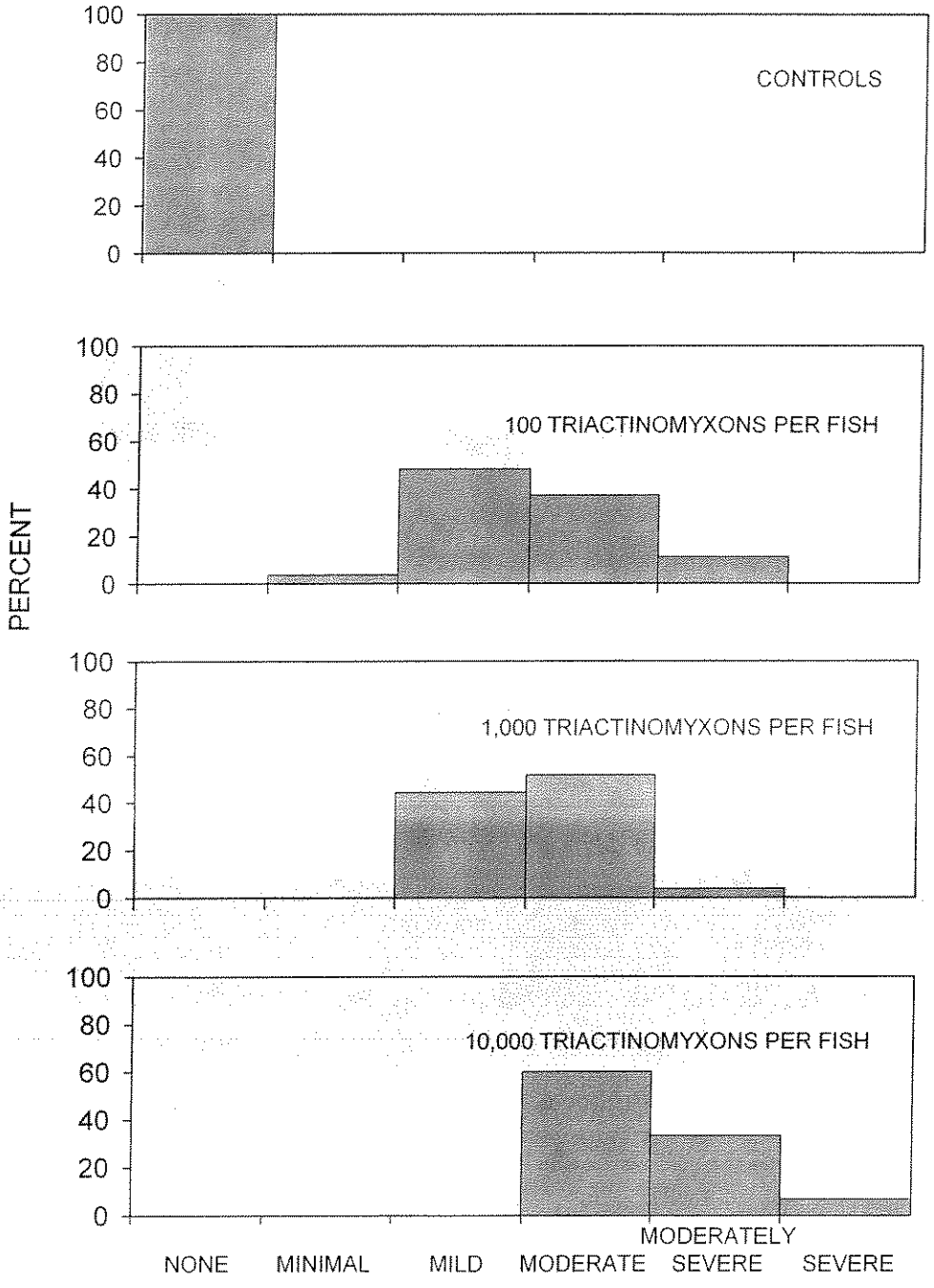


Figure 2.7—Frequency distributions of microscopic pathology category at 33 weeks posthatch by parasite dose level for rainbow trout exposed at 5 weeks posthatch.

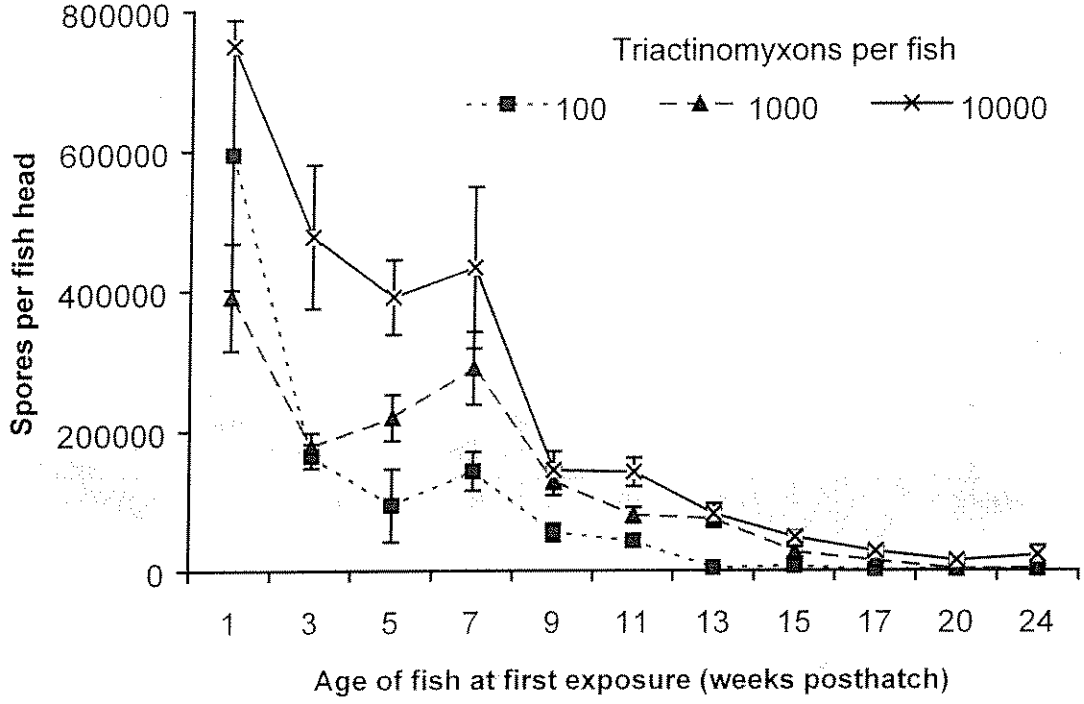


Figure 2.8—Mean (±SE) number of *Myxobolus cerebralis* spores per head of rainbow trout at 33 weeks posthatch, exposed at varying ages to 100, 1,000 or 10,000 triactinomyxons of *M. cerebralis* per fish.

Clinical Signs

Whirling disease clinical signs were not observed among any of the control fish and no clinical signs were observed among any fish exposed at 13 weeks posthatch or older, regardless of parasite dose level to which they were exposed.

The prevalence of clinical signs (blacktail, skeletal deformities and whirling behaviour combined) at 33 weeks posthatch decreased significantly with increasing age at exposure ($F = 475.16$, $P < 0.0001$; Figure 2.9). The prevalence of clinical signs decreased significantly with increasing age at exposure for fish exposed to the same

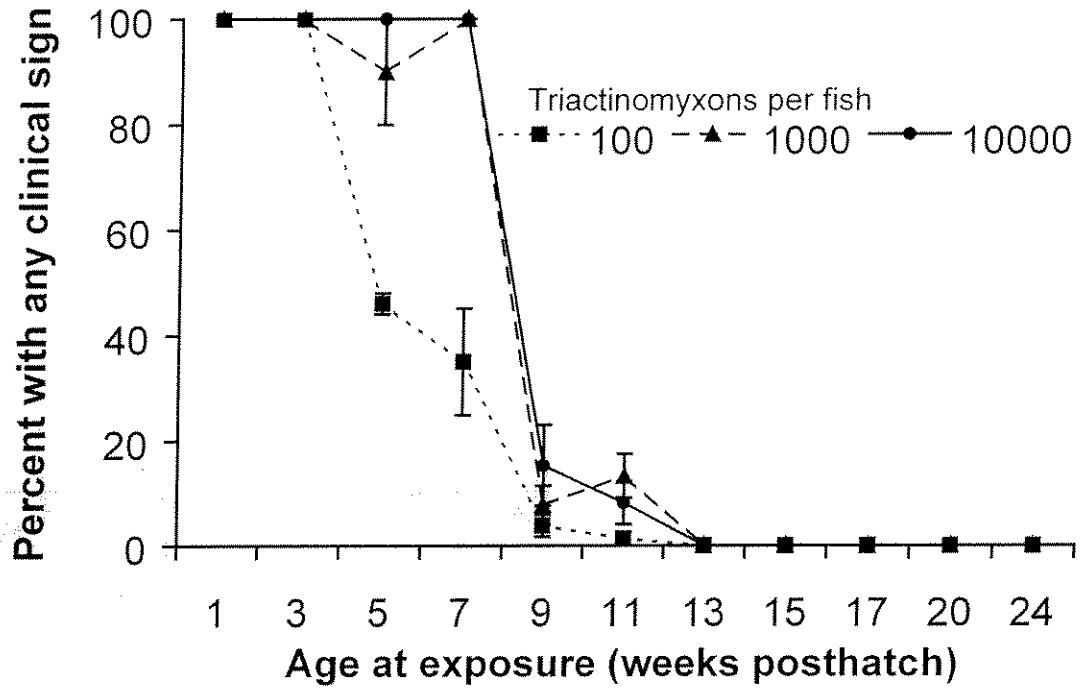


Figure 2.9—Mean (±SE) percent of rainbow trout with any clinical sign at 33 weeks posthatch, exposed at varying ages to 100, 1,000 or 10,000 triactinomyxons of *Myxobolus cerebralis* per fish.

parasite dose level when exposed at 9 weeks posthatch or younger. Fish exposed at 1 and 3 weeks posthatch to 100 triactinomyxons per fish had significantly higher prevalences of clinical signs than all other age groups exposed to the same dose. Fish exposed at 5 and 7 weeks posthatch to 100 triactinomyxons per fish had the same prevalence of clinical signs, but had significantly more clinical signs than all groups exposed to the same parasite dose at older ages. No significant differences in the prevalence of clinical signs occurred among fish exposed between 1 and 7 weeks posthatch to either 1,000 or 10,000 triactinomyxons per fish, although they all had significantly higher prevalences of clinical

signs than the fish exposed at 9 weeks posthatch or older. No significant differences in the prevalence of clinical signs occurred among the different age groups when exposed at 9 weeks posthatch or older, regardless of parasite dose level.

Level of parasite dose to which the fish were exposed also affected the percentage of fish with clinical signs at 33 weeks posthatch ($F = 415.33$, $P < 0.0001$; Figure 2.9). Prevalence of clinical signs increased with increasing parasite dose, but only for fish exposed at 7 weeks posthatch or younger. Tanks of fish exposed at 1 or 3 weeks posthatch to 100, 1,000 or 10,000 triactinomyxons per fish all had 100% of fish with clinical signs. The prevalence of clinical signs was significantly different for all four levels of parasite dose for fish exposed at 5 weeks posthatch with the prevalence increasing with increasing level of parasite dose. No significant difference was present between the fish exposed at 1,000 or 10,000 triactinomyxons per fish at 7 weeks posthatch although the prevalence was greater in these fish than the controls and those exposed at 100 triactinomyxons per fish, and those exposed to 100 triactinomyxons per fish had a significantly higher prevalence than the controls. Parasite dose level failed to significantly affect the prevalence of clinical signs for fish exposed at 9 weeks posthatch or older. A significant interaction was also present between parasite dose level at exposure and age of fish at exposure ($F = 73.97$, $P < 0.0001$) further indicating that the effects of the parasite dose level were not the same for all levels of age at exposure, as described above.

The prevalence of blacktail varied with age and dose in a similar pattern to the clinical signs combined. Age at exposure significantly influenced the prevalence of blacktail ($F = 21.70$, $P < 0.0001$; Figure 2.10) with the prevalence decreasing with

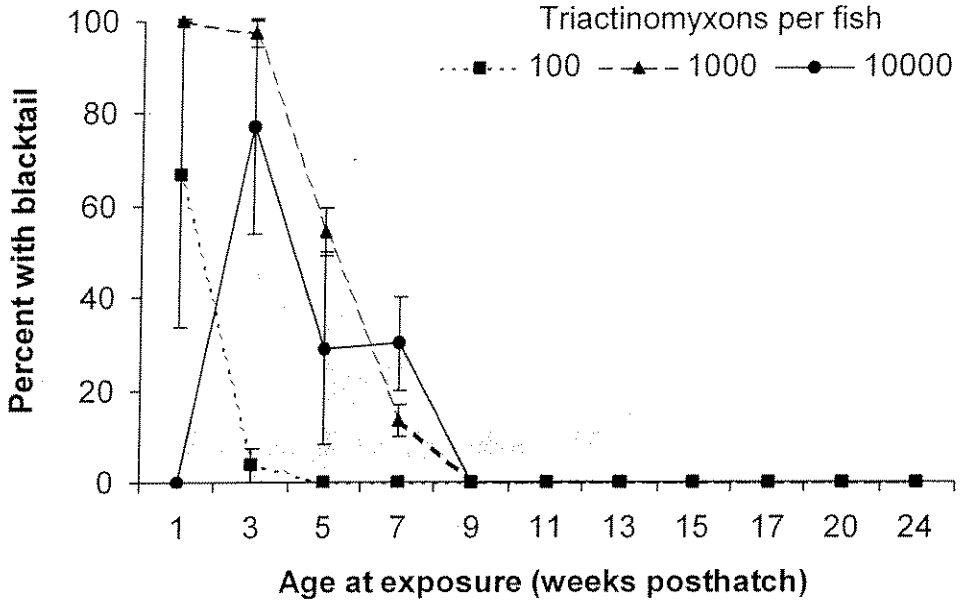


Figure 2.10—Mean (±SE) percent of rainbow trout with blacktail at 33 weeks posthatch, exposed at varying ages to 100, 1,000 or 10,000 triactinomyxons of *Myxobolus cerebralis* per fish.

increasing age at exposure. Increasing age at exposure resulted in a decrease in the prevalence of blacktail only in fish exposed at 7 weeks posthatch or younger. No blacktail was observed among fish exposed at 9 weeks posthatch or older. One exception to the relationship of age at exposure and the occurrence of blacktail was that the fish exposed to 10,000 triactinomyxons per fish at 1 week posthatch displayed no blacktail at 33 weeks posthatch. This group had the highest level of mortality and blacktail was present in these fish earlier in the experiment indicating that perhaps the most diseased fish, those with blacktail, died before the end of the experiment. Level of parasite dose to which the fish were exposed significantly affected the presence of blacktail ($F = 24.72$, $P < 0.0001$; Figure 2.10) with the prevalence of blacktail generally increasing with

increasing parasite dose, but only for fish exposed at 7 weeks posthatch or younger. This was also indicated by the presence of a significant interaction between age and dose ($F = 8.84$, $P < 0.0001$) further demonstrating that the effects of dose are not the same for all levels of age, as described above.

The prevalence of major skeletal deformities (mostly scoliosis) in rainbow trout also varied with age and dose. Age at exposure significantly influenced the prevalence of the major skeletal deformities ($F = 6.62$, $P < 0.0001$; Figure 2.11) with the prevalence decreasing with increasing age at exposure. Rainbow trout exposed at 1 week posthatch to 100 triactinomyxons per fish had a higher prevalence of skeletal deformities than all other age groups exposed to the same dose; however, no significant differences were present among any of the other age groups at this dose. No fish exposed at 3 weeks posthatch or older to 100 triactinomyxons per fish showed any signs of major skeletal deformities. A similar pattern was present with the fish exposed to 1,000 triactinomyxons per fish. At 1,000 triactinomyxons per fish the prevalence of skeletal deformities increased with increasing age up to 5 weeks posthatch. The fish exposed at 10,000 triactinomyxons per fish followed this same pattern with the exception that those exposed at 1 week posthatch did not show any signs of skeletal deformities. The level of parasite dose to which the fish were exposed also affected the prevalence of skeletal deformities ($F = 6.74$, $P = 0.0004$; Figure 2.11) but only for fish exposed at 1 and 3 weeks posthatch. A significant interaction between age and dose was present ($F = 3.75$, $P < 0.0001$) further indicating that the effects of level of parasite dose were not the same at all levels of age at exposure.

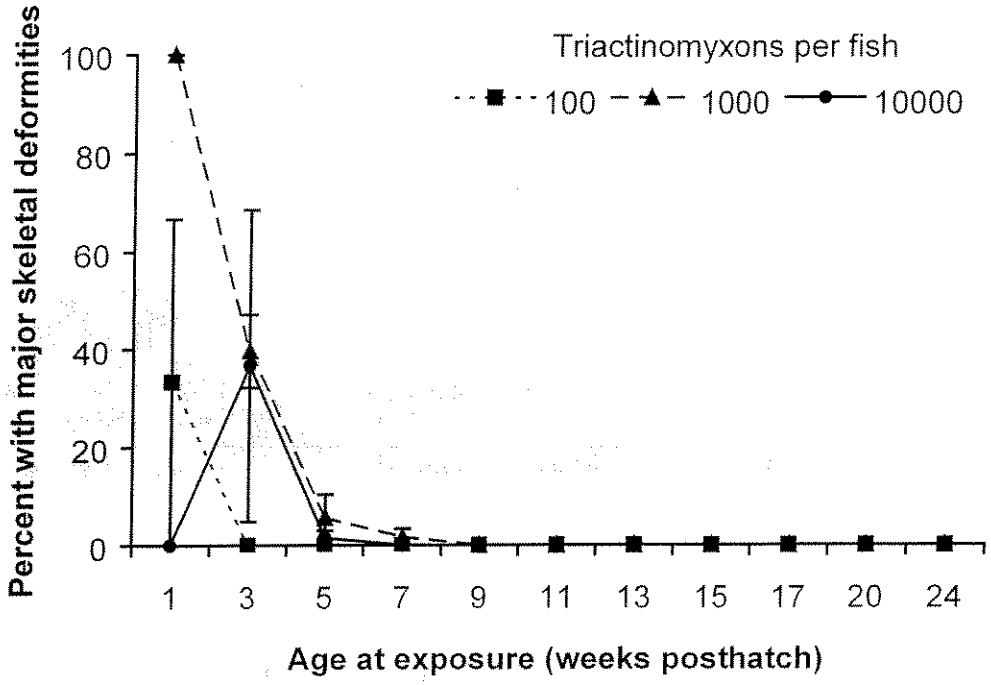


Figure 2.11—Mean (±SE) percent of rainbow trout with major skeletal deformities at 33 weeks posthatch, exposed at varying ages to 100, 1,000 or 10,000 triactinomyxons of *Myxobolus cerebralis* per fish.

The prevalence of minor skeletal deformities (primarily cranial deformities) followed a similar pattern to that of the clinical signs combined (Figure 2.12) with the exception that no minor deformities were recorded in fish exposed at 1 or 3 weeks posthatch to 10,000 triactinomyxons per fish. Increasing age at exposure caused a decrease in the prevalence of this sign ($F = 34.88$, $P < 0.0001$; Figure 2.12) as did decreasing levels of parasite dose ($F = 31.61$, $P < 0.0001$; Figure 2.12). Minor deformities were observed in fish exposed at 9 and 11 weeks posthatch; however, the prevalence of the signs in the groups exposed to a triactinomyxon load was not significantly different from the controls (no clinical signs were observed on any of the

controls). No deformities were observed on any of the fish exposed at 13 weeks posthatch or older, regardless of dose. Age and dose also significantly interacted ($F = 10.35$, $P < 0.0001$), further indicating that the effects of dose on the presence of the clinical sign were not the same at all levels of age at exposure, as described above.

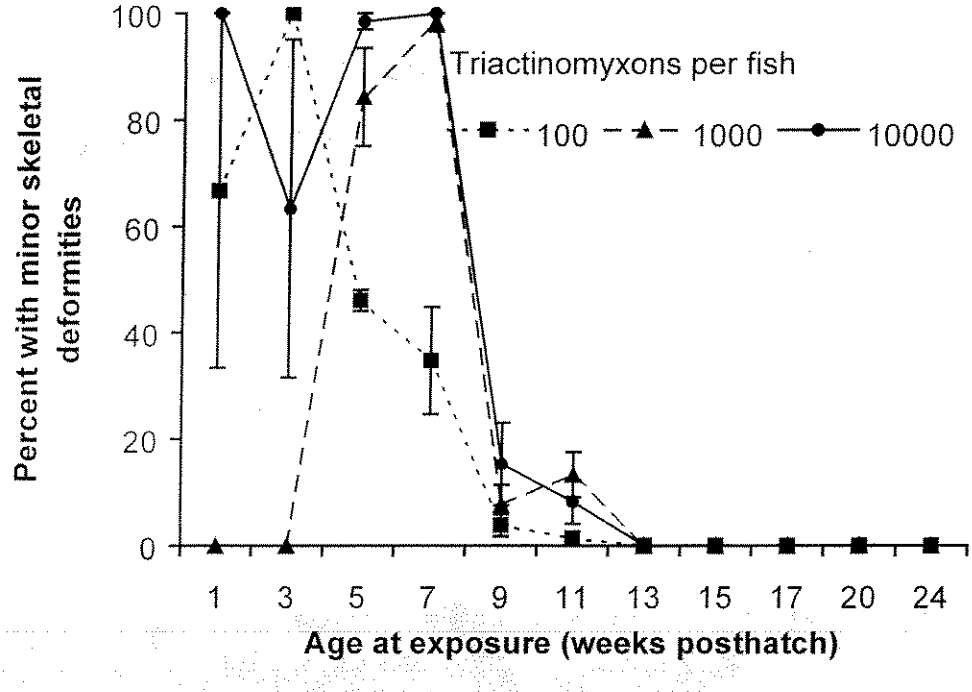


Figure 2.12—Mean (±SE) percent of rainbow trout with minor skeletal deformities at 33 weeks posthatch, exposed at varying ages to 100, 1,000 or 10,000 triactinomyxons of *Myxobolus cerebralis* per fish.

Whirling behaviour was the least prevalent of all the clinical signs recorded. The highest prevalence of whirling behaviour was less than 10% (Figure 2.13). However, age and dose were both found to significantly affect its prevalence ($F = 3.28$, $P = 0.0013$ and $F = 2.73$, $P = 0.0496$, respectively for age and dose); no significant interaction was present ($F = 1.37$, $P = 0.1352$). Significant differences in the prevalence of whirling were

only present among the different age groups when exposed to either 1,000 or 10,000 triactinomyxons per fish. Age did not significantly affect the prevalence when exposed at 9 weeks posthatch or older when exposed at 1,000 triactinomyxons per fish, and no differences were present among the fish exposed at 5 weeks posthatch or older at 10,000 triactinomyxons per fish. Whirling bahaviour was not observed among any of the fish exposed at 1 week posthatch. Parasite dose only influenced the prevalence of whirling bahaviour in fish exposed at 3, 5 or 7 weeks posthatch with increasing dose giving a general pattern of increasing prevalence. No whirling bahaviour was observed with fish exposed at 9 weeks posthatch or older.

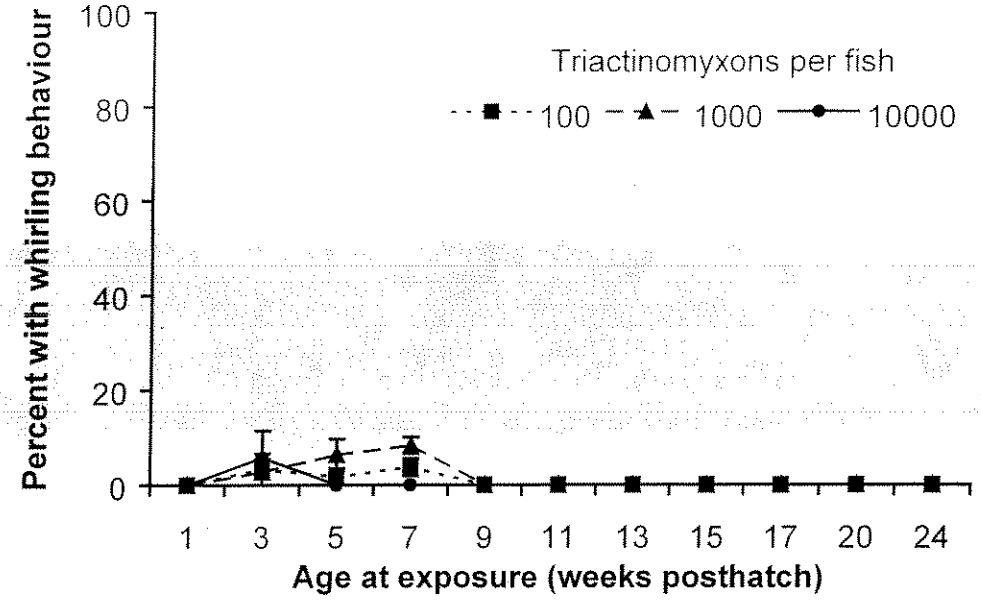


Figure 2.13—Mean (±SE) percent of rainbow trout with whirling behaviour at 33 weeks posthatch, exposed at varying ages to 100, 1,000 or 10,000 triactinomyxons of *Myxobolus cerebralis* per fish.

Discussion

The development of whirling disease in rainbow trout was dependent on both the age of fish at first exposure to *M. cerebralis* triactinomyxons and on the dose of triactinomyxons to which the fish were exposed. Hypotheses 1.1 and 1.2 of this dissertation were therefore rejected. Mortality, the presence of clinical signs, spore counts, and microscopic pathology decreased with increasing age of fish at exposure and decreasing level of parasite dose. These findings confirm what others had previously demonstrated about the relationship of fish age at first exposure to the parasite (Hoffman 1961; O'Grodnick 1979; Wolf 1986; Lom 1987; Markiw 1991; Markiw 1992a; Thompson et al. 1999), and the effects of parasite dose on the development of the disease (Hoffman 1974; Hoffman 1976; O'Grodnick 1979; Wolf 1986; Markiw 1992a; Markiw 1992b; Thompson et al. 1999). Unlike earlier studies, this study not only demonstrated the effect of fish age at exposure and parasite dose on the development of whirling disease but also identified the age when rainbow trout become resistant to the development of the disease. Although this experiment demonstrated the age of rainbow trout at which resistance to whirling disease develops, whether this relationship was a factor of fish age or size could not be determined, as the ages and sizes of fish used in the experiment were significantly correlated. The second objective of this dissertation was developed to determine the effects of age versus size of rainbow trout at time of exposure to the parasite on the development of whirling disease. This objective was addressed in Chapter 3 of this dissertation.

An age threshold at which whirling disease does not develop in rainbow trout, i.e., the age at which rainbow trout become resistant to the development of the disease, could be determined by comparing the effect of parasite dose within each age group. For instance, if fish exposed to triactinomyxons had no difference in disease response when compared to control fish (those given 0 triactinomyxons per fish) then exposure to *M. cerebralis* at that particular age did not result in the development of that particular disease response. The age threshold for mortality was 7 weeks posthatch; i.e., groups of fish exposed to *M. cerebralis* triactinomyxons at 7 weeks posthatch or older had the same amount of mortality as if the fish had never been exposed to *M. cerebralis* triactinomyxons. The threshold was 9 weeks posthatch for swimming performance and clinical signs, and for *M. cerebralis* spore counts the threshold was 13 weeks posthatch. Although the threshold for *M. cerebralis* spores was 13 weeks posthatch, all fish exposed to 100 triactinomyxons at 9 weeks posthatch or older did not have significantly more spores than the control fish. Rainbow trout exposed at 9 weeks posthatch or older, regardless of dose, had a modal microscopic pathology grade of mild or lower. When all of the responses of whirling disease were considered, the effects of whirling disease on rainbow trout were substantially reduced, or the same as in rainbow trout not exposed to the pathogen, when exposed to the parasite at 9 weeks posthatch or older, as compared to fish exposed at younger ages. Rainbow trout reared in *M. cerebralis*-free waters for 9 weeks posthatch or longer, whether in the wild or in a hatchery situation, should therefore exhibit enhanced survival and swimming performance, and reduced prevalence of clinical signs, spore counts and severity of microscopic pathology compared to fish first exposed to the parasite at younger ages.

Clinical signs are quick and easy to measure indicators of whirling disease severity. However, not all clinical signs are accurate measures of disease severity. Aggregated clinical signs showed the same pattern of whirling disease severity as the other indices (survival, spores, etc.). However, this pattern was not as obvious when the signs were analysed individually. Blacktail was the best individual clinical sign. Its presence can be quickly and easily determined. No blacktail was observed among fish exposed at 9 weeks posthatch or older. Only minor skeletal deformities were observed among these fish and the prevalence of this sign in these fish was not significantly different from that of the controls, in which no clinical signs were observed. Therefore, using blacktail alone could result in the presence of clinical signs in some fish may be overlooked; however, the clinical signs that would be missed are not good indicators of disease severity. Major skeletal deformities were considered to be less useful than blacktail because the effects of the parasite dose on major skeletal deformities were only present in fish exposed at either 1 or 3 weeks posthatch. Whirling behaviour was the least useful of the signs as it failed to follow the same patterns of disease severity as shown by the other indices.

Understanding the factors that affect the pathogenesis of any pathogen is crucial to its control. The control of whirling disease in hatcheries has relied on rearing fry and fingerlings in parasite-free water for as long as possible before transfer into earthen ponds or into waters known to contain the parasite; however, required durations were unknown. Recommendations ranged from 4 to 8 months after hatch (5 to 13 cm in length) (Halliday 1976; Hoffman 1976; Schaperclaus 1991; Garden 1992), which were generally based on the untested premise that resistance to whirling disease increases as a function of

ossification of the skeleton. Based on my work, the recommended time that juvenile rainbow trout should be maintained in *M. cerebralis*-free waters to reduce the severity of whirling disease is 9 weeks after hatch, which is substantially shorter than the previous recommendations. Whether the rainbow trout being reared are for stocking into infected waters or are for food production, if the fish are maintained in *M. cerebralis*-free water for the first 9 weeks after hatch the production and survival of the fish will be greatly increased.

Knowing the age threshold at which rainbow trout become resistant to the development of whirling disease has important implications not only for hatchery management but also for the management of wild trout fisheries. Management strategies that take into account the time period rainbow trout are most susceptible to the development of whirling disease can be used to better control the pathogen and the disease it manifests. The situation is simple in hatcheries however, controlling the pathogen and the disease it manifests in the wild is more complicated. When and where rainbow trout spawn, when the fry leave the redd, where the fry rear, and triactinomyxon abundance both spatially and temporally, are all essential to any management strategy (Downing et al. 2002). Habitats, known to be *M. cerebralis*-free, and where rainbow trout spawn and fry rear, should be maintained and conserved to encourage the fish to rear in these areas, especially if later in their life history they are known to travel into areas where the parasite is present. Flushing flows, such as an increased release from a dam, may be used to dilute triactinomyxons, and reduce the likelihood of infection and disease during periods when high triactinomyxon abundances are known to coincide with the rearing of susceptible juvenile salmonids. Early spawning strains may also be

stocked in areas where emergence and rearing of susceptible juveniles currently coincides with times of high triactinomyxon abundance. Introducing early spawners into the system will result in fry hatching earlier and theoretically rearing and developing to an age that provides them with protection against the development of the disease before triactinomyxon abundances are at their highest. Alternatively, eggs could be collected and fertilized from adult rainbow trout returning to the spawning areas earlier than the average spawners in the population. The fry from these collections could subsequently be reared in a hatchery and stocked back into the system to increase the numbers of early spawners within the population. The fry of early spawning fish have a greater chance of survival; therefore, early spawning fish may also become dominant within the population through the process of natural selection.

Although rainbow trout exposed to the whirling disease pathogen at 9 weeks posthatch or older will be resistant to the development of the disease and show no signs of disease, they have the potential to be carriers of the pathogen. Fish with no *M. cerebralis* spores were present in all exposure groups exposed at 9 weeks posthatch or older; however, only the groups exposed to 100 triactinomyxons per fish at either 17 or 24 weeks posthatch had no fish present with any *M. cerebralis* spores. Therefore, the potential exists for fish exposed to the parasite at ages older than 9 weeks posthatch to be infected with the parasite and become carriers of the pathogen while displaying no adverse signs of the disease. However, the potential for fish to become carriers of the pathogen decreases with increasing age at exposure and also with decreasing levels of parasite exposure. Fish exposed at 13 weeks posthatch or older to 100 triactinomyxons per fish or less would have a low potential for becoming carriers of the pathogen. The

fish exposed at 24 weeks posthatch to 100 triactinomyxons per fish developed no *M. cerebralis* spores, it could therefore be hypothesized that the risk of being a carrier of the pathogen can be reduced to zero if the fish are exposed at 24 weeks posthatch or older to low levels of the disease. Therefore, if fisheries managers knew the age at which fish would be first exposed to the pathogen and the level of parasite exposure the fish would receive it would allow for the estimation of the risk of those fish becoming carriers for the pathogen. A means to measure the density of *M. cerebralis* triactinomyxons present in a body of water (the "tamometer") has been developed at the BFTC and Montana State University, Bozeman, Montana (H. Lukins, Montana Cooperative Fishery Research Unit, personal communication). How the triactinomyxon densities equate to whirling disease in fish would require sentinel fish exposures to be carried out at the same time as the density measurements are made. These fish could then be compared to those from a controlled laboratory exposure where fish were exposed to a range of parasite doses. Fisheries managers would thereby be able to use the tamometer to determine the densities of triactinomyxons present in a body of water and be able to determine if their fish exposed to that density of triactinomyxons would have the potential to become carriers of the pathogen. However, if the goal of the fisheries manager or hatchery manager is purely to produce rainbow trout with no adverse effects of whirling disease, this could be simply achieved by preventing the exposure of the fish to the parasite until 9 weeks posthatch or older.

The information gained from this work can be applied to the management and control of whirling disease in wild and cultured rainbow trout populations. However, it may be less applicable to the management of other salmonid species. The rainbow trout

is the principal salmonid host for the parasite and is therefore the species most commonly studied in whirling disease research. Most other salmonid species are susceptible to the effects of the disease also, but to varying degrees (MacConnell and Vincent 2002). The relationship of increasing age or size at exposure and decreasing risk of development of disease is likely also present amongst the other whirling disease susceptible salmonid species, but the threshold level when the fish become resistant to the development of the disease is likely to be different. The general principles demonstrated in my work (increasing age or size and decreasing levels of parasite dose result in a decrease in the effects of whirling disease) can be applied loosely to the management of any salmonid species in *M. cerebralis*-positive areas. However, managing a mixed salmonid population or a population other than rainbow trout in a positive area may require more specific information as to when the particular species being managed becomes resistant to the development of the disease. In addition, further studies should be carried out to ascertain whether the same age thresholds would occur when fish are exposed to continual exposures of triactinomyxons such as would be found in the wild.

Several areas of future investigation arose from this work which would be beneficial to the management of the disease in both the wild and in hatcheries. Future investigations involving exposures of fish to a wider range of parasite doses than used in this experiment could be very beneficial to the management and understanding of whirling disease. Increasing parasite dose at exposure produced an increase in disease severity in rainbow trout exposed at 9 weeks posthatch or younger. However, the relationship of increasing parasite dose and subsequent disease severity has not been systematically determined. An increase in parasite dose exposure increased the disease

severity in rainbow trout, but this was only demonstrated on a very broad sense using triactinomyxon doses of different orders of magnitude. For the precise assessment of the effects of smaller differences in parasite dose we would need to know in more detail the effects of increasing parasite dose on the development of whirling disease severity. A parasite dose is likely to exist at which increasing levels fail to increase whirling disease severity. For several of the responses and age groups used in this experiment, increasing the exposure level from 1,000 triactinomyxons per fish to 10,000 triactinomyxons per fish did not result in an increase in severity of the response. However, for some of the responses, an increase in disease severity occurred between exposure to 1,000 triactinomyxons per fish and 10,000 triactinomyxons per fish. Therefore, the maximum parasite dose threshold where increasing levels fail to increase whirling disease severity is probably greater than 1,000 triactinomyxons per fish, but less than 10,000 triactinomyxons per fish. Similarly, a minimum triactinomyxon dose below which whirling disease does not develop in the fish is likely to exist. My lowest dose (100 triactinomyxons per fish) induced whirling disease in rainbow trout exposed during the susceptible age range; therefore, the minimum threshold must be lower than 100 triactinomyxons per fish.

Further areas of investigation, which became apparent from this work, included a refining of the histological grading scale used for determining the severity of whirling disease. The most commonly used index of whirling disease severity, both in laboratory exposures and in sentinel fish exposures, is the histological pathology index of severity (as used in this experiment) (MacConnell-Baldwin scale, Hedrick et al. 1999b). Although this histology scale gives the investigator an index of microscopic pathology

severity, it does not provide the investigator with absolute information on the dose of triactinomyxons to which the fish were exposed. Only relative relations can be inferred. The MacConnell-Baldwin scale provides qualitative information on microscopic pathology severity. However, it does not provide quantitative information on how the different categories relate to disease severity. For instance, the MacConnell-Baldwin scale would suggest that all fish graded as "mild" on the scale have all the same level of disease severity which would imply that the fish in this experiment exposed at 5 weeks posthatch with a median grade of "mild" on the scale have the same level of whirling disease severity as the fish exposed at 17 weeks posthatch with a median grade of "mild". The evidence from the other responses of disease severity used in this experiment show that this is clearly untrue. An additional common mistake made in whirling disease research is to treat the MacConnell-Baldwin grades as if they were quantitative continuous data with grades commonly being reported as means. This is statistically incorrect because the scale is not technically continuous and the difference in pathology between the grades is not known to be equal in size. Therefore, a great need is present for this scale to be studied in more detail and to be calibrated so that the data can be treated more powerfully using parametric techniques, instead of the current situation where the data can only be correctly used as categorical data with non-parametric statistical tests.

The histological pathology index of severity is by far the most commonly used index of whirling disease severity; however, the results in comparable studies vary widely. Most commonly the histology categories are reported as mean numbers with none = 0, minimal = 1, mild = 2, moderate = 3, moderately severe = 4, and severe = 5. In

laboratory studies where rainbow trout were exposed at about 60 days posthatch to 1,000 to 2,000 triactinomyxons per fish, mean lesion scores were reported as 3.5 to 4.2 (Hedrick et al. 1999b), 4.2 to 5.0 (Sollid et al. 2002), 2.24 to 4.29 (Vincent 2002), 1.8 (Hedrick et al. 1999a), and in my study the mode for comparable fish was 2. Some of this variability can be explained by the use of different strains of fish and may also be explained by differences in histology or grading technique. Significant differences in grading existed among histologists provided with identical slides (George Schisler, Colorado Cooperative Fish and Wildlife Research Unit, presented at the 1999 Whirling Disease Symposium, Fort Collins, Colorado). Direct comparisons of histological grading among different studies could be misleading unless the histology and grading techniques are standardized among researchers. Therefore, it is important to use other indices of whirling disease severity in addition to histological grading, such as those used in this study, and it is critical to standardize the techniques used within an experiment to ensure that the histology grading is comparable among experimental groups. Although the microscopic pathology results from my study do not match exactly those of similar studies, the techniques were standardized within the study and all slides were graded relative to the others so that comparisons could be made among the treatments. Higher microscopic pathology grades may have been expected among the two youngest groups, but the low scores may have been caused by high mortality. The most severely diseased fish likely died before the conclusion of the experiment.

One, or a combination of physiological factors at 9 weeks posthatch (raised at 12 °C), provides rainbow trout with protection against the development of whirling disease after exposure to *M. cerebralis* triactinomyxons. Whether in the wild or in a hatchery

situation, rainbow trout maintained in *M. cerebralis*-free water for at least 9 weeks posthatch will have a significant resistance to the development of whirling disease after they are exposed to the parasite.

The development of resistance with increasing age or size at exposure has generally been thought to be a result of increasing ossification of the skeleton, although this hypothesis has never been tested. *Myxobolus cerebralis* primarily targets fish cartilage (El-Matbouli et al. 1992). The trophozoites of the parasite digest cartilage and destroy the structural framework needed for healthy bone formation, leaving fish with permanent disfiguration. The abundant cartilage in the skeleton of young trout is thought to render them highly susceptible to the effects of the disease (El-Matbouli et al. 1992). It can be hypothesized that at 9 weeks posthatch the rainbow trout skeleton has become ossified sufficiently to provide the fish with protection against the parasite. The significant reductions in clinical signs, microscopic pathology and spore counts in fish exposed for the first time after 9 weeks posthatch, when compared to fish exposed at younger ages, maybe attributable to increased skeletal ossification and reduced amounts of available cartilage. Rainbow trout exposed at 9 weeks posthatch or older also had increased swimming performance. If the fish are less damaged structurally and under less stress caused by the effects of the disease they will be able to perform better. The increased survival found in fish exposed at 9 weeks posthatch or older can be attributed to a combination of all of the other factors. A healthy fish will be less stressed, will not be compromised by structural damage, and will have good swimming performance. These factors in combination will elicit increased performance and survival. The role of

skeletal ossification in the development of whirling disease in rainbow trout was studied in more detail in Chapter 3 of this dissertation.

Exposure to an infectious microorganism does not necessarily result in infection or the manifestation of clinical disease. The occurrence of disease results from a series of complex interactions of the host, pathogen and the environment (Snieszko 1973). These complex interactions have to be understood to determine if an interaction between host and pathogen will result in disease. We now know that the age of the host is critical for the development of whirling disease, as is the density of the pathogen to which the host is exposed, and that both of these factors significantly interact in such a way that the effect of age on the development of the disease is not the same at all levels of parasite dose, and vice versa.

Resistance to a pathogen can be caused by several different factors: penetration of the host may be reduced, there maybe an induced mechanism such as antibody or interferon production, or the pathogen may be inactivated by serum components, phagocytic cells, acute-phase proteins, or killer cells (Chevassus and Dorson 1990). Besides the increase in ossified skeleton found in rainbow trout as they age or grow, immune responses may also be present in the fish that could provide them with protection against the development of the disease. Rainbow trout do develop a humoral (antibody) and cellular immune response to the whirling disease pathogen (Hedrick et al. 1998). However, an active cellular immune response is not evident in the fish until after significant cartilage damage has occurred (Hedrick et al. 1998) and specific anti-*M. cerebralis* antibodies are not present until 12 weeks after exposure to the parasite (Chapter 4). The induction of cellular or humoral immune responses can therefore not be

responsible for providing the fish with an increased resistance against development of the disease with increasing age because the cellular response is not induced until after damage has occurred, and the humoral response takes too long after infection to occur to provide the fish with protection. However, there maybe other non-specific immune mechanisms that rainbow trout develop with time that could provide them with resistance against development of the disease. The immune response of rainbow trout to *M. cerebralis* was studied in more detail in Chapter 4 of this dissertation.

Another possible explanation for increased resistance to the development of whirling disease with increasing age at exposure is related to the damage the parasite causes to the nervous system of its host. Significant neuropathology is associated with the presence of whirling disease in rainbow trout (Rose et al. 2000). Younger fish at first exposure would have a less mature nervous system that would be more vulnerable to dysfunction caused by the whirling disease pathogen (Rose et al. 2000). Therefore, development of a more mature and well-developed nervous system may coincide with the development of resistance to whirling disease.

Several examples exist in human medicine where age is a critical factor for the determination of disease development. Infants and toddlers are more susceptible than older children to many infectious agents because of limited immune defenses or because of physiologic or anatomic factors (Klein 1986). For example, the development of otitis media (inflammation or infection of the middle ear) is most common in infancy, which can be related to the development of the eustachian tube. In infancy, nasopharyngeal secretions readily reach the middle ear because the eustachian tube is short, wide, and horizontal. By school age, the tube has elongated, narrowed, and is oblique;

nasopharyngeal secretions then have limited access to the middle ear (Teele et al. 1980). Infants in their first month of life are also particularly predisposed to bacterial meningitis, which is related to their immature immune systems and also anatomical ease of infection, one route being through the eustachian tube and middle ear infections (Linton 1982; Mandell et al. 1995). In human infectious diseases it is rare for an individual infected early in life to contract the same disease a second time because of the immunity established by the previous infections. The immune response of rainbow trout to *M. cerebralis*, and the protection it provides is covered in detail in Chapter 4. However, because rainbow trout are only susceptible to the effects of whirling disease during a relatively short time frame, in addition to being resistant to subsequent exposure because of an acquired immune response, they will also be resistant to the development of the disease because of their physiological resistance that develops after 9 weeks posthatch.

Myxobolus cerebralis has a complex two-host life cycle. Control and management of the parasite and the disease it manifests will only be achieved after complete understanding of the processes involved in the life cycle. Although in North America the rainbow trout is considered to be the primary host of *M. cerebralis* (MacConnell and Vincent 2002), the rainbow trout was not the primary host that the parasite evolved with. *Myxobolus cerebralis* is endemic to Eurasia (Hoffman 1970; Hoffman 1990) and is thought to have co-evolved with the European brown trout (Hoffman 1970). Brown trout show a greater resistance to the development of whirling disease than rainbow trout and will only develop the disease when exposed to high triactinomyxon densities (Hedrick et al. 1999a). Whirling disease has resulted in significant declines in wild rainbow trout populations in the Madison River, Montana

(Vincent 1996), and in the Colorado River, Colorado (Nehring and Walker 1996). However, in the Madison River no declines were reported in the brown trout population (Vincent 1996). The mechanisms by which brown trout are more resistant to the development of the disease than rainbow trout have yet to be identified. Other explanations focus more on the immune response of the brown trout providing them with protection. Resistant salmonid species may be more effective at immune recognition and destruction of *M. cerebralis* (MacConnell and Vincent 2002). However, current knowledge on the immune response to *M. cerebralis* in the salmonid host is limited. Microscopic lesions in brown trout contain more multinucleated giant cells than rainbow trout and rarely progress beyond small, discrete foci (Baldwin et al. 2000), whereas, rainbow trout mount an extensive cell-mediated response that contributes to disease. Additionally, parasites and lesions in rainbow trout are found in cartilage throughout the body but are consistently located in the cranial regions, primarily the ventral calvarium. In contrast, infections in brown trout most commonly occur in gill arches and rarely in the ventral calvarium (Hedrick et al. 1999a; Baldwin et al. 2000).

In addition to physiological and immunological differences that occur between brown trout and rainbow trout, life history attributes may contribute to differences in disease susceptibility. The most critical period for salmonids in whirling disease-positive areas is the time between hatching and emergence when the fish are most susceptible to the disease (MacConnell and Vincent 2002). Rainbow trout have evolved to spawn during the spring season, stimulated by rising water temperatures, and hatch in early summer (Behnke 1992), whereas brown trout spawn in the autumn and hatch in late winter to early spring (Hunter 1991). The release of the triactinomyxon stage of *M.*

cerebralis in the wild has a seasonal pattern, with the highest density of parasites occurring during June through September (Thompson and Nehring 2000, Downing et al. 2002). Therefore, it would be expected that in comparison to brown trout, rainbow trout would be more susceptible to the effects of whirling disease in the wild because their hatching and emergence generally coincides with the periods of high triactinomyxon densities in the wild.

Because rainbow trout are a relatively new host of *M. cerebralis* it can be hypothesized that over time rainbow trout will also evolve with the parasite to become more resistant to the effects of the disease. This may occur through physiological and or immunological changes, or it may result from shifts in life history strategies to produce populations that are better able to co-exist with the parasite. Rainbow trout most susceptible to the effects of whirling disease are those that rear in areas where triactinomyxon abundances are high. In these areas, selection pressure may result in changes of spawning timing so hatching and emergence occur when the triactinomyxon abundances are not at their peak. Alternatively, rainbow trout that spawn in areas where triactinomyxon abundances are not at levels lethal to their young may be selected for. Rainbow trout with an innate immunity towards the parasite may likewise be selected for. Recently, rainbow trout strains in Europe have been identified as being more resistant to whirling disease than rainbow trout strains from North America (Mansour El-Matbouli, University of Munich, personal communication). These "resistant' rainbow trout from Germany, although originally imported from North America, have been raised in Germany in *M. cerebralis*-positive waters for up to 110 years. *Myxobolus cerebralis* has been present in North America for about 50 years and in the Rocky Mountain States for

less than 10 to 15 years. If given another 100 years, the rainbow trout in the Rocky Mountains may also become resistant to the disease; however population reductions may continue to occur as seen in the Madison River, Montana, if no management strategies are taken to prevent such losses.

We can only speculate as to what will happen with the *M. cerebralis*-rainbow trout relationship over time. As scientists and fisheries managers, we have two options; we can either let nature take its course and let the equilibrium develop between *M. cerebralis* and rainbow trout, or we can intervene and try to manage and control the relationship. If our goal is to preserve the fisheries resource and to limit the effects of the disease as much as possible, then we should intervene and use the information we have on the relationships between host and pathogen to better control the parasite and the disease it manifests.

CHAPTER 3

OBJECTIVE 2: EFFECTS OF AGE VERSUS SIZE AT TIME OF EXPOSURE ON THE DEVELOPMENT OF WHIRLING DISEASE IN RAINBOW TROUT

Introduction

The development and severity of whirling disease pathology in salmonids has long been known to be dependent generally on the age, or size, of fish when first exposed to the triactinomyxon spores of *Myxobolus cerebralis* (Hoffman 1961; O'Grodnick 1979; Wolf 1986; Lom 1987; Markiw 1991;Markiw 1992a, Thompson et al. 1999). The intensity of whirling disease decreases with increasing size (O'Grodnick 1979), or age (Markiw 1992a), and trout exposed to the parasite with larger mean weights will have better survival (Thompson et al. 1999). Hoffman (1961), Wolf (1986), and Lom (1987) suggested that fish older than 6 months, or larger than the fingerling stage, would not become diseased. The youngest age at which trout can become infected with the parasite is 2 days posthatch (Markiw 1991). No studies, prior to my dissertation work described in Chapter 2, had been conducted to systematically determine the age of trout when they become resistant to the effects of the parasite. Consequently, effective management of wild salmonid fisheries in whirling disease-positive areas has been prevented because fisheries managers do not know when resistance to the disease develops in fish and thus, do not know how long trout must rear in *M. cerebralis*-free waters to reduce or eliminate the effects of the disease. Similarly, the control of whirling disease in hatcheries has relied on rearing fry and fingerlings in parasite-free water for as long as possible before

transfer into earthen ponds or into waters known to contain the parasite, but required durations were unknown. Recommendations ranged from 4 to 8 months after hatch (5 to 13 cm in length) (Halliday 1976; Hoffman 1976; Schaperclaus 1991; Garden 1992), which were generally based on the untested premise that resistance to whirling disease increases as a function of ossification of the skeleton. Narrowing this range of recommended rearing times of rainbow trout in parasite-free water would make for better management of rainbow trout in whirling disease-positive areas, whether in the wild or in hatcheries.

I systematically demonstrated in Chapter 2 (objective 1) that rainbow trout become resistant to the development of whirling disease at 9 weeks posthatch. However, the ages and sizes of fish used in that experiment were significantly correlated. Therefore, I could not determine whether the development of resistance with increasing age was a factor of age or size of the fish at exposure, or perhaps a co-variate of one or both, such as skeletal ossification. Therefore, my second objective was to determine whether age or size is more important in the development of resistance to the disease. Rainbow trout of same size but different age, and same age but different size, were exposed to the parasite. Such determination is critical for effective management and control of whirling disease, as is determination of the specific age or size at which resistance is conferred. If age were the more critical factor for determining when fish become resistant, fisheries managers would then know that if the fish were maintained in parasite-free water for their first 9 weeks after hatch (see Chapter 2) that their chances of developing whirling disease would be greatly decreased. However, if size is more important than the temporal age of fish, then a smaller fish 9 weeks after hatch would be

more susceptible to development of the disease than one of the same age but larger size. The duration, or size, to which a fish must be maintained in parasite-free water can therefore only be specified if it is known whether the age or the size of the fish at first exposure to the parasite is more critical for the development of the disease.

Myxobolus cerebralis primarily targets fish cartilage (El-Matbouli et al. 1992). After penetration of the host epidermis, the parasite reaches the cartilage via peripheral nerves and the central nervous system (El-Matbouli et al. 1995). The trophozoites of the parasite digest cartilage and destroy the structural framework needed for healthy bone formation, leaving fish permanently disfigured. The abundant cartilage in the skeleton of young trout is thought to render them highly susceptible to the effects of the disease (El-Matbouli et al. 1992). However, the hypothesis that resistance to whirling disease increases directly as a function of ossification of the skeleton has never been tested. In addition to determining whether the age or size of rainbow trout is more important for the development of resistance to whirling disease, I compared the level of skeletal ossification in rainbow trout at time of exposure to the subsequent severity of whirling disease. Determination of the factors responsible for why age or size influences the development of resistance to whirling disease in rainbow trout may enhance our understanding of this parasite, and therefore aid in the management of wild and hatchery fish in whirling disease-positive areas.

Methods

Whirling disease severity was compared in rainbow trout first exposed at different combinations of age and size to determine which factor was more influential in enhancing development of resistance to the disease. The effect of age was determined by exposing fish of the same size but different ages to the parasite. Similarly, size was evaluated by exposing rainbow trout of the same age but different sizes. These combinations were achieved by rearing fish at different water temperatures prior to exposure. Whirling disease severity was measured using mortality, swimming performance, clinical signs, microscopic pathology, and spore counts as response variables.

Experimental Procedures

Myxobolus cerebralis triactinomyxons were produced in the laboratory. Cultures of *Tubifex tubifex* worms were exposed to myxospores of *M. cerebralis,* and triactinomyxons were collected using a procedure similar to that described by Hedrick et al. (1999b).

Erwin strain rainbow trout were hatched from eggs supplied by the Ennis National Fish Hatchery, United States Fish and Wildlife Service, Ennis, Montana, and were maintained at the Bozeman Fish Technology Center, Bozeman, Montana, until exposure. After hatch, the fish were reared at one of three different water temperatures (9.3, 12.0, or 15.4 °C) to produce three groups of fish with different growth rates and therefore different sizes at the same age. The fish were exposed at 7 or 9 weeks posthatch.

Exposures at each of the six age-size combinations (Table 3.1) included a control exposure (three replicates) and one exposure to 1,000 triactinomyxons per fish (three replicates). Each replicate included 40 fish.

Table 3.1. Lengths and degree-days of development at exposure for fish reared at different temperatures. No significant difference existed between mean lengths of fish of different ages with the same degree-days of development (588 or 756 degree-days).

Temperature (°C)	Age at exposure (weeks posthatch)	Degree-days at exposure	Mean (±SE) fork length at exposure (mm)
9.3	7	456	28 ± 0.99
	9	588	36 ± 0.47
12	7	588	36 ± 0.83
	9	756	40 ± 0.64
15.4	7	756	40 ± 0.53
	9	970	46 ± 0.69

Exposures were conducted at the Wild Trout Research Laboratory (WTRL), Montana State University, Bozeman, Montana. Each lot was exposed to *M. cerebralis* triactinomyxons in aerated 5-liter exposure chambers for 2 hours. Control replicates were subjected to a sham exposure. Following exposure, lots were maintained separately at 13 °C in 38-liter glass aquaria supplied with filtered and oxygenated water by a

recirculating process system at the WTRL. The fish were fed a commercial trout diet at 2 to 3 percent body weight per day. Mortalities were counted and removed daily.

The swimming stamina of individual fish was tested 20 weeks after exposure in a stamina tunnel (Figure 2.1). Swimming stamina was measured as the length of time a fish could maintain its position in the tunnel at a constant water velocity (35cm/s). The times to fatigue were recorded for nine randomly selected fish from each replicate.

The fish used in the stamina test and the remaining fish from the replicates were examined for clinical signs typical of whirling disease (blacktail, skeletal and cranial deformities, and whirling behavior), euthanatized and their fork lengths (mm) were measured. The heads of the euthanatized fish from the stamina test were removed, cut in half along the midsagittal plane, preserved in Davidson's fixative, and prepared for microscopic examination using standard histological techniques. From each fish, one head half and the tail were prepared for histology. Microscopic pathology was categorized according to Hedrick et al. (1999b), in which cartilaginous tissue was examined for the presence of the parasite and associated lesions. The abundance of parasites, cartilage damage, inflammation, extent of lesions, involvement of other tissues, and bone distortion were evaluated and categorized into one of six categories: no infection, minimal, mild, moderate, moderately severe or severe (E. MacConnell, United States Fish and Wildlife Service, personal communication, Table 2.2). Two head sections and two tail sections were evaluated histologically for each fish collected.

The other halves of the heads were used to obtain spore counts. The standard plankton centrifuge method (O'Grodnick 1975) was used for spore extraction. After extraction, spores were resuspended in a known volume of deionized water, and 1-ml

aliquots were placed on both sides of a standard 1-ml hemocytometer counting chamber. Total spores per original head were calculated as follows: (2 × total number of spores counted × 10^4 × volume of suspension) / (number of 1-mm^2 areas counted). Three counts of spores were made from each suspended sample; the mean of the three was used in analyses.

Bias was reduced throughout the experiment wherever possible. Fish were randomly assigned to lots and lots were assigned to tanks randomly. Samples were collected randomly from the tanks for swimming performance tests, histology, and spore counts. Histology slides and spore samples were examined blindly and in random order. The exposure designation of each sample was not determined until all samples had been examined and recorded.

The percentage of cartilage at time of exposure was estimated in 10 randomly selected fish from each age-size group. The fish were euthanatized, fixed in 10 % buffered formalin and preserved in 75 % ethanol. Cartilage and bone were differentially stained using alcian blue and alizarin red S. My protocol was adapted from those described by Webb and Byrd (1994), Fritzsche and Johnson (1980), and Song and Parenti (1995) and allowed for easy identification of cartilage and ossified bone structures after staining. Both eyes were removed and discarded before staining. A midsagittal cut was made along the length of the head and the left side of the head was removed; both sides were retained for staining. The head was cut in half to allow for better identification of the skeletal structures within both halves. All entrails and the operculum on the right side of the head were removed and discarded. Removing the operculum allowed more light to

pass through the specimen after staining to allow for better definition of the skeletal structures.

Specimens were washed in several changes of de-ionized water to remove all traces of the fixative. The cartilage was stained using an alcian blue solution (10 mg alcian blue 8GN, 80 ml 95 % ethanol, and 20 ml glacial acetic acid) for 48 hours. After cartilage staining, the specimens were rehydrated using two 2-hour immersions in 95 % ethanol followed by one immersion each of 75 %, 50 %, and 30 % ethanol, and finally to de-ionized water until the specific gravity of the specimens exceeded 1.0. Trypsin in a 30 % sodium borate buffer (1 g trypsin, 30 ml saturated aqueous sodium borate, 70 ml de-ionized water) was used to digest (clear) muscle. The specimens in the trypsin solution were placed on a warming tray at 30 °C for 4 to 15 days, depending on the size of the specimens. Frequent observation of the specimens during this period was critical to avoid over-digestion. Digestion was considered complete when light could pass freely through the fish and stained cartilaginous structures could be seen clearly. The specimens were then placed in 0.5 % aqueous potassium hydroxide for 1 hour to wash away any remaining enzyme and also to build a potassium hydroxide environment to help the alizarin red S penetrate bone. The specimens were placed in an alizarin red S solution for 18 hours to stain ossified bone. The solution was made by adding alizarin red S powder to 0.5 % potassium hydroxide until the solution turned purple. After staining, the specimens were placed in 0.5 % potassium hydroxide with 2 drops of hydrogen peroxide for 30 minutes for a final clearing. The specimens were placed in successive solutions of 30 %, 50 %, and 70 % ethanol for 30 minutes in each to dehydrate them and prepare them for storage. The specimens were left in 0.5 % potassium hydroxide overnight and then

into 30 % glycerol (diluted with 0.5 % potassium hydroxide) followed by 70 % glycerol until the specific gravity of the specimens exceeded 1.0. The specimens were stored in 100% glycerol.

A digital image of each fish (Figure 3.1) was processed using image analysis software (Image-Pro Express 4.0) to calculate the proportions of cartilage and bone present in the fish skeleton. Each digital image was broken into 20 to 30 smaller pieces to allow for more accurate analysis of the images. In each of the smaller images regions of the skeleton were designated as blue (cartilage) or red (ossified bone) and the area of each color was calculated using the image analysis software. This procedure allowed the ratio of cartilage to bone in the whole fish skeleton to be calculated.

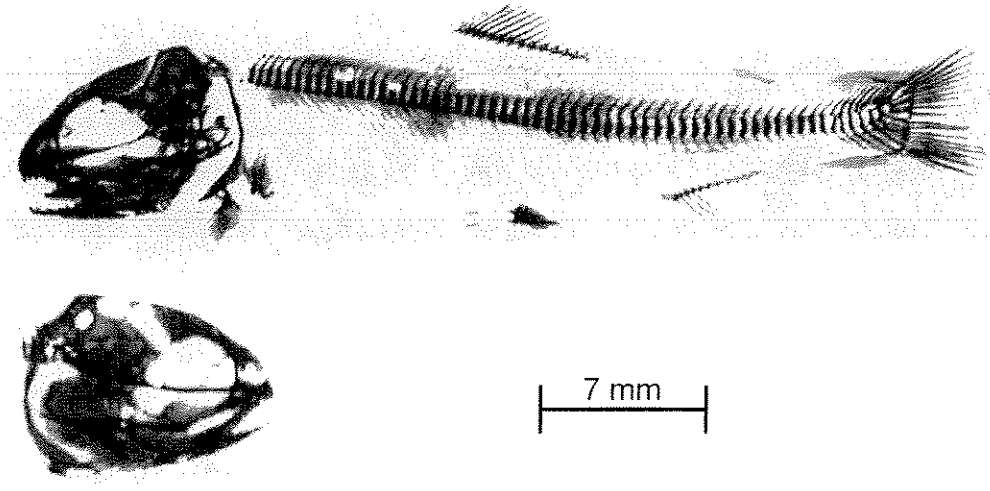


Figure 3.1—Rainbow trout differentially stained using alcian blue and alizarin red S. Cartilage is blue and ossified bone is red.

Statistical Analyses

The experiment was designed and analyzed as a three-way factorial. The three factors, or treatments, were age of fish at exposure (7 or 9 weeks posthatch), size at exposure (28, 36, 40, or 46 mm) and parasite dose (0 or 1,000 triactinomyxons per fish). Size of fish is designated as the mean forklength for the group. Forklength was used to describe the size of fish instead of degree-days as the latter was considered more a measure of age and period of development rather than actual size of fish. The number of mortalities, percent of fish with clinical signs, spore counts, swimming performance and microscopic pathology were compared among the 12 treatment groups (i.e., six age-size combinations and two parasite dose levels). All responses were treated parametrically with the exception of microscopic pathology.

Spore counts and swimming performance were analyzed with random factors included in the model for tank and fish, and the fish were treated as the experimental unit. Mortality and clinical signs were analyzed in the same way as spore counts and swimming performance, with the exceptions that tanks were the experimental units and no factor for fish was included in the model. A mixed linear model was used that combined both the fixed (age and size of fish at exposure and parasite dose) and random (tank and fish) effects. Type 3 *F*-statistics were used (Montgomery 1997). ANOVA tables are in Appendix B. The important assumptions supporting this analysis are that the data are normally distributed and that they are independent with constant variance. Visual inspection of residual plots of data for all responses confirmed that these assumptions were met. The units of measure (fish or tank) were not independent, however, this assumption could be dropped by modeling statistical correlation into the

analysis, which assumes constant variance and constant covariance (PROC MIXED, compound symmetry covariance option; Littell et al. 1996). The model used for the analysis was the following:

$$y_{ijklmn} = \mu + \alpha_i + \beta_j + \tau_k + (\alpha\beta)_{ij} + (\alpha\tau)_{ik} + (\beta\tau)_{jk} + (\alpha\beta\tau)_{ijk} + \gamma_{l(ijk)} + \delta_{m(ijkl)} + \varepsilon_{ijklmn},$$

where

$i = 1,\ldots,a,$

$j = 1,\ldots,b,$

$k = 1,\ldots,c,$

$l = 1,\ldots,d,$

$m = 1,\ldots,e,$

$n = 1,\ldots,f,$

and

μ = the overall mean,

α_i = the effect of the *i*th level of the fixed factor A (age of fish at exposure),

β_j = the effect of the *j*th level of the fixed factor B (size of fish at exposure),

τ_k = the effect of the *k*th level of the fixed factor C (parasite dose),

$(\alpha\beta)_{ij}$ = the interaction effect between the *i*th level of factor A and the *j*th level of factor B,

$(\alpha\tau)_{ik}$ = the interaction effect between the *i*th level of factor A and the *k*th level of factor C,

$(\beta\tau)_{jk}$ = the interaction effect between the *j*th level of factor B and the *k*th level of factor C,

$(\alpha\beta\tau)_{ijk}$ = the interaction effect between the *i*th level of factor A, *j*th level of factor B and *k*th level of factor C,

$\gamma_{l(ijk)}$ = the effect of the *l*th level of the random factor D (tank nested in factors A, B, and C),

$\delta_{m(ijkl)}$ = the effect of the *m*th level of the random factor E (fish nested in factors A-D; this effect is not included in the model when analyzing the mortality or clinical signs response), and

ε_{ijklmn} = a random error caused by sampling.

Bonferroni's multiple comparison procedure was used to compare all pairwise differences of the least-square means. For each significance test, $\alpha = 0.05$. The percentage of cartilage in the skeleton was compared among the six age-size combinations as a two-way factorial. Cartilage percentage was analyzed in the same way as the other parametric responses with the exception that the only treatments were age and size of the fish.

Pearson correlation coefficients were calculated for size of fish at the end of the experiment (fork length, mm) and response variable (swimming performance and spore counts), to determine if the size of individual fish within a treatment influenced the development of whirling disease after exposure to *M. cerebralis*. This assumes that all fish within a treatment are growing at the same rate.

The chi-square test of homogeneity was used to determine whether age or size at exposure significantly affected microscopic pathology (Daniel 1990). This non-parametric procedure was used because these data were categorical. All statistical analyses were conducted with the statistical software program SAS/STAT (SAS Institute 1996).

Results

Cumulative Mortality

The age (weeks posthatch) of rainbow trout at first exposure, size (length) at first exposure and level of triactinomyxon dose did not significantly affect the cumulative mortality of fish at 20 weeks after exposure (age $F = 1.56$, $P = 0.2236$; size $F = 0.59$, $P = 0.6249$; dose $F = 3.34$, $P = 0.0802$, Figure 3.2).

Swimming Performance

Size (length) at exposure did not significantly affect the swimming performance of the fish ($F = 0.14$, $P = 0.9338$; Figure 3.3), but significant differences in swimming performance did exist among groups exposed at different ages (weeks posthatch) ($F = 5.20$, $P = 0.0318$; Figure 3.3). Swimming performance only increased with increasing age at exposure for fish exposed at 40 mm fork length, and not for those exposed at 36 mm fork length. Swimming performance was also significantly affected by the dose of triactinomyxons (either 0 or 1,000 triactinomyxons per fish) to which the fish were

exposed ($F = 18.82$, $P = 0.0002$; Figure 3.3); exposure to 1,000 triactinomyxons per fish significantly reduced swimming performance when compared to that of the controls.

No significant correlations were present between size of fish at the end of the experiment and swimming performance within any of the treatment groups (Figure 3.4 and Figure 3.5). Assuming that all fish within a treatment grew at an equal rate after exposure to *M. cerebralis*, the size of the fish at exposure, within a treatment group, does not influence their swimming performance.

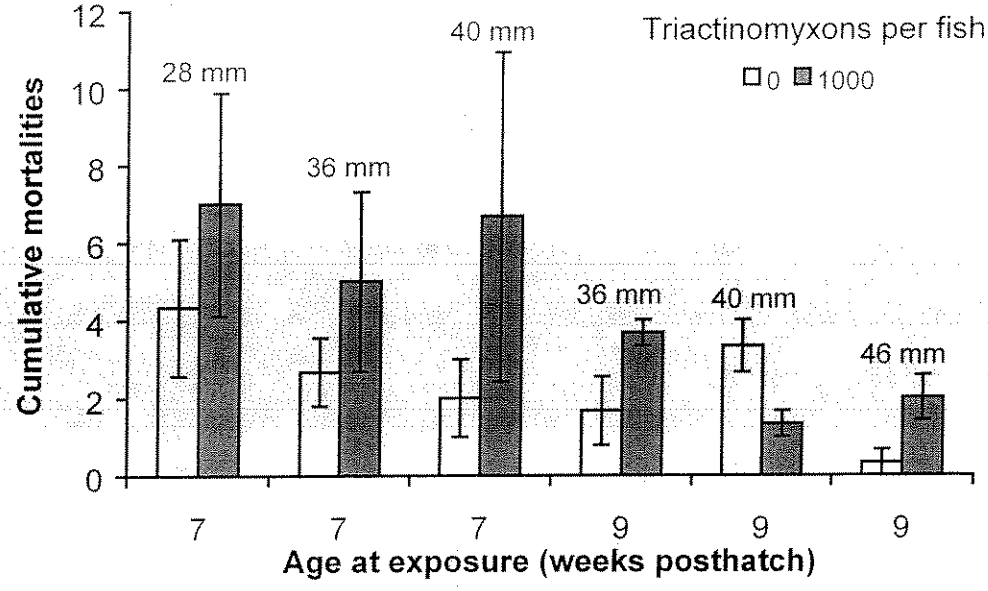


Figure 3.2—Mean (±SE) number of mortalities of rainbow trout at 20 weeks after exposure, exposed at different ages and sizes to either 0 or 1,000 *Myxobolus cerebralis* triactinomyxons per fish. Numbers above bars represent average fork lengths of fish at exposure.

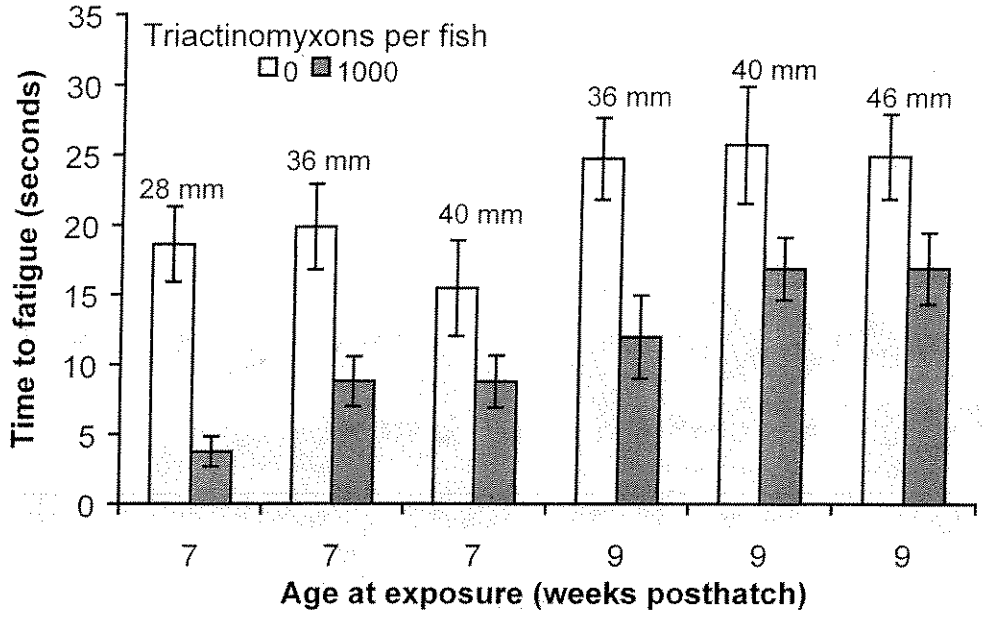


Figure 3.3—Mean (±SE) time to fatigue of rainbow trout at a water velocity of 35 cm/s 20 weeks after exposure, exposed at different ages and sizes to either 0 or 1,000 *Myxobolus cerebralis* triactinomyxons per fish. Numbers above bars represent average fork lengths of fish at exposure.

Clinical Signs

None of the control fish exposed to 0 triactinomyxons per fish exhibited clinical signs of whirling disease, but clinical signs were present among all groups exposed to 1,000 triactinomyxons per fish. Therefore, dose of triactinomyxons to which the fish were exposed significantly affected the percent of fish with clinical signs at 20 weeks after exposure ($F = 405.96$, $P < 0.0001$). All groups exposed to 1,000 triactinomyxons per fish had a higher percentage of fish with clinical signs than the control groups.

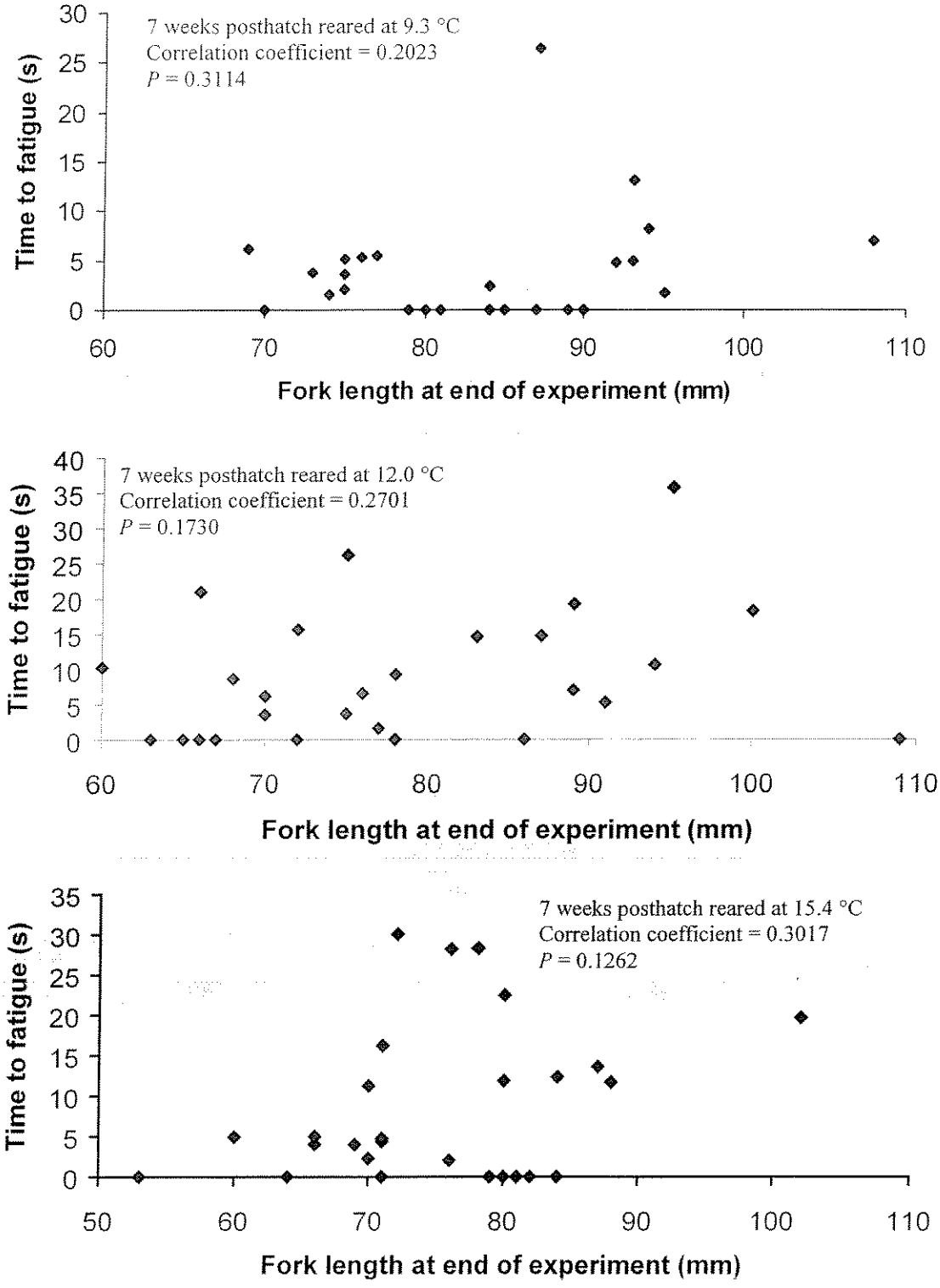


Figure 3.4—Scatter plots of time to fatigue and fork length of fish at the end of the experiment for each of the three groups exposed at 7 weeks posthatch.

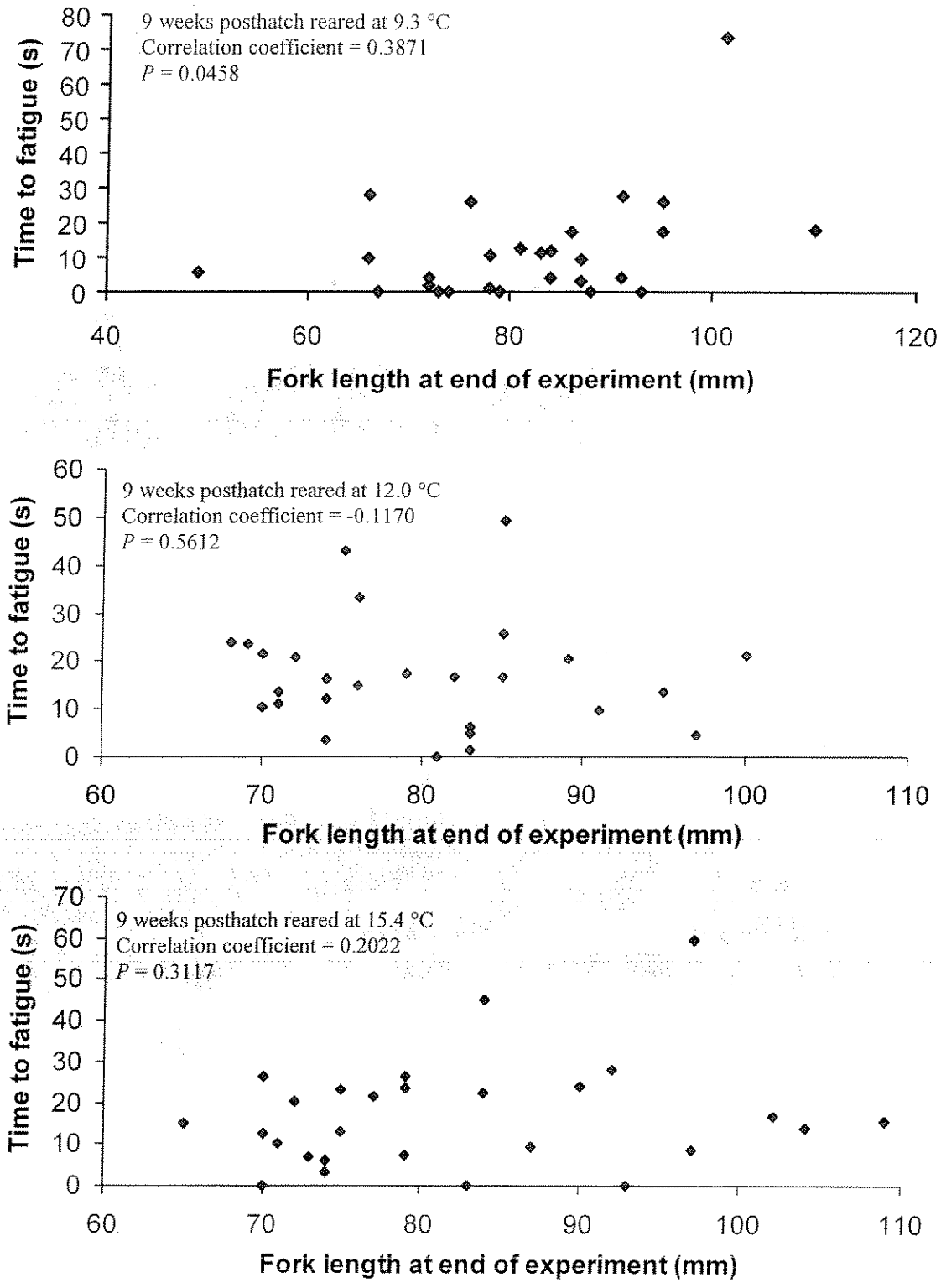


Figure 3.5—Scatter plots of time to fatigue and fork length of fish at the end of the experiment for each of the three groups exposed at 9 weeks posthatch.

Significant differences in the percent of fish with clinical signs existed among groups exposed at different ages (weeks posthatch) ($F = 23.10$, $P < 0.0001$; Figure 3.6). Increased age at exposure decreased the percent of fish with clinical signs when exposed at 40 mm fork length; however, no significant difference existed in the percentage of fish with clinical signs of different ages when exposed at 36 mm fork length.

Significant differences in the percent of fish with clinical signs also existed among groups exposed at different sizes (length) ($F = 4.41$, $P = 0.0132$; Figure 3.6); increasing size at exposure decreased the percent of exposed fish with clinical signs, but only when the fish were exposed at 9 weeks posthatch. Size at exposure did not significantly affect the percent of fish with clinical signs when the fish were exposed at 7 weeks posthatch.

Significant interactions in response were present between age and size of fish at exposure ($F = 10.91$, $P = 0.0030$), between age of fish at exposure and parasite dose ($F = 23.10$, $P < 0.0001$), and among age and size at exposure and parasite dose ($F = 10.91$, $P = 0.0030$). An interaction occurs when the difference in response between the levels of one factor is not the same at all levels of the other factor. An increase in age at exposure resulted in a decrease in the percentage of fish with clinical signs but only when the fish were exposed at 40 mm and not when they were exposed at the smaller size of 36 mm. Similarly, the effect of size at exposure on the percentage of fish with clinical signs was not the same at all levels of age at exposure; an increase in size at exposure caused a decrease in clinical signs but only when the fish were exposed at 9 weeks posthatch and not when they were exposed at 7 weeks posthatch. The magnitude of difference in percentage of fish with clinical signs between the controls and those exposed to 1,000

triactinomyxons per fish also changed with varying levels of age and size of fish at exposure. An increase in size and age at exposure caused a decrease in the difference between the percentage of fish with clinical signs in the control group and in the group exposed to 1,000 triactinomyxons per fish.

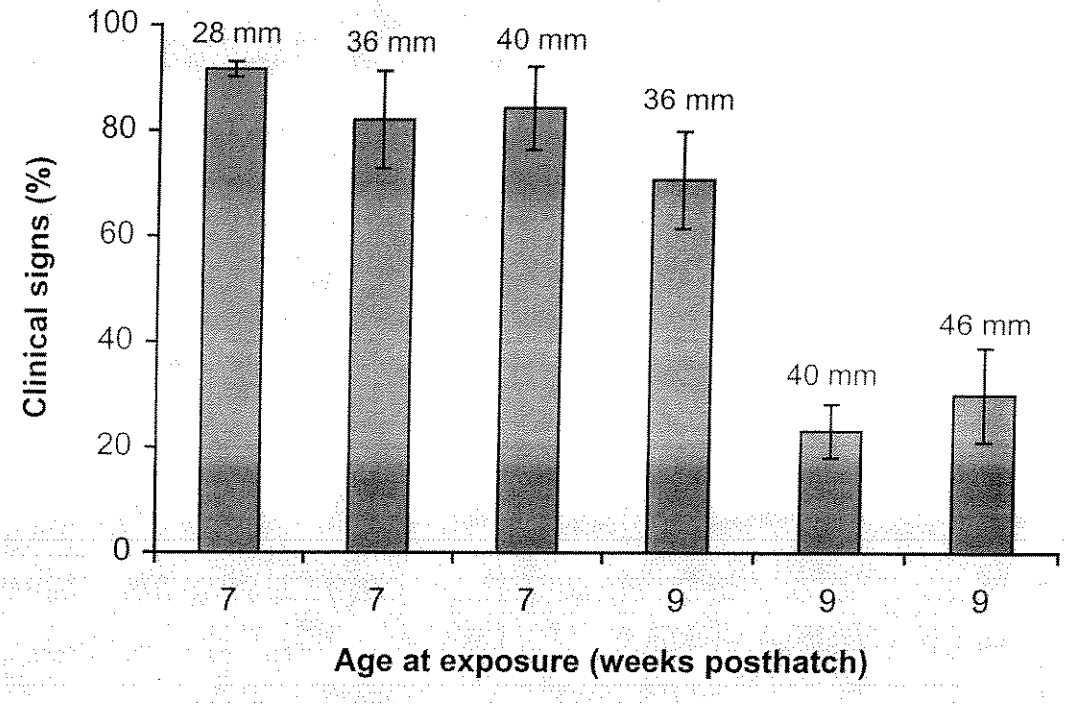


Figure 3.6—Mean (±SE) percent of rainbow trout with clinical signs at 20 weeks after exposure, exposed at different ages and sizes to 1,000 *Myxobolus cerebralis* triactinomyxons per fish. Numbers above bars represent average fork lengths of fish at exposure.

Microscopic Pathology

Frequency distributions of microscopic pathology category were significantly different at $P < 0.05$ among the groups exposed at different sizes within each of the two age classes (Figure 3.7). However, regardless of the size at exposure, the modal microscopic pathology category was moderate for the fish exposed at 7 weeks posthatch. Whereas when exposed at 9 weeks posthatch the mode decreased from moderate for the smallest size at exposure to mild for the two larger sizes at exposure.

Frequency distributions of microscopic pathology category were also significantly different between the different ages at exposure when exposed at the same size (Figure 3.7). However, both groups exposed at 36 mm had a modal category of moderate; whereas, when the fish were exposed at 40 mm the modal category decreased with increasing age at exposure from moderate to mild.

Spore Counts

Myxobolus cerebralis spores were not found in any of the control fish. Dose of triactinomyxons to which the fish were exposed (either 0 or 1,000 triactinomyxons per fish) significantly affected the number of *M. cerebralis* spores in the fish 20 weeks after exposure ($F = 47.74$, $P < 0.0001$, Figure 3.8); however, only fish exposed to 1,000 triactinomyxons per fish at 7 weeks posthatch at 28 mm, 9 weeks posthatch at 36 mm, and at 9 weeks posthatch at 46 mm had significantly more *M. cerebralis* spores than the controls. No significant difference was present in spore counts between fish exposed to 1,000 triactinomyxons per fish and the controls when exposed at 7 weeks posthatch at either 36 mm or 40 mm, or when exposed at 9 weeks posthatch at 46 mm length.

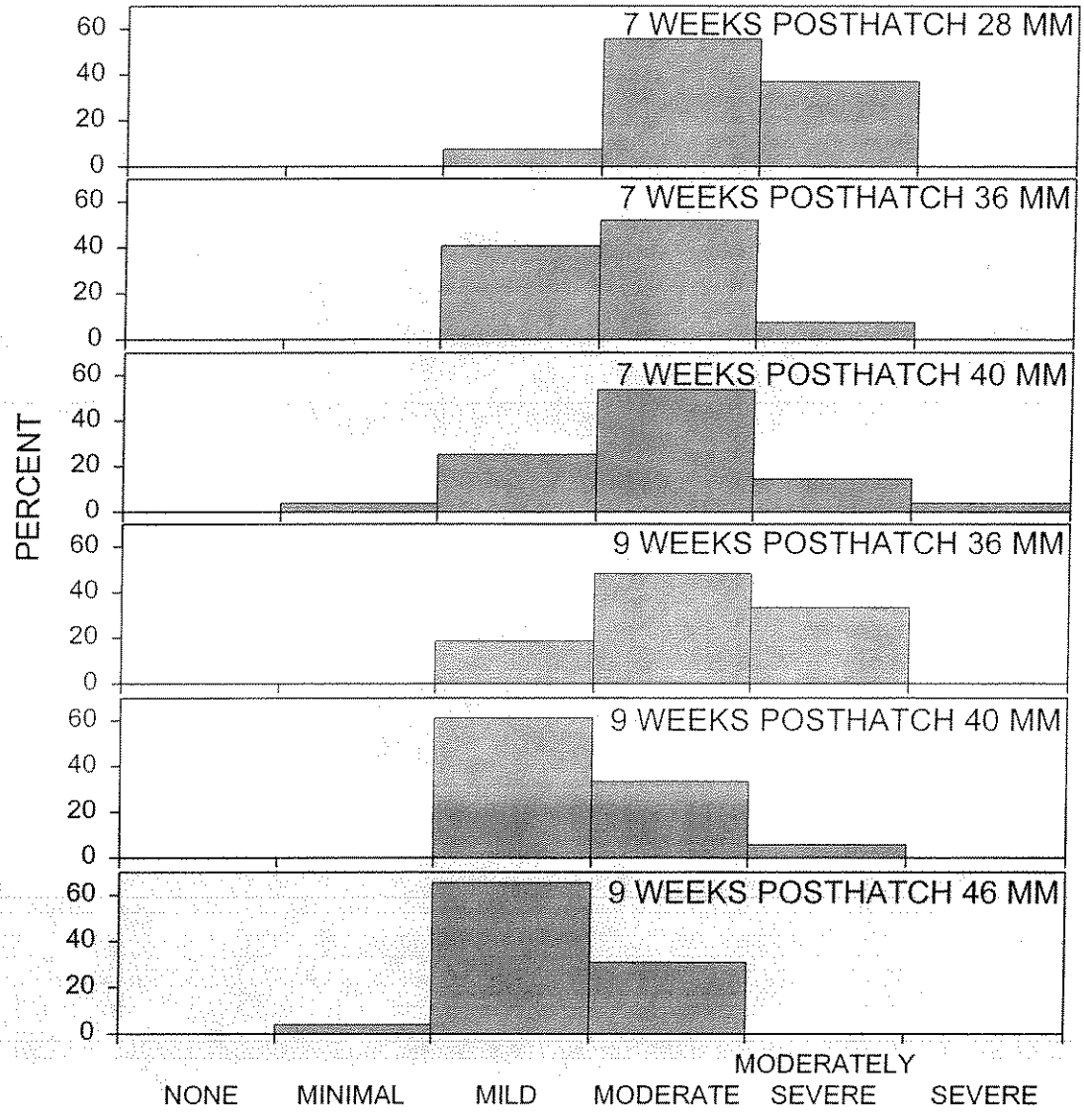


Figure 3.7—Frequency distributions of microscopic pathology category for rainbow trout exposed to 1,000 triactinomyxons per fish at different ages and sizes.

A significant interaction was present between age of fish at exposure and parasite dose ($F = 7.46$, $P = 0.0117$). Therefore, the differences in response between the levels of age at exposure were not the same at all levels of parasite dose.

Age (weeks posthatch) of fish at exposure significantly affected the number of *M. cerebralis* spores present in the fish 20 weeks after exposure ($F = 7.46$, $P = 0.0117$; Figure 3.8). Increasing age at exposure increased the number of *M. cerebralis* spores found in the fish; the fish exposed at 9 weeks posthatch had significantly more spores than the corresponding fish exposed at the same size but at 7 weeks posthatch.

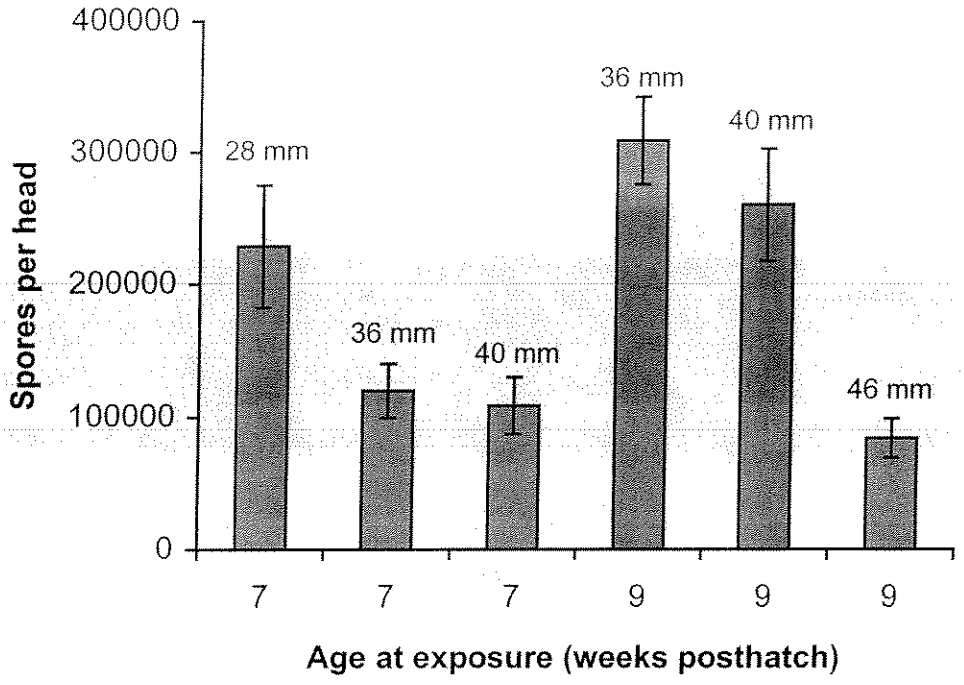


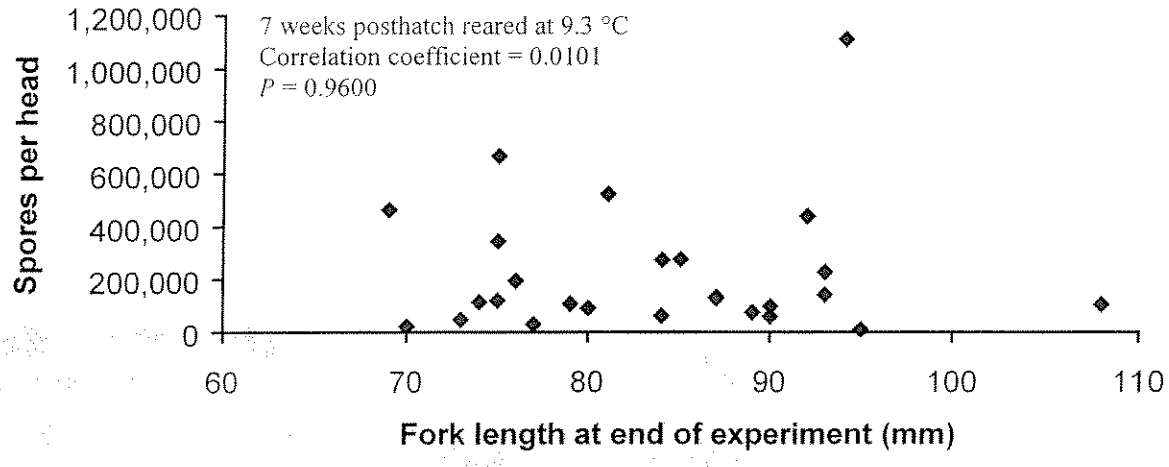
Figure 3.8—Mean (±SE) number of *Myxobolus cerebralis* spores per head of rainbow trout at 20 weeks after exposure, exposed at different ages and sizes to 1,000 *Myxobolus cerebralis* triactinomyxons per fish. Numbers above bars represent average fork lengths of fish at exposure.

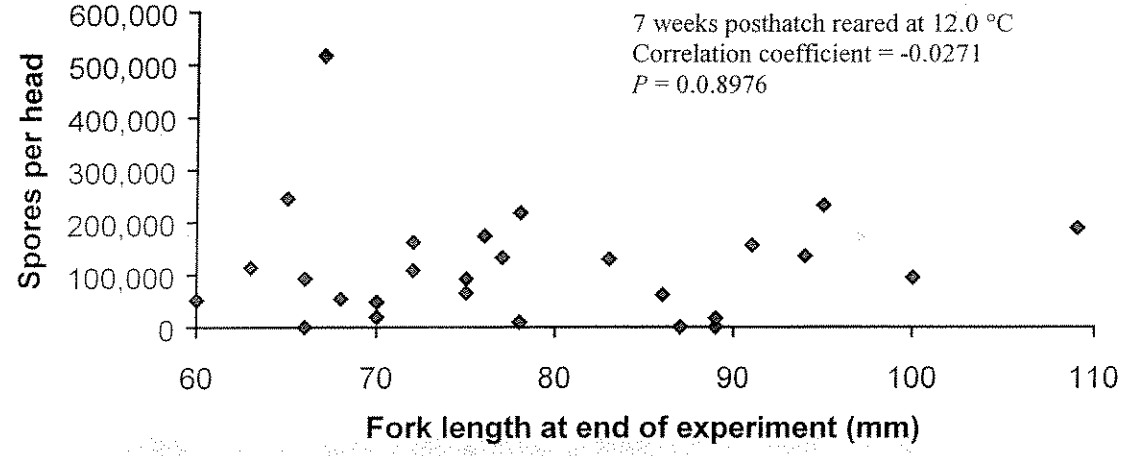
Significant differences in the number of *M. cerebralis* spores present in the fish 20 weeks after exposure also existed among groups exposed at different sizes (length) ($F = 3.08$, $P = 0.0466$; Figure 3.8). Increasing size of fish at exposure significantly decreased the number of spores present in fish, but only for fish exposed at 9 weeks posthatch. Size at exposure did not significantly affect the number of spores present in fish that were exposed at 7 weeks posthatch.

No significant correlations were present between size of fish at the end of the experiment and number of *M. cerebralis* spores within any of the treatment groups (Figure 3.9 and Figure 3.10). Assuming that all fish within a treatment grew at an equal rate after exposure to *M. cerebralis*, the size of the fish at exposure, within a treatment group, does not influence the number of *M. cerebralis* spores that develop within the fish.

Cartilage

The amount of cartilage present in the skeletons of fish was significantly affected by degree-days of development ($F = 20.74$, $P < 0.0001$; Figure 3.11) but not by age in weeks posthatch ($F = 0.05$, $P = 0.8287$; Figure 3.11). Rainbow trout reared at 9.3 °C sampled at 7 weeks posthatch (456 degree-days) had a significantly higher percentage of cartilage in the skeleton than the other two groups sampled at 7 weeks posthatch with more degree-days of development. No significant difference in cartilage existed between fish reared at 12.0 °C and 15.4 °C sampled at 7 weeks posthatch (588 and 756 degree-days). No significant difference in cartilage was found in fish sampled at 9 weeks posthatch, regardless of their degree-days of development. The fish with 456 degree-





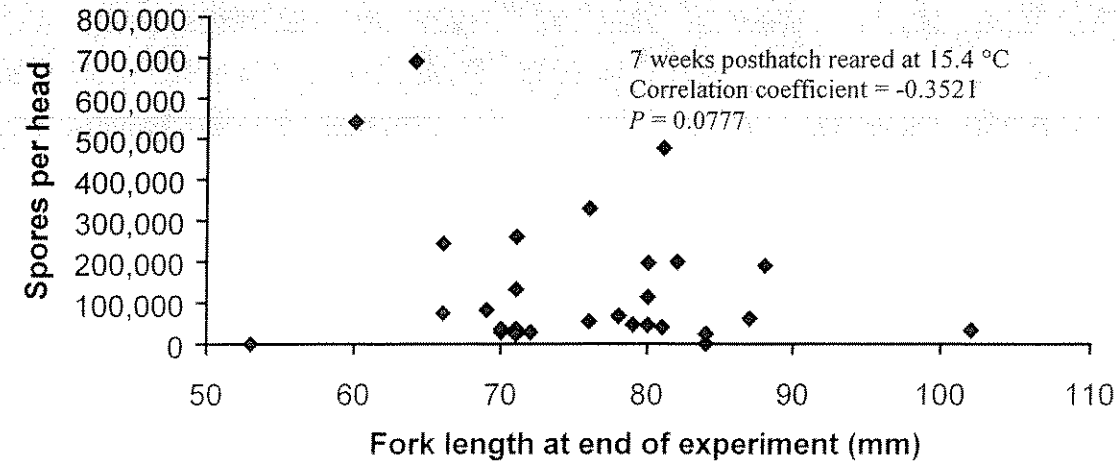


Figure 3.9—Scatter plots number of *Myxobolus cerebralis* spores per head and fork length of fish at the end of the experiment for each of the three groups exposed at 7 weeks posthatch.

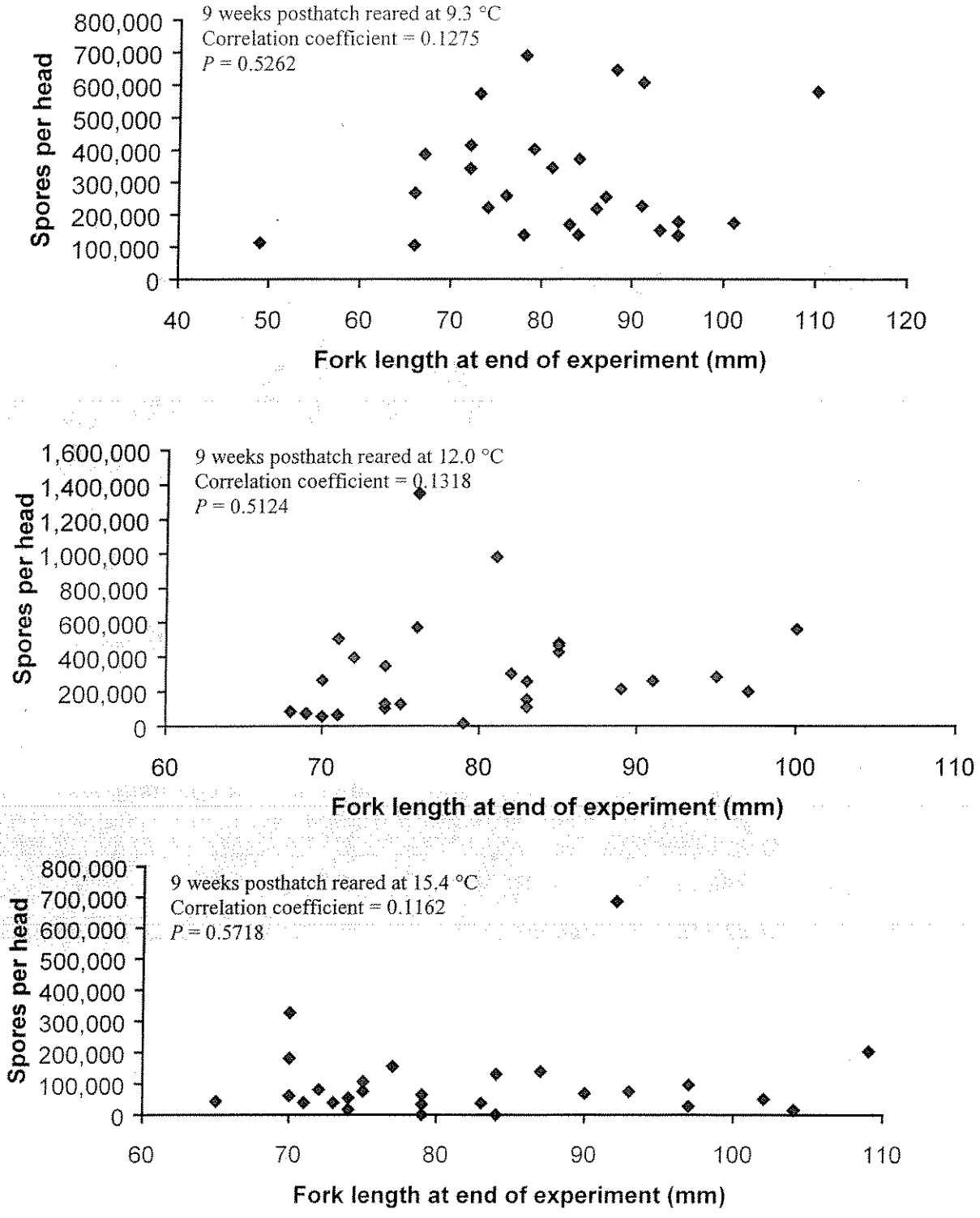


Figure 3.10—Scatter plots of number of *Myxobolus cerebralis* spores per head and fork length of fish at the end of the experiment for each of the three groups exposed at 9 weeks posthatch.

days of development had about twice the percent of cartilage in their skeleton than all other groups.

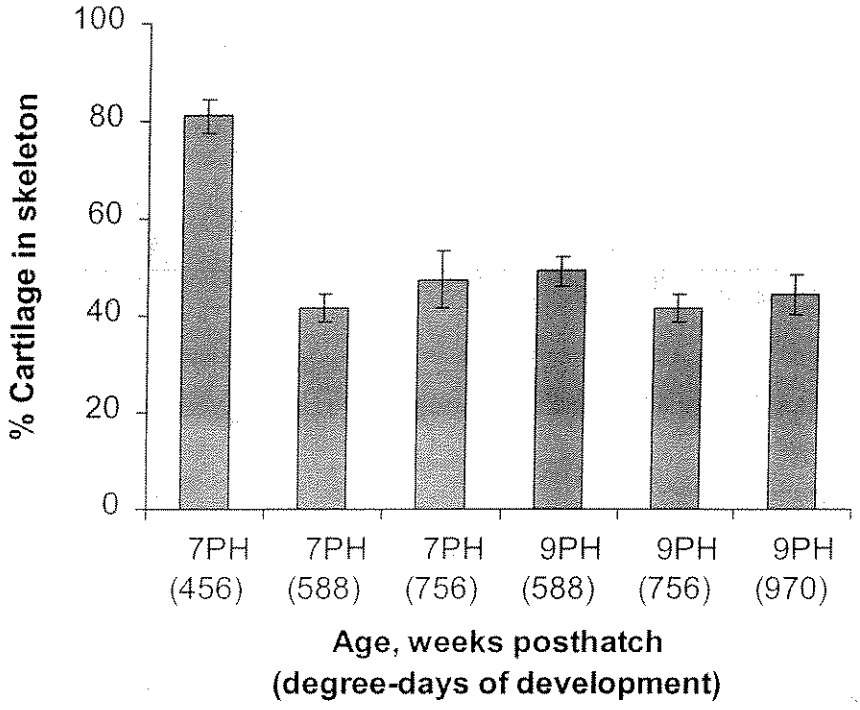


Figure 3.11—Mean (±SE) percent of cartilage in the skeleton of fish of different ages and degree-days of development.

Comparisons were made for each response (i.e., mortality, swimming performance, clinical signs, microscopic pathology, and spore counts) to determine whether the differences in percent cartilage could explain the differences found in response. If the percent cartilage at time of exposure to *M. cerebralis* is solely responsible for the subsequent intensity of whirling disease the fish develops, it would be expected that the group with the largest amount of cartilage would develop the most

severe whirling disease symptoms and the remaining five groups with no difference in the percent of cartilage in their skeleton would show equal intensities of whirling disease symptoms.

The group of fish with the highest percent of cartilage (81%; exposed at 7 weeks posthatch, 456 degree-days) had the same number of mortalities when exposed to 1,000 triactinomyxons per fish as all of the other groups, with the exception of those exposed at 9 weeks posthatch and 756 degree-days (Table 3.2, Figure 3.2). The differences in swimming performance among the groups could also not be completely explained by differences in percent cartilage at time of exposure. The group with the highest percentage of cartilage had the same swimming performance after exposure to 1,000 triactinomyxons per fish as three of the other groups (Table 3.2, Figure 3.3). Clinical signs were not more prevalent in the group with the highest percent of cartilage than in all of the other groups with less cartilage in their skeleton (Table 3.2, Figure 3.6). The two groups with significantly less clinical signs than all the other groups had no less cartilage than three of the other groups with significantly more clinical signs (Table 3.2, Figure 3.6). Three out of the five groups with the least percent of cartilage also had significantly fewer *M. cerebralis* spores in their heads than all of the other groups (Table 3.2, Figure 3.8). However, the group with the significantly highest amount of cartilage had the same number of spores as four of the other groups (Table 3.2, Figure 3.8). Frequency distributions of microscopic pathology were significantly different for the different age and size groups (Figure 3.7). However, the fish with the lowest amount of cartilage had the same modal pathology category as three groups with a higher percentage of cartilage (Table 3.2, Figure 3.7) and the two groups with the lowest modal pathology category had

the same percentage of cartilage as three of the other groups with different modal pathology categories (Table 3.2, Figure 3.7). Therefore, whirling disease severity correlates poorly to the percent of cartilage present in the fish skeleton at time of exposure.

Table 3.2. Results of Bonferroni's multiple comparison procedure for the responses of whirling disease intensity. Comparisons were made between the six groups of fish based only on their percent of cartilage in their skeletons at time of exposure. Comparing down the columns, different letters indicate a significant difference in response ($P < 0.05$). All groups were exposed to 1,000 triactinomyxons per fish. Age is weeks posthatch.

Stage at exposure			Response of whirling disease intensity			
Age	Degree-days	Cartilage (%)	Mortality	Swimming performance	Clinical signs	Spore counts
7	456	81	z	z	z	zy
7	588	42	z	zy	zy	yx
7	756	47	z	zy	zy	yx
9	588	49	z	zy	y	z
9	756	41	y	y	x	z
9	970	44	zy	y	x	x

Discussion

Whirling disease severity in rainbow trout was affected by both age and size at first exposure. The hypothesis that size of rainbow trout at first exposure to *M. cerebralis* does not affect the development of whirling disease (hypothesis 2) is therefore rejected.

The effects of size on the development of whirling disease in rainbow trout were however, not the same for fish exposed at different ages. Size at exposure was important for the development of whirling disease in the older age group, with an increase in size at exposure resulting in a decrease in severity of whirling disease that developed. However, size at exposure did not affect the development of whirling disease in the younger age group. Similarly, the effects of age on the development of whirling disease in rainbow trout were not the same for fish exposed at different sizes. Age at exposure was important for the development of whirling disease in the larger sized fish with an increase in age at exposure resulting in a decrease in severity of whirling disease.

The susceptibility of the youngest and the smallest fish to whirling disease could not be altered by changing their size, or age, respectively for the youngest and smallest fish, at exposure. In contrast whirling disease susceptibility of older and larger fish could be reduced by either increasing their age at exposure, or increasing their size at exposure. The younger (7 weeks posthatch) and the smaller fish (36 mm) used in this experiment seem to be so susceptible to the effects of the *M. cerebralis* parasite that the development of the disease can not be reduced by exposing them at either larger sizes (for the 7 weeks posthatch fish) or older ages (for the 36 mm fish). The mechanisms needed to provide the fish with protection against development of the disease must therefore not be able to develop within such young or small fish. Although, for the older and larger fish only a slight increase in age or size was all that was needed to induce the mechanisms which protect the fish against the development of the disease. The possible mechanisms present in the fish, which result in a resistance against the development of whirling disease, will be discussed in more detail later in this section.

Rainbow trout exposed at 9 weeks posthatch had a greater number of *M. cerebralis* spores than fish exposed at 7 weeks posthatch. This result contradicts findings in Chapter 2, wherein spore counts decreased with increasing age at exposure, and does not follow the pattern among the other measures of whirling disease severity used in this experiment. In the experiment described in Chapter 2, all of the fish were raised at 12 °C before exposure; therefore, fish exposed at 7 weeks posthatch were exposed at 588 degree-days and those exposed at 9 weeks posthatch were exposed at 756 degree-days. In the experiment used in this chapter, the rainbow trout exposed at 9 weeks posthatch and 756 degree-days of development had significantly more spores than those exposed at 7 weeks posthatch and 588 degree-days of development, contradictory to the results in Chapter 2. The temperatures that the fish were reared at in the current experiment can perhaps explain this anomaly. The 36 mm, 9 week posthatch fish were reared at 9.3 °C before exposure and had significantly more *M. cerebralis* spores present in their heads than the fish exposed at 36 mm and 7 weeks posthatch, which were reared at 12.0 °C. Temperature is known to affect all aspects of the physiology of the teleosts. It could be possible that the fish held at the lower temperature prior to exposure to the parasite may have compromised defense mechanisms, in comparison to the fish held at the warmer temperature, which may have put them at a greater risk of developing the disease. For instance, decreasing water temperatures are known to reduce the defensive properties of the skin, and lower water temperatures are also known to slow down the immune system of teleosts (Roberts 1975). Therefore, the higher number of spores in the 36 mm fish reared at 9.3 °C, when compared to those exposed at the same size but reared at 12 °C, could perhaps be explained by the fact that prior to exposure the defense mechanisms of

the fish reared at the colder temperature were compromised. The fish exposed at 40 mm that developed the most *M. cerebralis* spores was the 9-week posthatch group, which were also reared at a cooler temperature than the 40 mm and 7-week posthatch fish. However, if the water temperature prior to exposure did affect the susceptibility of the fish to the development of the disease we would expect to see the same relationship of lower temperatures prior to exposure resulting in an increase of disease susceptibility in all of the responses. The only response that showed this pattern was the number of *M. cerebralis* spores present in the fish heads. The anomaly of why the older fish at exposure developed more *M. cerebralis* spores than the size-matched younger fish at exposure is therefore more likely to be explained by experimental error or bias either during the spore extraction process or during the spore counts.

I discussed in Chapter 2 that the development and severity of whirling disease pathology in rainbow trout is dependent on the age of fish when first exposed to the triactinomyxon stage of *M. cerebralis*, and that the effects of whirling disease on rainbow trout are substantially reduced when they are exposed to the parasite for the first time at 9 weeks posthatch or older, as compared to fish exposed at younger ages. Age may no longer be the best measurement of when young trout become resistant to the effects of the disease because we now know that both the age of fish at first exposure and the size of fish at first exposure are important for the development of whirling disease. Prior to the exposures for the experiment to determine the effects of age or size on the development of the disease, varying the water temperature, which the fish were held at, varied the size of fish; the groups reared to the same degree-days showed no difference in size, regardless of age. The fish used in the exposure experiment described in this chapter,

which correspond to the 9-week fish used in the experiment described in Chapter 2, were the 40-mm fish (i.e., those exposed at 756 degree-days). Within those groups that had a mean length of 40 mm at exposure no difference in the development of whirling disease based on the actual size of fish at exposure was present. Therefore, the recommendation made in Chapter 2 can now be adapted to reflect this. The recommendation can now read: rainbow trout reared in *M. cerebralis*-free waters for 756 degree-days of development or until they are 40 mm in length, whether in the wild or in a hatchery situation, should exhibit enhanced survival and swimming performance, reduced prevalence of clinical signs, spore counts and severity of microscopic pathology, compared to fish first exposed to the parasite at an earlier stage of development.

It has long been hypothesized that the decreasing severity of whirling disease with increasing age or size of fish at first exposure occurs because of an increase in ossification of the skeleton (Halliday 1973; Schaperclaus 1991; El-Matbouli et al. 1992; Garden 1992). If this hypothesis were true we would expect to find a decrease in susceptibility to whirling disease severity with a decrease in the percentage of cartilage present in the skeleton. No relationship was found between the percentage of cartilage in the skeleton at time of exposure and susceptibility to the severity of whirling disease. The group of fish in this experiment with the shortest period of development (456 degree-days) had the highest percentage of cartilage in their skeleton. However, this group was not more susceptible to the development of whirling disease than some of the other treatment groups with lower percentages of cartilage in their skeleton. The three groups of fish exposed at 7 weeks posthatch at different developmental stages (456, 588, and 756 degree-days of development) did not show any difference in susceptibility to

development of whirling disease, although they did not all have the same percentage of cartilage in their skeleton. No difference in the presence of clinical signs, *M. cerebralis* spores, swimming performance or microscopic pathology was found among these three groups even though the group exposed at the earliest stage of development (456 degree-days) had a significantly higher percentage of cartilage than the other two groups. The percentage of cartilage present in the fish was not different among the groups with different degree-days of development at 9 weeks posthatch. However, the susceptibility of rainbow trout to whirling disease increased with decreasing degree-days of development for fish exposed at 9 weeks posthatch. This suggests that the percentage of cartilage present in the skeleton of rainbow trout does not affect their susceptibility to *M. cerebralis* triactinomyxons and the subsequent development of whirling disease after exposure.

The role of cartilage in rainbow trout susceptibility to the whirling disease pathogen cannot be ruled out. The tissue primarily targeted by *M. cerebralis* is the cartilage (El-Matbouli et al. 1992). The trophozoites of the parasite digest the cartilage and destroy the structural framework needed for healthy bone formation, leaving the fish with permanent skeletal disfiguration. Logic would then follow that the abundant cartilage in the skeleton of young trout would render them susceptible to the effects of the disease, but it may not be as simple as just the quantity of cartilage present in the young fish at exposure that controls their susceptibility to the development of the disease. Future investigations should study the relationship of cartilage and whirling disease susceptibility of rainbow trout in more detail to try to determine the role, if present, that cartilage plays in the development of resistance against the disease in rainbow trout. One

would expect that if the trophozoites can find areas of cartilage more easily that it would result in an increase in whirling disease susceptibility. Perhaps the absolute amount of cartilage in relation to the dose of parasites to which the fish are exposed is more important than the percentage of cartilage in the skeleton. Alternatively, it may be the proportion of cartilage to total body mass that determines the susceptibility of rainbow trout to the development of whirling disease. Several questions remain unanswered in determining the role cartilage plays in the development of whirling disease in salmonids. For instance, the amount of cartilage needed for a trophozoite to mature is unknown. How many trophozoites it takes to induce disease and consequently how much cartilage is needed to support that number of trophozoites is also unknown. Therefore, it cannot yet be determined if disease severity will increase, decrease, or neither with increasing or decreasing quantities of cartilage.

Other processes, currently unknown, may also explain why the susceptibility of rainbow trout to whirling disease decreases with increasing age or size at first exposure. Resistance to a pathogen can be caused by several different factors: penetration of the host may be reduced, there maybe an induced mechanism such as antibody or interferon production, or the pathogen may be inactivated by serum components, phagocytic cells, acute-phase proteins, or killer cells (Chevassus and Dorson 1990).

Rainbow trout do develop a humoral and cellular immune response to the whirling disease pathogen (Hedrick et al. 1998). However, an active cellular immune response is not evident in the fish until after significant cartilage damage has occurred (Hedrick et al. 1998) and specific anti-*M. cerebralis* antibodies are not present until 12 weeks after exposure to the parasite (Chapter 4). Therefore, it can be assumed that

neither a cellular or humoral immune response is responsible for the relationship of increasing fish age or size at exposure resulting in a decrease in disease severity. However, there could be other non-specific immune mechanisms that rainbow trout develop with time that could provide them with resistance against development of the disease. The immune response of rainbow trout to *M. cerebralis* will be discussed in more detail in the next chapter.

Significant neuropathology is related to the presence of whirling disease in rainbow trout (Rose et al. 2000). An explanation for increased whirling disease severity with decreased age at exposure could be that the neuropathology has a greater effect in young fish. Younger fish at first exposure would have a less mature nervous systems that would be more vulnerable to dysfunction caused by the whirling disease pathogen (Rose et al. 2000). Therefore, development of a more mature and well-developed nervous system may coincide with the development of resistance to whirling disease.

One, or a combination of physiological factors at about 756 degree-days of development or 40 mm in length, provides rainbow trout with protection against the development of whirling disease after exposure to *M. cerebralis* triactinomyxons. Whether in the wild or in a hatchery situation, rainbow trout maintained in *M. cerebralis*-free water for at least 756 degree-days of development or until they are at least 40 mm in length, will have a significant resistance to the development of whirling disease after they are exposed to the parasite.

CHAPTER 4

OBJECTIVE 3: EFFECTS OF AN INITIAL EXPOSURE TO *MYXOBOLUS CEREBRALIS*, AND INDUCTION OF AN ACQUIRED IMMUNE RESPONSE, ON THE DEVELOPMENT OF RESISTANCE TO WHIRLING DISEASE IN RAINBOW TROUT

Introduction

The two main types of immune response are the innate and the specific immune responses. The innate immune response provides the first line of defense against common microorganisms and also plays a crucial part in the initiation and subsequent direction of the specific immune response. Moreover, because a delay exists before the initial specific immune response takes effect, the innate immune response has a critical role in controlling infections during this period. A specific immune response, such as the production of antibodies against a particular pathogen, is known as an acquired (or adaptive) immune response because it occurs during the lifetime of an individual as a reaction to infection by that pathogen. Acquired immune responses depend upon lymphocytes, which provide a more versatile means of defense than the innate immune response, and provide an increased level of protection from a subsequent re-infection with the same pathogen (Janeway et al. 1999; Goldsby et al. 2002).

The acquired immune system is capable of generating tremendous diversity in its recognition molecules, allowing it to recognize billions of uniquely different structures on foreign antigens. When the immune system has recognized and responded to an antigen,

it exhibits immunologic memory; that is, a second encounter with the same antigen induces a heightened state of immune reactivity. Because of this attribute, the immune system can confer lifelong immunity to many infectious agents after an initial encounter. The immune system normally responds only to foreign antigens, because it is capable of self/nonself recognition (Janeway et al. 1999; Goldsby et al. 2002).

An effective acquired immune response involves two major groups of cells: lymphocytes and antigen-presenting cells. Lymphocytes produce antigen-binding cell-surface receptors on their membranes and are capable of the immunologic attributes of specificity, diversity, memory, and self/nonself recognition. The two major types of lymphocytes involved in the immune response are B lymphocytes (B cells) and T lymphocytes (T cells). Each B cell expresses a unique antigen-binding receptor on its membrane. The B-cell receptor is a membrane-bound antibody molecule. When a naïve B cell, which has not previously come in contact with the antigen it is specific for, first encounters the antigen that matches its membrane-bound antibody, the binding of the antigen to the antibody causes the cell to divide rapidly. The progeny of an activated B cell differentiate into memory B cells and plasma cells. Memory B cells have a longer life span than naïve cells; they continue to express the same membrane-bound antibodies as their parent naïve B cell. Plasma cells produce antibody in a form that is secreted instead of expressing membrane-bound antibody. Secreted antibodies are the major effector molecules of humoral immunity. The secreted antibodies bind to the antigen facilitating its clearance from the body (Janeway et al. 1999; Goldsby et al. 2002).

T cells express a unique antigen-binding molecule, called the T-cell receptor, on their membrane. T-cell receptors can only recognize an antigen that is bound to cell-

membrane proteins called major histocompatibility complex (MHC) molecules. Class I MHC molecules are expressed by nearly all nucleated cells. Class II MHC molecules are expressed only by antigen-presenting cells such as macrophages and B lymphocytes. When a naïve T cell encounters an antigen combined with an MHC molecule on a cell, the T cell proliferates and differentiates into memory T cells and various effector T cells (Janeway et al. 1999; Goldsby et al. 2002).

Two types of T cells exist: T helper and T cytotoxic cells. After a T helper cell recognizes and interacts with an antigen-MHC class II molecule complex, the cell is activated and becomes an effector cell that secretes various growth factors known as cytokines. The secreted cytokines play an important role in activating B cells, T cytotoxic cells, macrophages, and various other cells that participate in the immune response. Under the influence of T helper cell-derived cytokines, a T cytotoxic cell that recognizes an antigen-MHC class I molecule complex proliferates and differentiates into an effector cell called a cytotoxic T lymphocyte (CTL). The CTL exhibits cytotoxic activity and mediates the killing of altered self-cells (Janeway et al. 1999; Goldsby et al. 2002) (Figure 4.1).

The whirling disease pathogen induces a strong cellular host immune response during active cartilage destruction (Hedrick et al. 1998). However, the interactions of host immune cells and *Myxobolus cerebralis* are poorly understood. Indirect fluorescent antibody tests have revealed evidence that rainbow trout produce antibodies against *M. cerebralis* (Griffin and Davis 1978) and antibodies to triactinomyxon and myxosporean antigens have been recognized in serum from infected rainbow trout using an enzyme-linked immunosorbent assay (ELISA) and Western blotting assays (Hedrick et al. 1998).

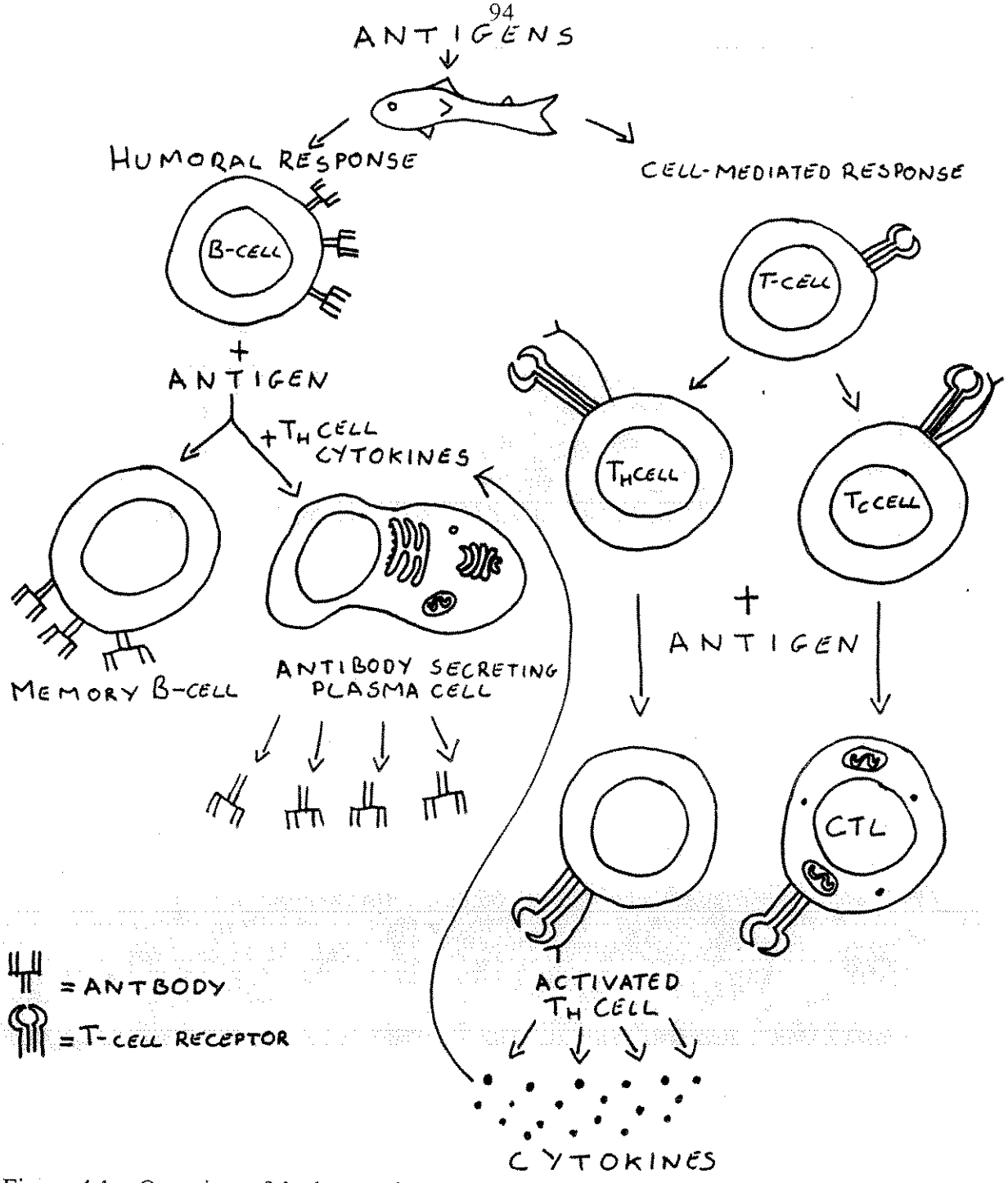


Figure 4.1—Overview of the humoral and cell-mediated branches of the acquired immune system. In the humoral response, B cells interact with antigen (Ag) and then differentiate into antibody-secreting plasma cells. In the cell-mediated response, subpopulations of T cells recognize antigen presented on self-cells. T helper cells (T_H cells) respond to antigen by producing cytokines. Cytotoxic T cells (T_C cells) respond to antigen by developing into cytotoxic T lymphocytes (CTL), which mediate killing of altered self-cells (adapted from Goldsby et al. 2002).

Other studies have failed to detect the presence of anti-*M. cerebralis* antibodies in infected fish (Halliday 1974; Pauley 1974). However, the instances in which *M. cerebralis* infected fish did not produce specific anti-*M. cerebralis* antibodies have been attributed to the use of a nonspecific fluorescent antibody conjugate, rather than an insufficiency on the part of the infected animal (Griffin and Davis 1978).

Attempts to demonstrate that serum with anti-*M. cerebralis* antibodies from infected trout might provide passive protection to young rainbow trout have shown only marginal efficacy (Hedrick et al. 1998). However, some evidence exists that rainbow trout can acquire resistance to reinfection after a prior exposure (Hedrick et al. 1998). An exposure of 1,000 triactinomyxons per fish provided rainbow trout with protection against reinfection as early as 35 days after initial exposure, demonstrated by the absence of sporoplasms in epidermal tissue sections at 2 hours post-re-exposure. Fish exposed to 100 triactinomyxons per fish failed to develop resistance to reinfection. These experiments demonstrated resistance only by the absence of sporoplasms in the fish epidermis after exposure to the parasite; no demonstration of reduced susceptibility to the development of whirling disease was made.

The presence of an acquired immune response after the first contact with triactinomyxons of *M. cerebralis* could allow for the development of management strategies to minimize the effects of whirling disease on wild trout populations, hatchery trout and stocked trout. For instance, if a light infection can provide fish with immunity or resistance against a subsequent higher parasite dose, a young wild trout in *M. cerebralis*-positive waters may gain a benefit from being infected with a low level of the parasite if it later enters waters where the risk of higher infectivity is great. Similarly, a

low level of exposure in a hatchery may be beneficial to fish stocked into positive waters, and critical to their subsequent survival and performance. Management and control of the pathogen in hatcheries could involve the use of a *M. cerebralis* vaccine. Vaccination is the deliberate induction of an acquired immune response to a dead or attenuated (non-pathogenic) form of the pathogen. Vaccination would provide protection for the fish only if an acquired immune response could be induced against the pathogen. The demonstration that rainbow trout could develop an acquired immune response to *M. cerebralis* would be necessary prior to the development of a vaccine against the pathogen. Vaccination could facilitate operation of rainbow trout hatcheries in whirling disease-positive areas, or where rainbow trout are cultured for stocking into whirling disease-positive waters.

My objective was to determine if rainbow trout develop an acquired immune response to *M. cerebralis* and if present, whether the immune response provided the fish with protection against subsequent exposures. I hypothesized that a low-level exposure or immunization with *M. cerebralis* would induce an antigen-specific antibody response, which would provide the fish with protection against subsequent higher levels of exposure. I tested whether immunization with *M. cerebralis* triactinomyxons provided the fish with protection against the development of whirling disease after a subsequent exposure, and secondly, I tested whether specific anti-*M. cerebralis* antibodies were produced in rainbow trout after immunization.

Methods

Rainbow trout were given an immunization dose of *M. cerebralis* triactinomyxons followed by a later re-exposure to determine if the immunization provided the fish with protection against subsequent exposures. Throughout this chapter, I refer to the first exposure as the immunization and the second exposure as the exposure. The dose of the immunization was varied to determine what level provided the fish with protection; the exposure dose was varied to determine what level of exposure the fish could be protected against. The timing between the immunization and exposure was varied to determine the time period required for protection to develop. Whirling disease severity was compared among treatments to determine if the immunization dose had provided the fish with protection against subsequent exposures. Blood serum samples were analyzed for the presence of anti-*M. cerebralis* antibodies to determine if immunized rainbow trout were able to mount a specific acquired immune response. The presence of anti-*M. cerebralis* antibodies was determined at different time periods after the exposure to determine when the immune response was mounted by the fish.

Experimental Procedures

Myxobolus cerebralis triactinomyxons were produced in the laboratory. Cultures of *Tubifex tubifex* worms were exposed to spores of *M. cerebralis,* and triactinomyxons were collected using a procedure similar to that described by Hedrick et al. (1999b).

Erwin strain rainbow trout, hatched from eggs supplied by the Ennis National Fish Hatchery, United States Fish and Wildlife Service, Ennis, Montana, were maintained

at the Bozeman Fish Technology Center, Bozeman, Montana, at 12 °C until scheduled for exposure. Immunizations and exposures were conducted at the Wild Trout Research Laboratory (WTRL), Montana State University, Bozeman, Montana. Each lot of fish was immunized or exposed to *M. cerebralis* triactinomyxons in aerated 5-liter exposure chambers for 2 hours. Following immunization or exposure, lots were maintained separately at 13 °C in 38-liter glass aquaria supplied with filtered and oxygenated water by a recirculating process system at the WTRL. The fish were fed a commercial trout diet at 2 to 3 percent body weight per day. Mortalities were counted and removed daily.

The fish were given an immunization dose of 0, 100, 1,000, or 10,000 triactinomyxons per fish at 5 weeks posthatch, and segregated into two equal groups; one group was exposed four weeks after the immunization (9 weeks posthatch), and the other eight weeks after the immunization (13 weeks posthatch). Exposure doses were 0, 100, 1,000, or 10,000 triactinomyxons per fish. Treatment groups included every possible combination of immunization and exposure dose (Table 4.1). Each immunization and exposure combination was carried out in triplicate with 55 fish in each replicate, for a total of 96 lots.

The swimming performances of three randomly selected fish from each replicate were tested 20 weeks after the immunization. Fish were tested individually in a stamina tunnel (Figure 2.1). Swimming stamina was measured as the length of time a fish could maintain its position in the tunnel (time to fatigue) at a constant velocity (35 cm/s).

Table 4.1. Experiment design. Each combination was carried out in triplicate; numbers are dose of triactinomyxons per fish.

Immunization 5 weeks posthatch	Exposure 9 weeks posthatch	Exposure 13 weeks posthatch
0	0 100 1000 10000	
0		0 100 1000 10000
100	0 100 1000 10000	
100		0 100 1000 10000
1000	0 100 1000 10000	
1000		0 100 1000 10000
10000	0 100 1000 10000	
10000		0 100 1000 10000

The fish used in the stamina test, and an additional six randomly selected fish from each replicate, were examined for clinical signs typical to whirling disease (blacktail, skeletal and cranial deformities, and whirling behavior) and then euthanatized. The heads of the euthanatized fish were removed, cut in half along a midsagittal plane, preserved in Davidson's fixative, and prepared for microscopic examination using standard histological techniques. One head half and the tail section of each fish were prepared for histology. Microscopic pathology was categorized according to Hedrick et al. (1999b), where cartilaginous tissue was examined for the presence of the parasite and associated lesions. The abundance of parasites, cartilage damage, inflammation, extent of lesions, involvement of other tissues, and bone distortion were evaluated and categorized into one of six categories: no infection, minimal, mild, moderate, moderately/severe or severe (E. MacConnell, United States Fish and Wildlife Service, personal communication, Table 2.2). Two head sections and two tail sections were evaluated histologically for each fish collected.

The other halves of the heads were used to obtain spore counts. The Colorado Division of Wildlife performed the spore counts using the enzymatic digestion method to extract the spores from the tissues (Markiw and Wolf 1974). After extraction, spores were resuspended in a known volume of deionized water, and 1-ml aliquots were placed on both sides of a standard 1-ml hemocytometer counting chamber. Total spores per original head were calculated as follows: (2 × total number of spores counted × 10^4 × volume of suspension) / (number of 1-mm^2 areas counted) (O'Grodnick 1975). Three counts of spores were made from each suspended sample; the mean of the three was used in analyses.

A blood serum sample was collected from five randomly selected fish from each replicate at 2 hours and at 4 weeks after the exposures and screened for anti-*M. cerebralis* antibodies. Blood serum was also collected from the 9 randomly selected fish sacrificed 20 weeks after the immunization. Total serum immunoglobulin was quantified by ELISA using triactinomyxon-specific antigens (Dr. M. A. Adkison, School of Veterinary Medicine, The University of California at Davis, personal communication). The level of anti-*M. cerebralis* antibodies was quantified using the following procedure:

1. ELISA plates were coated with 50 µl/well of homogenized *M. cerebralis* triactinomyxon antigen in bicarbonate coating buffer (4 µg/ml) and left overnight at 4 °C. The plates were wrapped in cellophane wrapper to prevent desiccation. Outside wells of the ELISA plates were not used in the analyses. Bicarbonate coating buffer: 0.795 g NA_2CO_3, 1.465 g $NaHCO_3$, and 500 ml distilled water. Bicarbonate coating buffer was stored at 4 °C for no longer than 2 weeks.
2. The wells were blocked with 300 µl/well of blocking buffer/diluent (2 % non-fat dairy milk in tris-base solution) for 1 hour at room temperature. Tris-base solution: 6.07 g 50 mM tris base, 0.409 g 1 mM ethylene diamine-tetraacetic acid, 8.7 g 150mM NaCl, and pH adjusted to 8.0 with HCl.
3. Plates were washed 3 times, at 3.0 PSI, with TTBS (0.1 % Tween-20 in tris-base solution).
4. Serum samples were diluted 1:50 with 1 % non-fat dairy milk in TTBS (0.05 % Tween-20 in tris-base solution). 50 µl of diluted serum solution was added to the wells. All samples were put on the plates in triplicate and each plate included a

known positive and negative control serum sample, and a no-serum control set. The serum samples were left on the plates for 2 hours at room temperature.

5. Plates were washed 5 times, at 3.0 PSI, with TTBS (0.1 % Tween-20 in tris-base solution).
6. Secondary antibody (purified Warrs) was diluted 1:10,000 in 1 % non-fat dairy milk-TTBS diluent, and 50 µl of diluted secondary antibody was added to each well for 1 hour at room temperature.
7. Plates were washed 5 times, at 3.0 PSI, with TTBS (0.1 % Tween-20 in tris-base solution).
8. Goat anti-mouse immunoglobulin-peroxidase was diluted 1:2,000 in 1 % non-fat dairy milk-TTBS diluent, and 50 µl added to each well for 1 hour at room temperature.
9. Plates were washed 10 times, at 3.0 PSI, with TTBS (0.1 % Tween-20 in tris-base solution).
10. Plates were developed with 100 µl/well of tetramethyl benzidine developer (12 ml citric acid, 60 µl tetramethyl benzidine stock, and 60 µl of 2 % H_2O_2). Tetramethyl benzidine stock: 3,3',5,5'-tetramethyl benzidine, 10 mg/ml in dimethylsulfoxide, stored at 4 °C. Plates were developed for 15 minutes at room temperature then the reaction was stopped by adding 50 µl/well of 1 M H_2SO_4. Plates were equilibrated for 5 minutes before reading.
11. Absorbance was read at 450 nm. The samples were blanked to the negative controls on each plate. The higher the absorbance the more anti-*M. cerebralis*

antibodies were present in the serum. The mean absorbance for the three replicates of each sample was used in analyses.

Bias was reduced throughout the experiment wherever possible. Fish were randomly assigned to lots and lots were randomly assigned to tanks. Samples were collected randomly from the tanks for swimming performance tests, histology, spore counts, and blood serum. Histology, spore count, and blood serum samples were examined blindly and in a random order. The treatment designation of each sample was not determined until all samples had been examined and the results recorded.

Statistical Analysis

A necessary precursor to the evaluation of the effectiveness of an immunization is determination that the exposure elicited disease. That is, positive controls (fish not immunized but exposed) must exhibit significantly more disease than negative controls (fish not immunized nor exposed) to allow attribution of differences in disease between immunized (treated) and un-immunized (positive control) exposed fish to the immunization. More explicitly, I needed to be able to show that exposures actually made fish tangibly sick if I was to show that immunization reduced disease. If un-immunized exposed fish were no sicker than un-immunized, un-exposed fish, then it would be impossible to tell if they were sicker than immunized, exposed fish. Analysis of variance (ANOVA) mixed models were used to determine if significant differences existed between the negative controls and each positive control for each of the responses with the exception of microscopic pathology.

Spore counts and swimming performance were analyzed by including random factors in the model for tank and fish, and the fish were treated as the experimental unit. Mortality and clinical signs were analyzed in the same way with the exceptions that tanks were the experimental units and no factor for fish was present in the model. A mixed linear model was used that combined both the fixed (parasite dose level of the exposure) and random (tank and fish) effects. Type 3 *F*-statistics were used in the analysis (Montgomery 1997). The important assumptions supporting this analysis are that the data are normally distributed and that they are independent with constant variance. Visual inspection of residual plots of data for all responses confirmed that these assumptions were met. The units of measure (fish or tank) were not independent; however, this assumption could be dropped by modeling statistical correlation into the analysis, which assumes constant variance and constant covariance (PROC MIXED, compound symmetry covariance option; Littell et al. 1996). The model used for the analysis was the following:

$$y = \mu + \alpha_i + \gamma_{j(i)} + \delta_{k(ij)} + \varepsilon_{ijkl},$$

where

$i = 1,\ldots,a,$

$j = 1,\ldots,b,$

$k = 1,\ldots,c,$

$l = 1,\ldots,d,$

and

μ = the overall mean,

α_i = the effect of the *i*th level of the fixed factor A (parasite dose level of the exposure),

$\gamma_{j(i)}$= the effect of the *i*th level of the random factor B (tank nested in factor A),

$\delta_{k(ij)}$ = the effect of the *k*th level of the random factor C (fish nested in factors A and B; this effect is not included in the model when analyzing the mortality or clinical signs response), and

ε_{ijkl} = a random error caused by sampling.

The chi-square test of homogenieity was used instead of the ANOVA for the microscopic pathology results because these data were non-parametric (Daniel 1990).

If it was determined from this initial analysis that that the positive controls were not more diseased than the negative controls, then the analysis was not taken any further. Alternatively, if the response showed that the positive controls were significantly more diseased than the negative controls, I determined what effect the immunization dose had on the disease caused by the exposure dose. Protection provided by the immunization against the exposure was determined by comparing the disease severity of the fish that received both the immunization and the exposure with that of the fish that just received the immunization. If complete protection were provided by the immunization the disease severity of the fish that received both the immunization and exposure would be equal to that of the fish that only received the immunization because the effect of the exposure would be eliminated by the immunization. The experiment was designed and analyzed as a two-way factorial, one each for the fish exposed at 9 or 13 weeks posthatch. The two factors, or treatments, were immunization parasite dose level (0, 100, 1,000, or 10,000 triactinomyxons per fish) and parasite dose level at the exposure (0, 100, 1,000, or 10,000

triactinomyxons per fish). The analysis was the same as is described above with the exception that a treatment group was added to the model for immunization dose level. The model used for the analysis was the following:

$$y = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + \gamma_{k(ij)} + \delta_{l(ijk)} + \varepsilon_{ijklm},$$

where

$i = 1,\ldots,a,$

$j = 1,\ldots,b,$

$k = 1,\ldots,c,$

$l = 1,\ldots,d,$

$m = 1,\ldots,e,$

and

μ = the overall mean,

α_i = the effect of the ith level of the fixed factor A (parasite dose level of the immunization),

β_j = the effect of the jth level of the fixed factor B (parasite dose level at the exposure),

$\alpha\beta_{ij}$ = the interaction effect between the ith level of factor A and the jth level of factor B,

$\gamma_{k(ij)}$= the effect of the kth level of the random factor C (tank nested in factors A and B),

$\delta_{l(ijk)}$ = the effect of the *l*th level of the random factor D (fish nested in factors A-C; this effect is not included in the model when analyzing the mortality or clinical signs response), and

ε_{ijklm} = a random error caused by sampling.

Bonferroni's multiple comparison procedure was used to compare the pairwise differences of the least-square means. The chi-square test of homogeneity test was again used to analyze the microscopic pathology data (Daniel 1990). The levels of anti-*M. cerebralis* antibodies were compared among the treatments using the same two-way factorial model as described above. ANOVA tables are in Appendix C.

For each significance test, $\alpha = 0.05$. All statistical analyses were conducted with the statistical software program SAS/STAT (SAS Institute 1996).

Results

Disease Indicators

The positive controls (fish not immunized but exposed) did not exhibit significantly more disease than the negative controls (fish not immunized nor exposed) for several of the disease indicators. These included swimming performance (Figure 4.2A) and spore counts (Figure 4.2B) of fish exposed at 9 weeks posthatch, and cumulative mortality (Figure 4.2C), swimming performance (Figure 4.2D) and spore counts (Figure 4.2E) of fish exposed at 13 weeks posthatch. These indicators of disease severity were deleted from further analysis because the effectiveness of the immunization could not be determined. Indicators for which the positive controls did exhibit

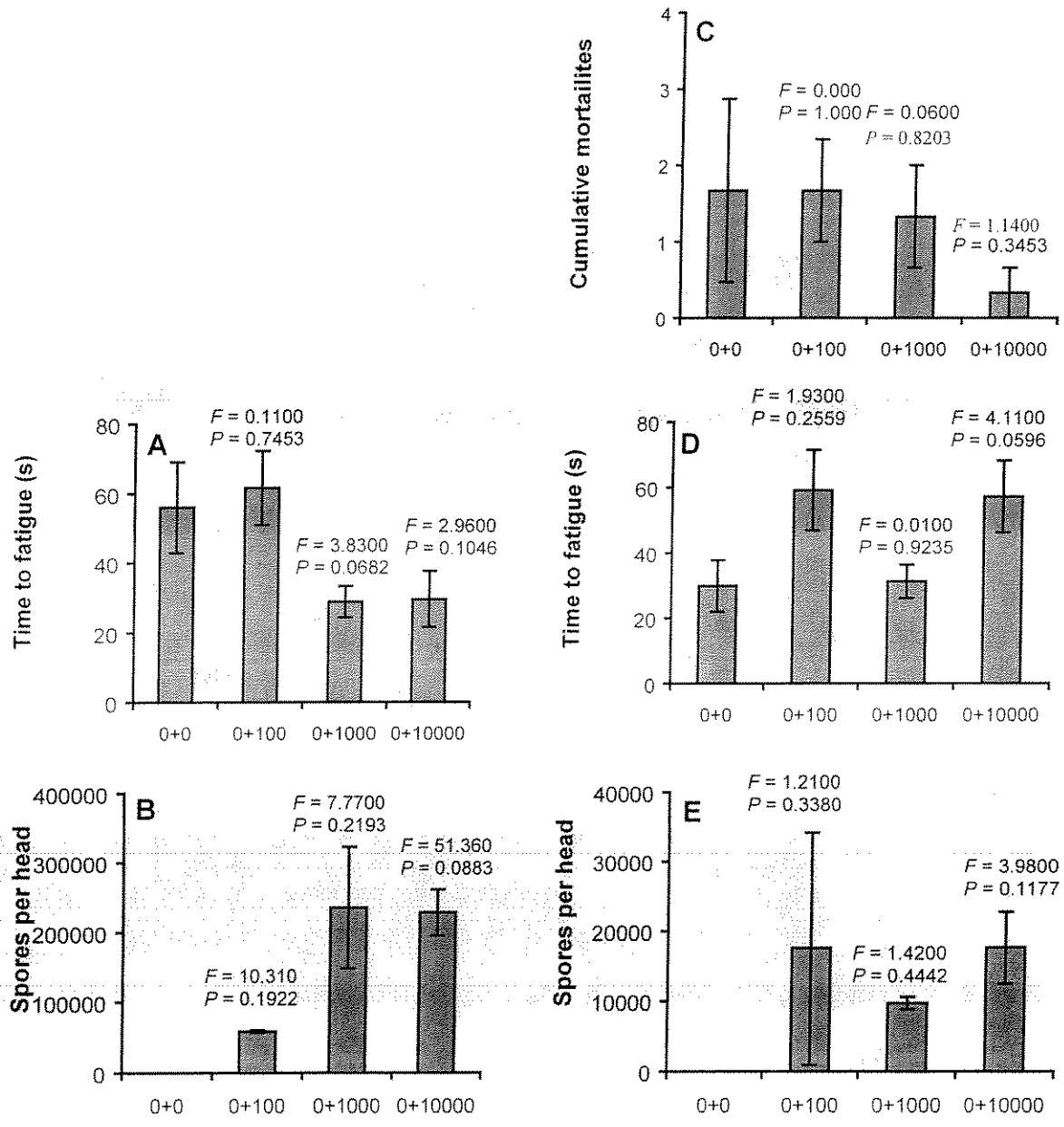


Figure 4.2A-E—Mean (±SE) value of disease indicator for responses which were not significantly different from the negative controls. X-axis is level of immunization and exposure dose (triactinomyxons per fish). Fish were immunized at 5 weeks posthatch with a sham exposure followed by an exposure at 9 or 13 weeks posthatch to a range of parasite doses (0, 100, 1,000, or 10,000 triactinomyxons per fish). Values above bars represent the results from the ANOVA comparisons between the negative controls (fish not immunized nor exposed) and the positive controls (fish not immunized but exposed).

significantly more disease than the negative controls were cumulative mortality (Figure 4.3A), the prevalence of clinical signs (Figure 4.3B), and microscopic pathology (Figure 4.3C) of fish exposed at 9 weeks posthatch, and clinical signs (Figure 4.3D) and microscopic pathology (Figure 4.3E) of fish exposed at 13 weeks posthatch.

If complete protection were provided by the immunization the disease severity of the fish that received both the immunization and exposure would be equal to that of the fish that only received the immunization because the effect of the exposure would be eliminated by the immunization. An immunization of either 100 or 10,000 triactinomyxons per fish provided complete protection against the effects of exposure at 9 weeks posthatch to all doses on cumulative mortality (Figures 4.4A and 4.4C). An immunization of 1,000 triactinomyxons per fish provided complete protection against the affects of exposure to 100 or 1,000 triactinomyxons per fish on mortality (Figure 4.4B), but not against exposure to 10,000 triactinomyxons per fish (Figure 4.4B). Because the mean cumulative mortality values were low (less than 6 per tank), it is possible that this anomalous result was caused by experimental error. For all levels of immunization and exposure, none of the groups which received both the immunization and exposure, regardless of timing of exposure, had higher levels of disease severity than the groups which just received the immunization dose, as measured by clinical signs (Figures 4.5 and 4.6) and microscopic pathology. Although the statistical analysis for microscopic pathology did indicate significant differences in frequency distributions, the median category failed to increase with the addition of the exposure when compared to fish that only received the immunization exposure. Additionally, visual inspection of the frequency distributions (Figures 4.7 to 4.12) lends very little evidence to suggest that the

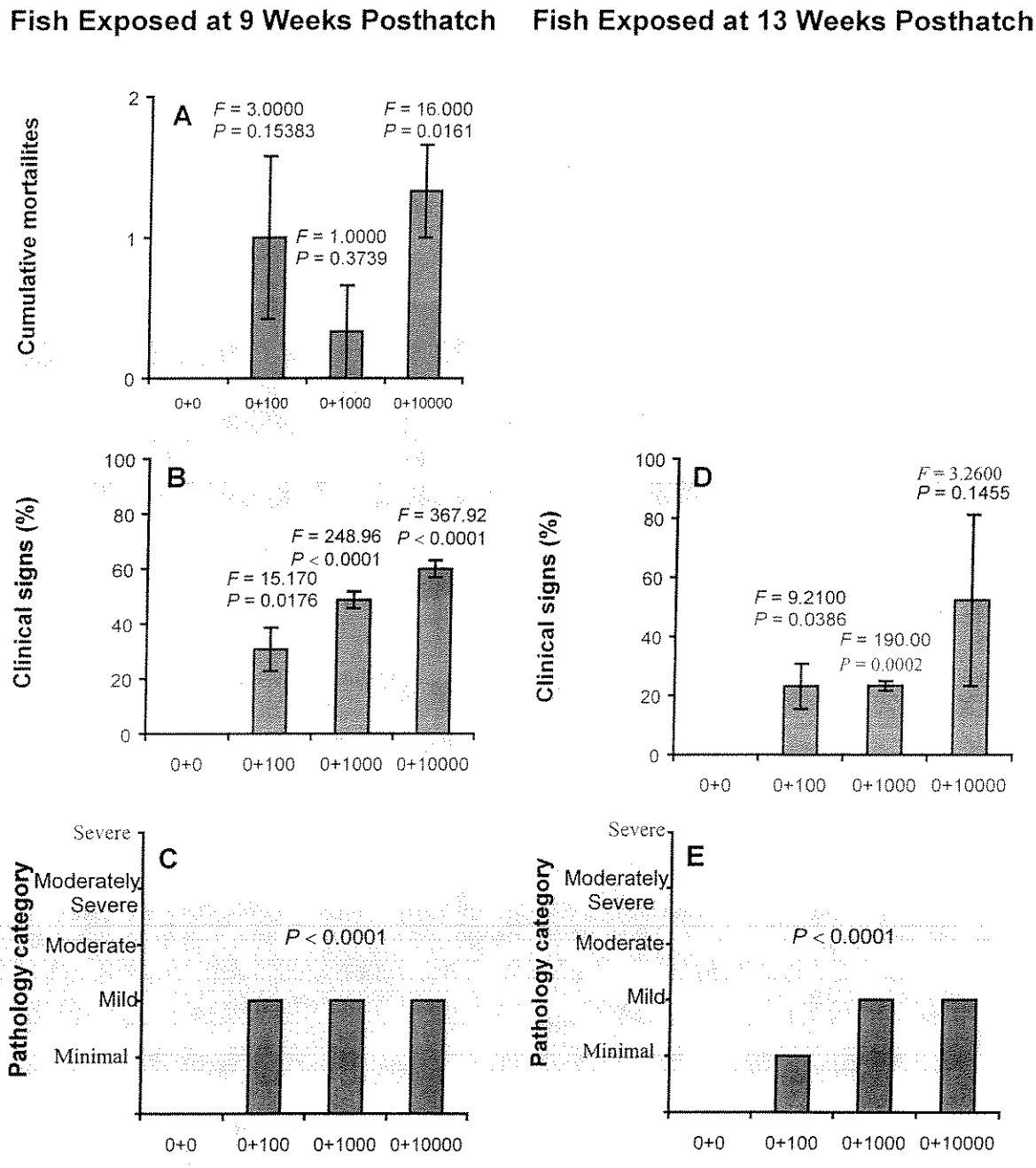


Figure 4.3A-E—Mean (±SE) value of disease indicator for responses which did differ significantly from the negative controls. X-axis is level of immunization and exposure dose (triactinomyxons per fish). Fish were immunized at 5 weeks posthatch with a sham exposure followed by an exposure at 9 or 13 weeks posthatch to a range of parasite doses (0, 100, 1,000, or 10,000 triactinomyxons per fish). Values above bars represent the results from the ANOVA comparisons between the negative controls (fish not immunized nor exposed) and the positive controls (fish not immunized but exposed). *P* values on graphs C and E are results from the chi-square test of homogeneity, $P < 0.05$ indicates a significant difference in frequency distributions within the group.

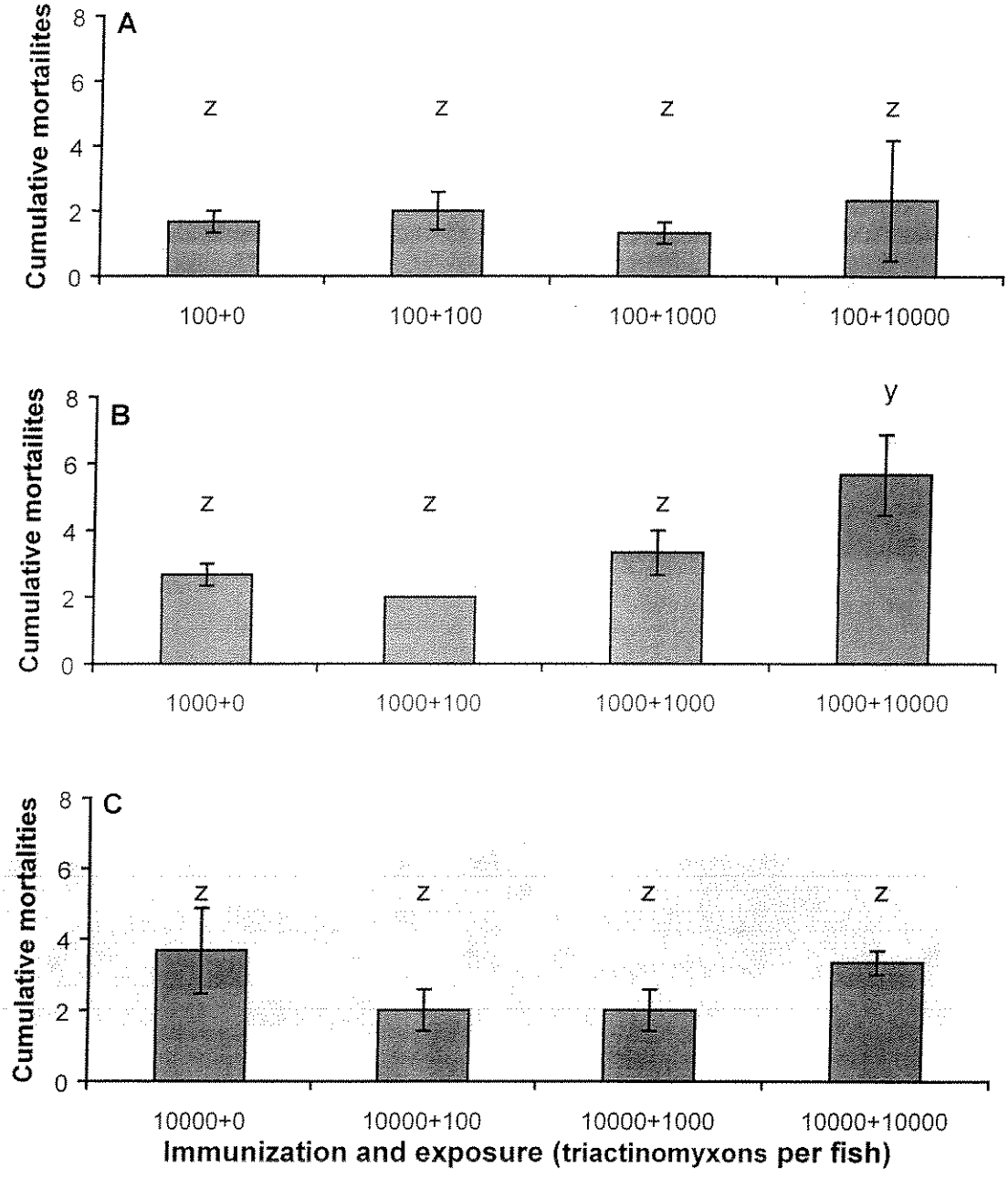


Figure 4.4A-C—Mean (±SE) cumulative number of mortalities 20 weeks after the immunization. Fish were immunized at 5 weeks posthatch to a range of parasite doses (0, 100, 1,000, or 10,000 triactinomyxons per fish) followed by an exposure at 9 weeks posthatch to a range of parasite doses (0, 100, 1,000, or 10,000 triactinomyxons per fish). Different letters on individual graphs indicate bars with a significantly higher response than those that just received the immunization (Bonferroni's multiple comparison procedure $P < 0.05$).

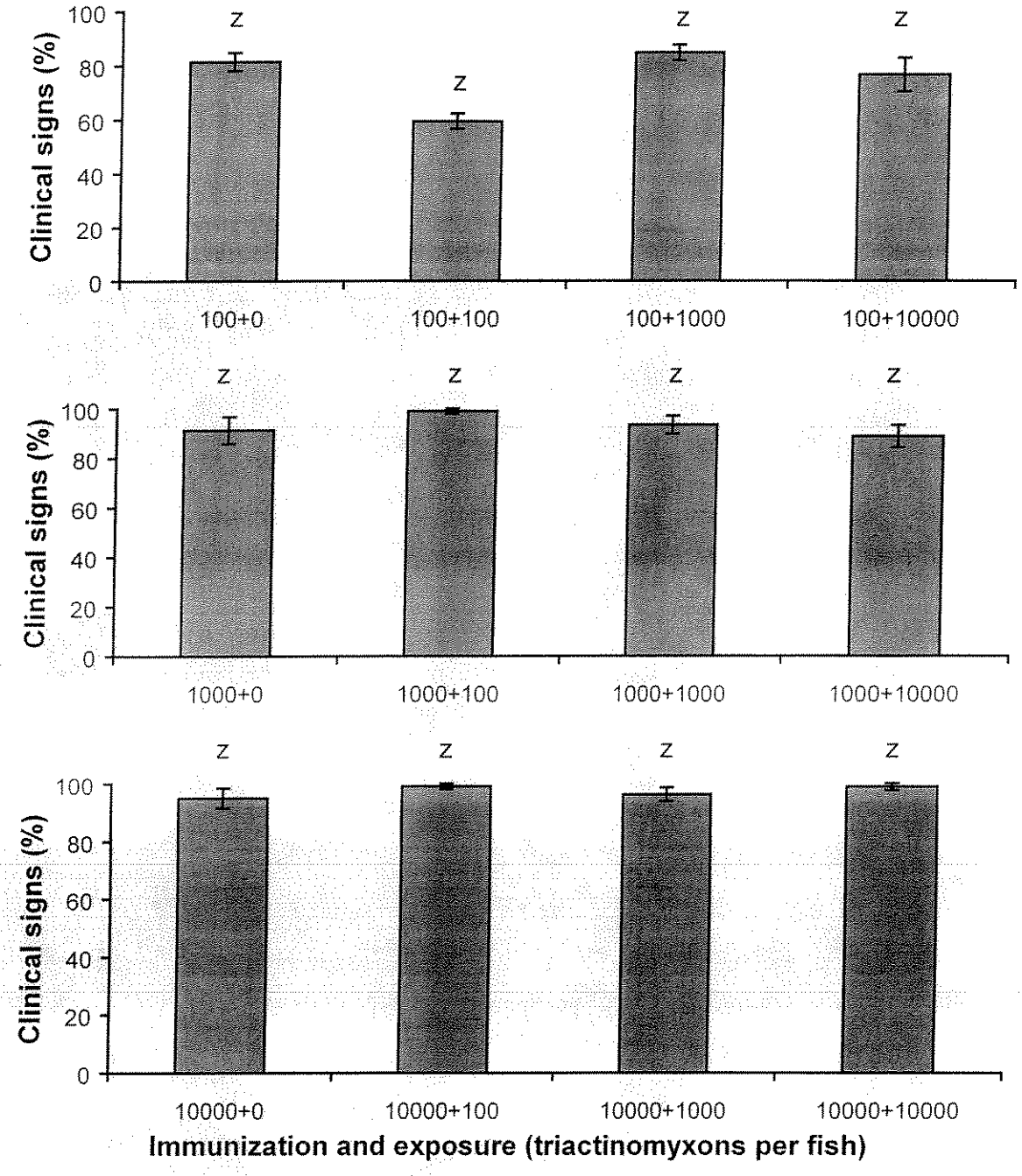


Figure 4.5—Mean (±SE) percent of fish with clinical signs 20 weeks after the immunization. Fish were immunized at 5 weeks posthatch to a range of parasite doses (0, 100, 1,000, or 10,000 triactinomyxons per fish) followed by an exposure at 9 weeks posthatch to a range of parasite doses (0, 100, 1,000, or 10,000 triactinomyxons per fish). Different letters on individual graphs indicate bars with a significantly higher response than those that just received the immunization (Bonferroni's multiple comparison procedure $P < 0.05$).

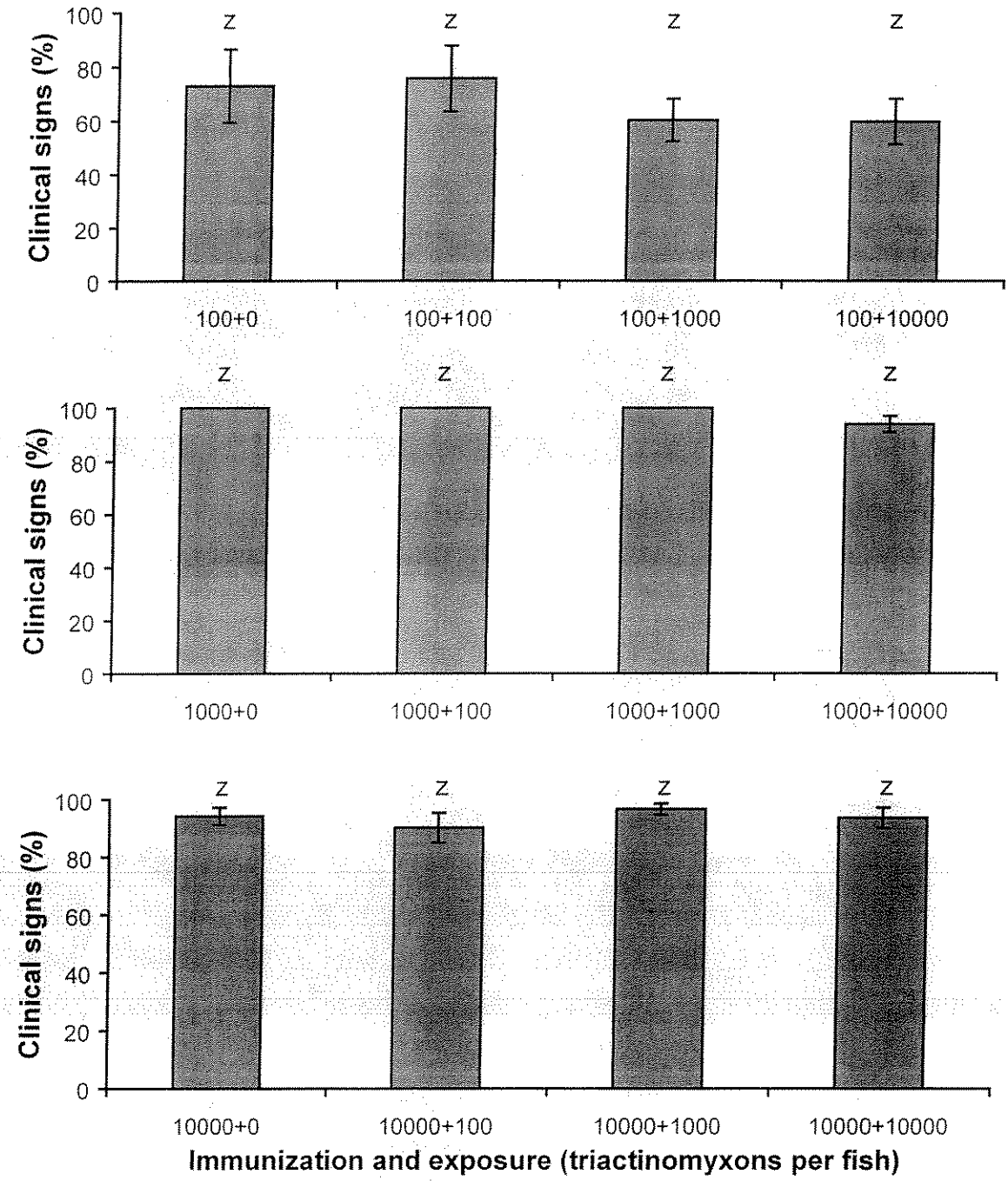


Figure 4.6—Mean (±SE) percent of fish with clinical signs 20 weeks after the immunization. Fish were immunized at 5 weeks posthatch to a range of parasite doses (0, 100, 1,000, or 10,000 triactinomyxons per fish) followed by an exposure at 13 weeks posthatch to a range of parasite doses (0, 100, 1,000, or 10,000 triactinomyxons per fish). Different letters on individual graphs indicate bars with a significantly higher response than those that just received the immunization (Bonferroni's multiple comparison procedure $P < 0.05$).

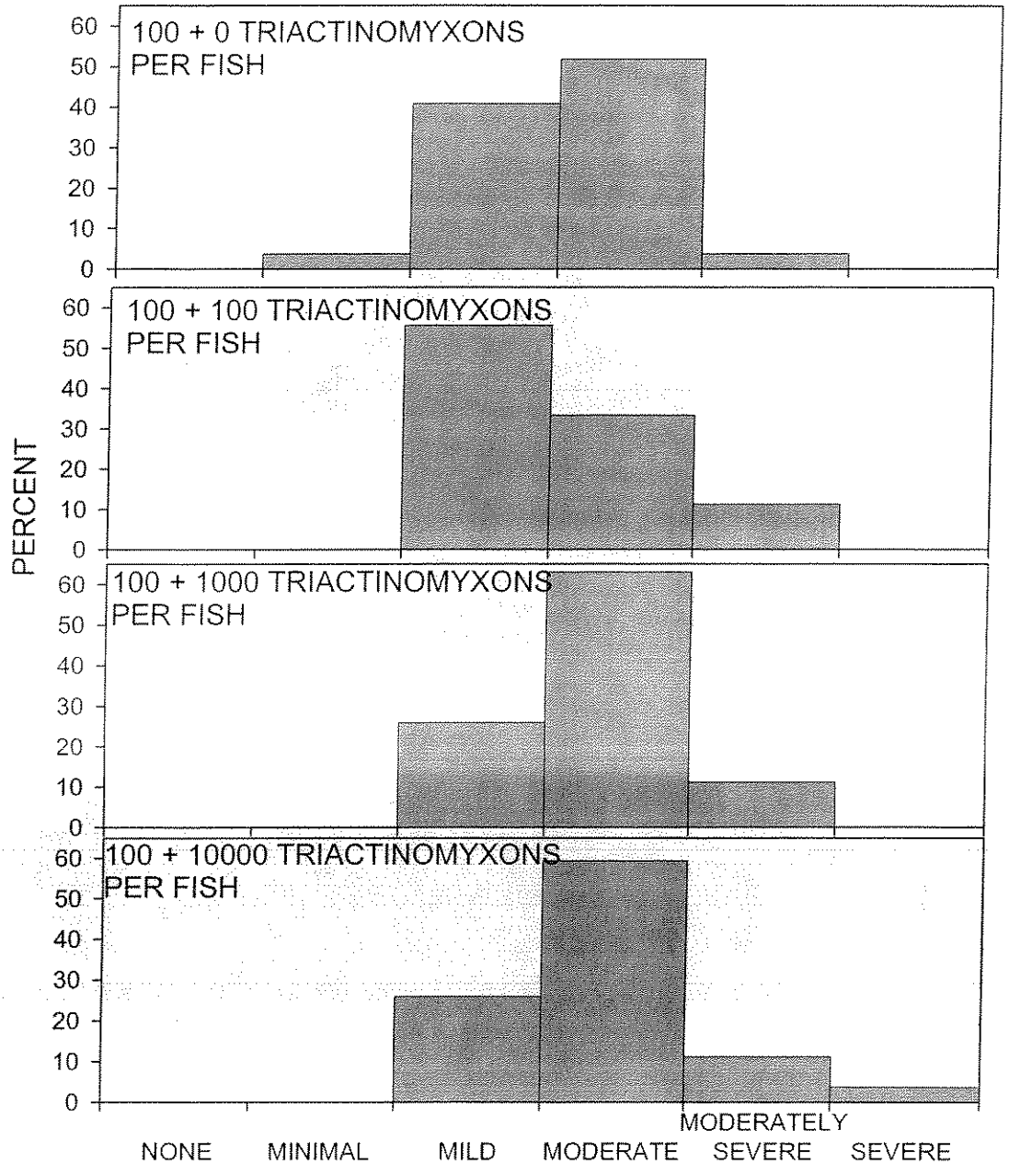


Figure 4.7—Frequency distributions of microscopic pathology category for rainbow trout immunized with 100 triactinomyxons per fish at 5 weeks posthatch and exposed to varying dose levels at 9 weeks posthatch. The frequency distributions were significantly different at $P < 0.0001$ (chi-square test of homogeneity).

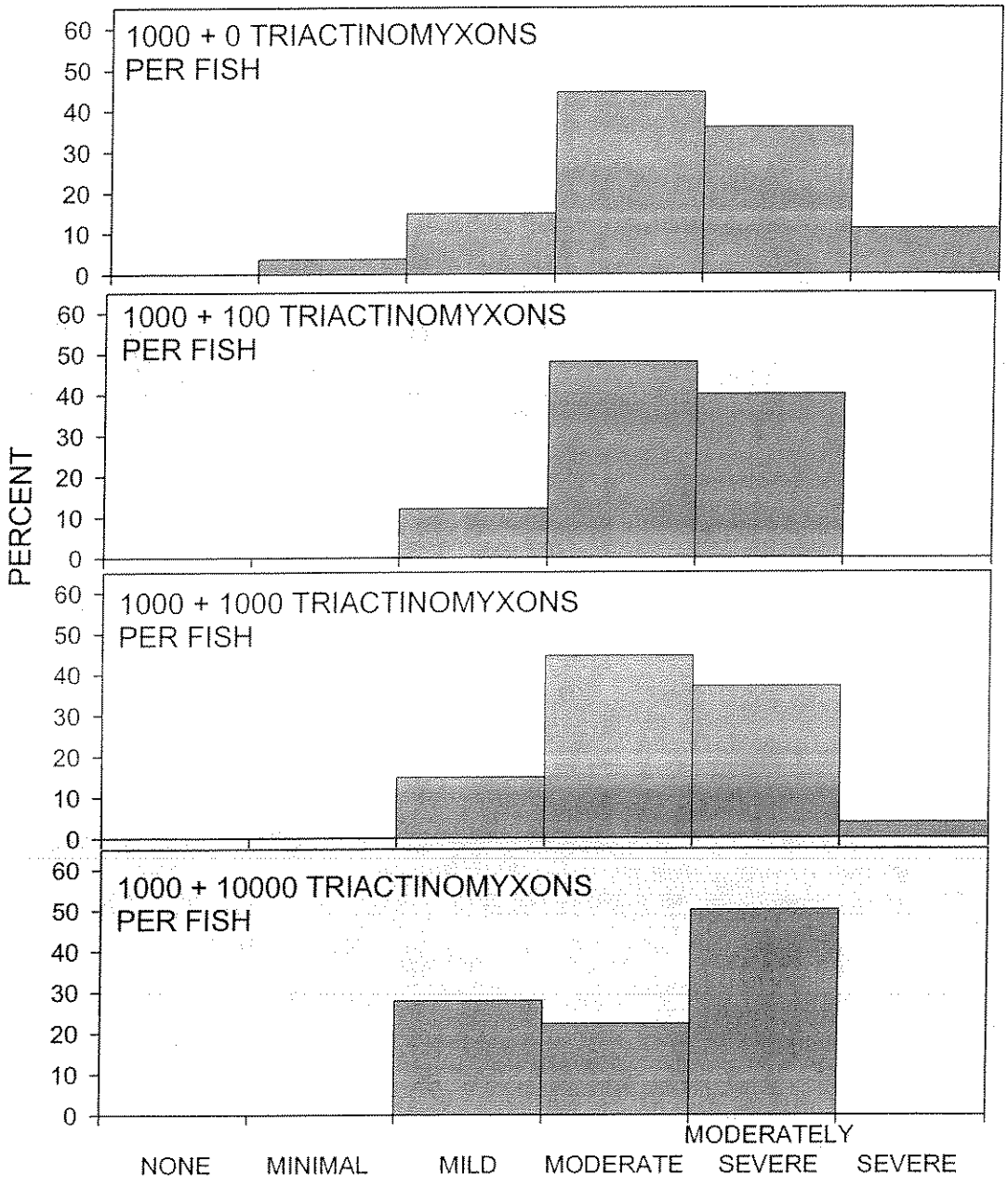


Figure 4.8—Frequency distributions of microscopic pathology category for rainbow trout immunized with 1,000 triactinomyxons per fish at 5 weeks posthatch and exposed to varying dose levels at 9 weeks posthatch. The frequency distributions were significantly different at $P < 0.0001$ (chi-square test of homogeneity).

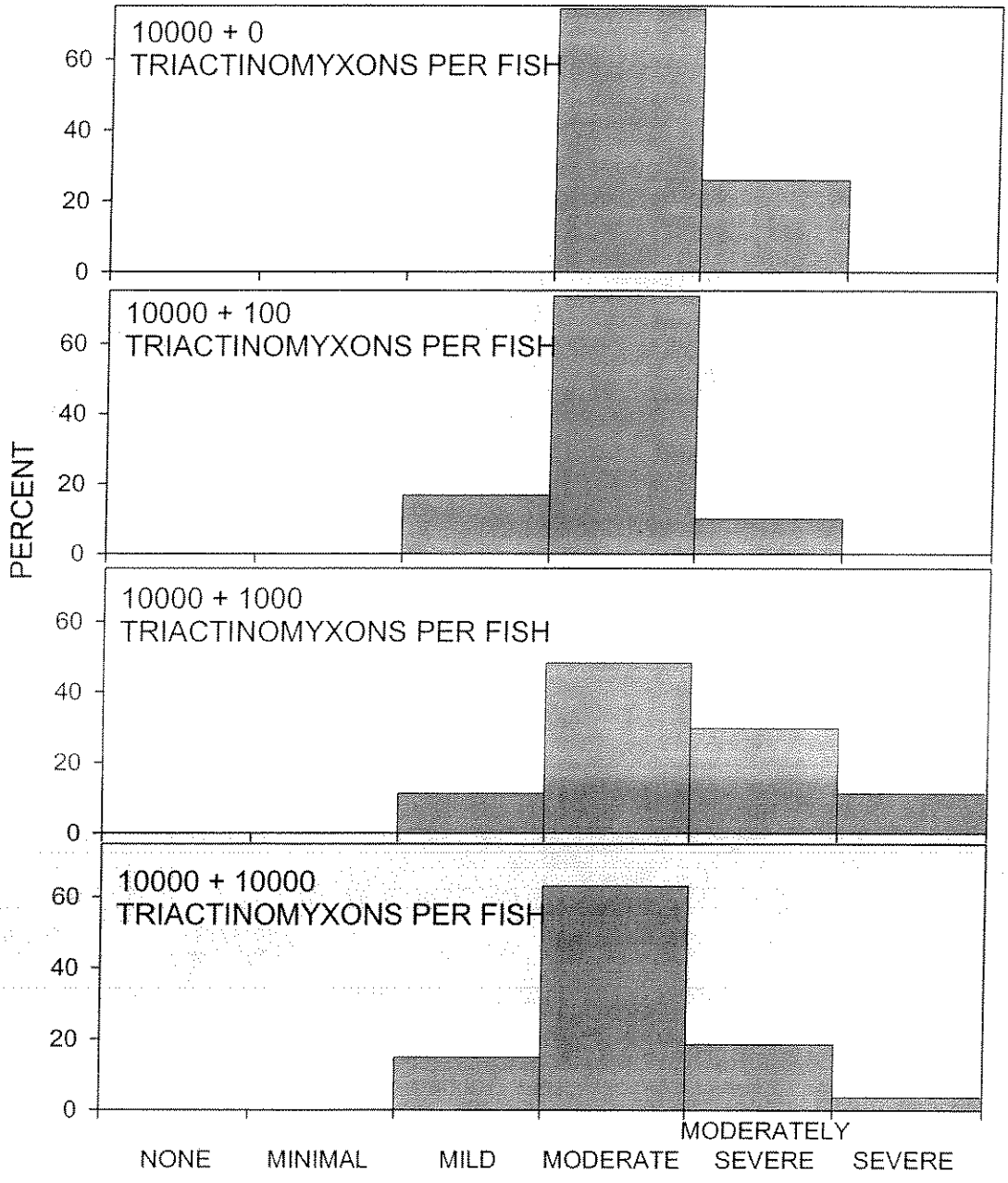


Figure 4.9—Frequency distributions of microscopic pathology category for rainbow trout immunized with 10,000 triactinomyxons per fish at 5 weeks posthatch and exposed to varying dose levels at 9 weeks posthatch. The frequency distributions were significantly different at $P = 0.0067$ (chi-square test of homogeneity).

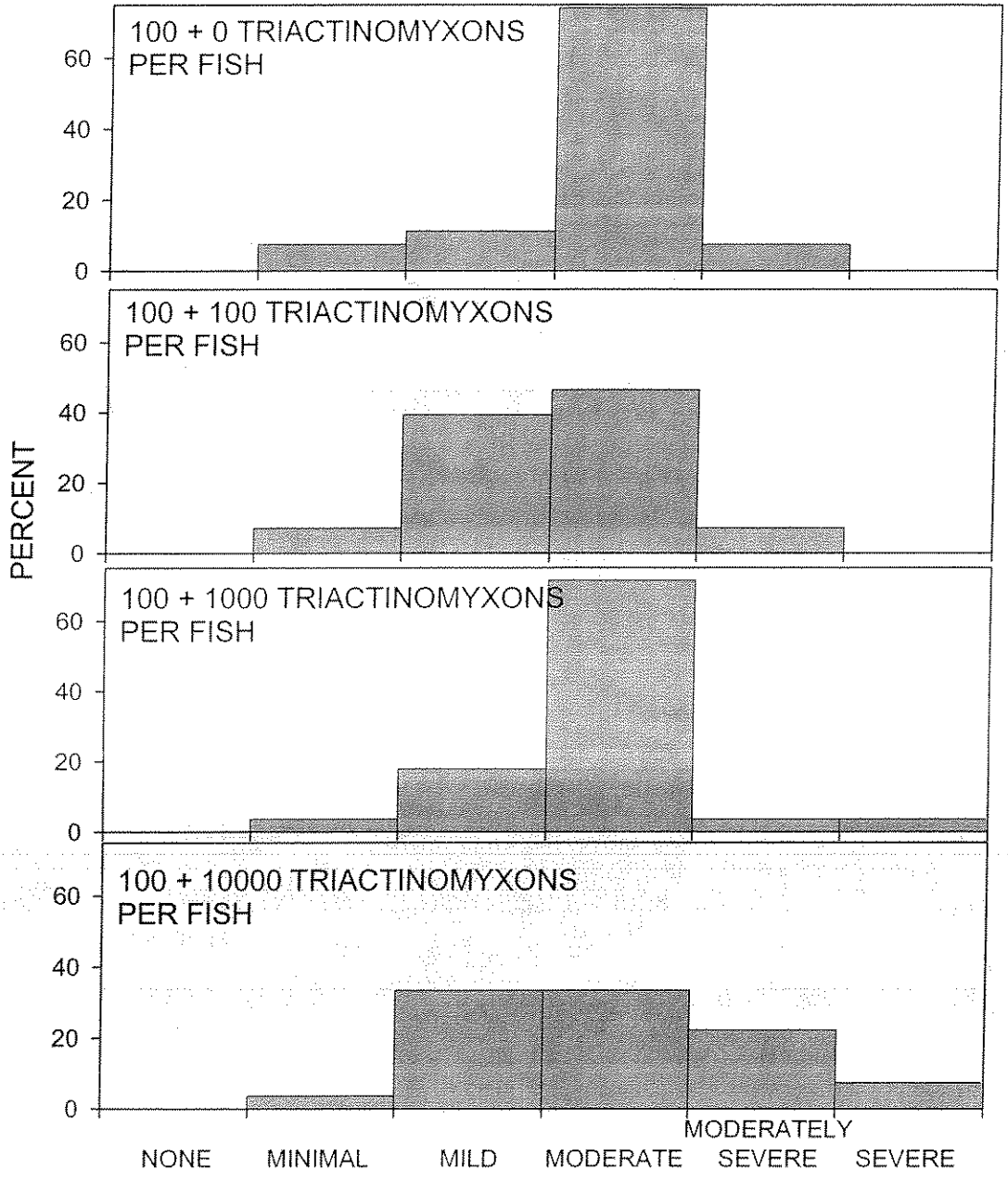


Figure 4.10—Frequency distributions of microscopic pathology category for rainbow trout immunized with 100 triactinomyxons per fish at 5 weeks posthatch and exposed to varying dose levels at 13 weeks posthatch. The frequency distributions were significantly different at $P < 0.0001$ (chi-square test of homogeneity).

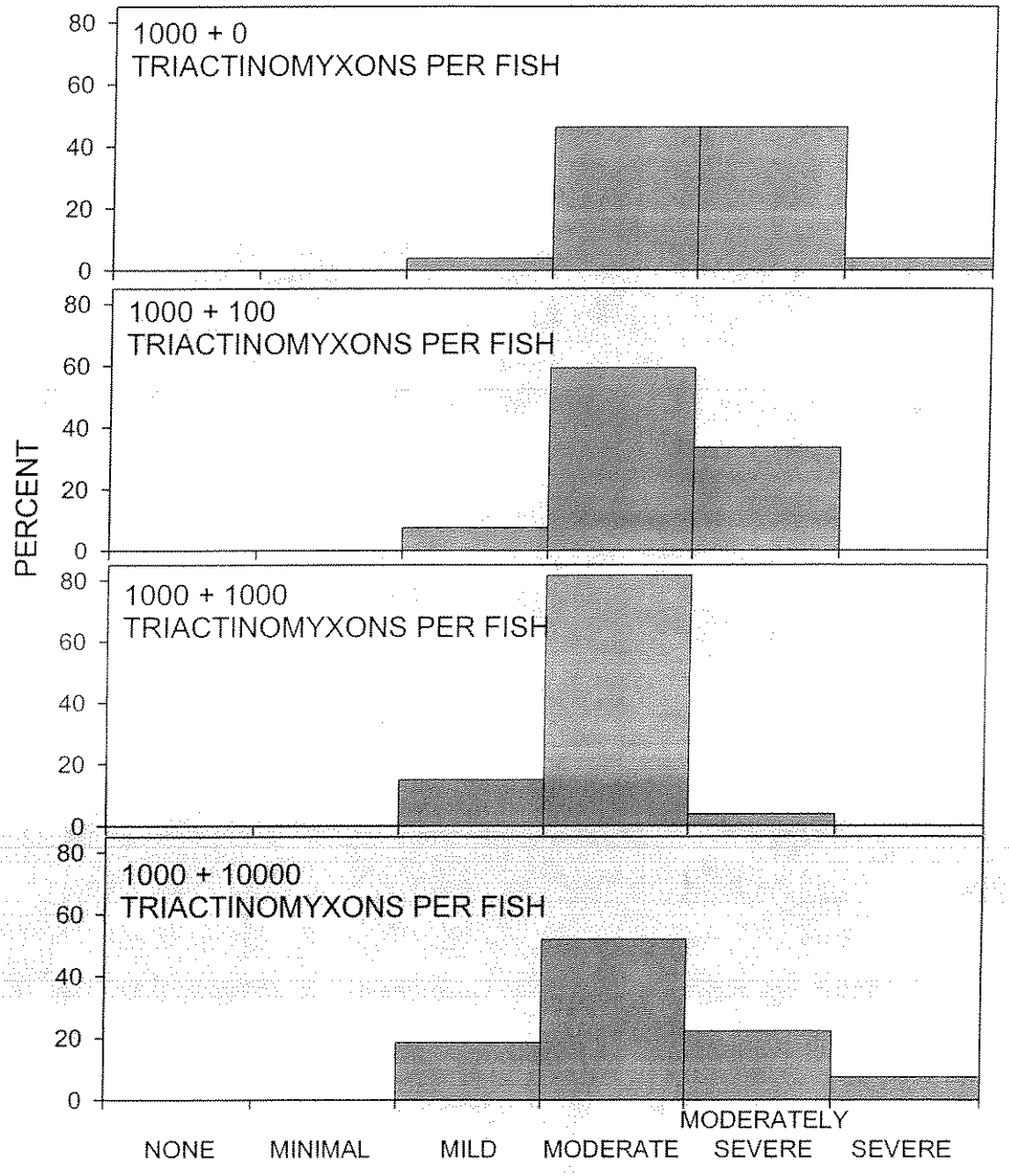


Figure 4.11—Frequency distributions of microscopic pathology category for rainbow trout immunized with 1,000 triactinomyxons per fish at 5 weeks posthatch and exposed to varying dose levels at 13 weeks posthatch. The frequency distributions were significantly different at $P < 0.0001$ (chi-square test of homogeneity).

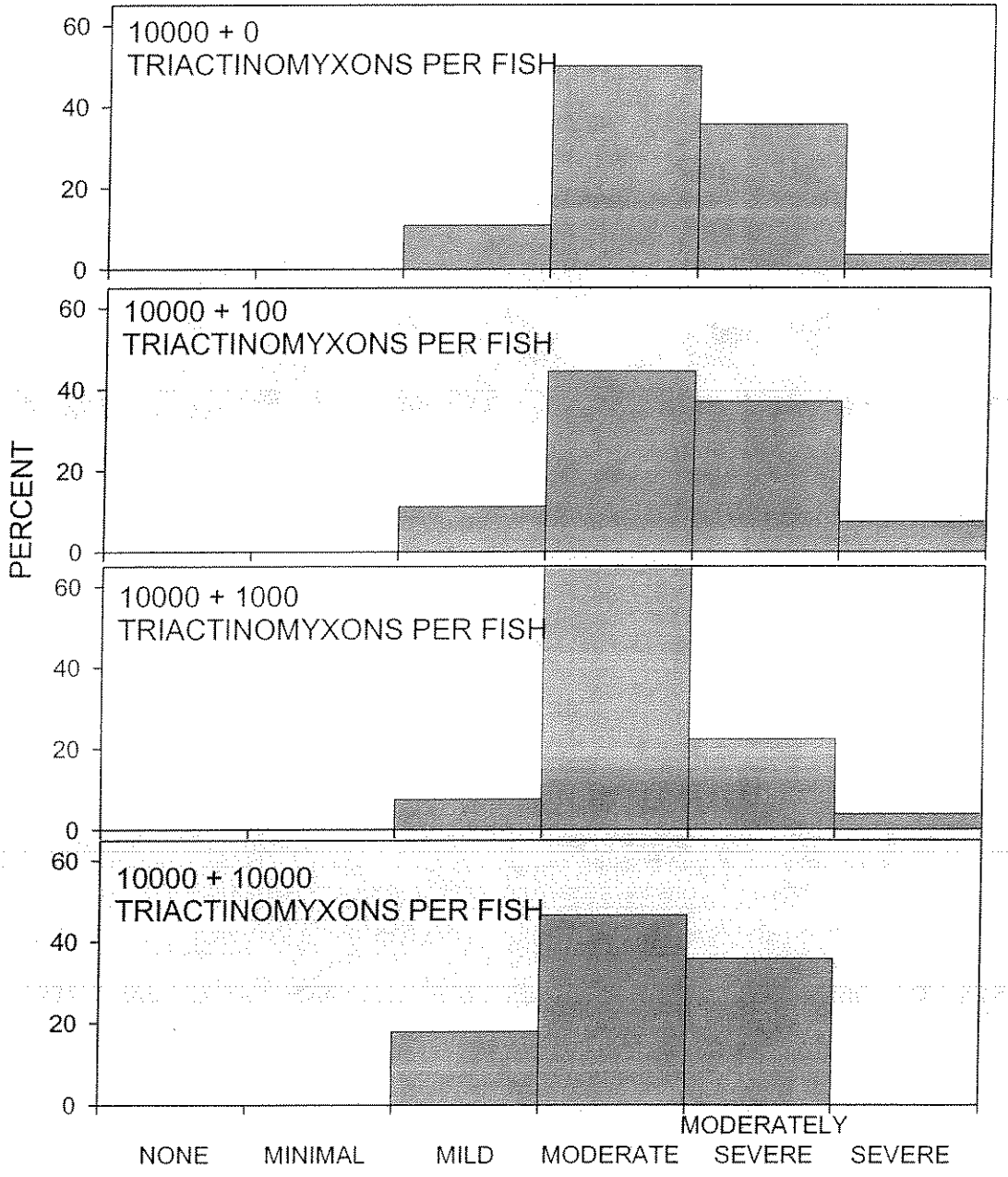


Figure 4.12—Frequency distributions of microscopic pathology category for rainbow trout immunized with 10,000 triactinomyxons per fish at 5 weeks posthatch and exposed to varying dose levels at 13 weeks posthatch. The frequency distributions were significantly different at $P < 0.0001$ (chi-square test of homogeneity).

microscopic pathology scores are increasing with the addition of the exposure dose. Therefore, all levels of immunization dose provided protection against all levels of exposure dose, regardless of when the exposure dose was given.

Disease severity, as measured by cumulative mortality (sham exposure at 9 weeks posthatch) (Figure 4.13A), swimming performance (Figures 4.13C and 4.13D), clinical signs (Figures 4.13E and F), microscopic pathology (Figures 4.13G and H) and spore counts (Figures 4.13I and 4.13J), was significantly increased in fish that received the immunization dose compared to the negative controls. Therefore, although the immunization dose provided the fish with protection against disease caused by the exposure, the immunization dose also caused the fish to become diseased. Cumulative mortality measured from the fish given the sham exposure at 13 weeks posthatch was not significantly affected by the immunization dose (Figure 4.13B).

Serum antibody levels: 9-week posthatch exposure

Two hours after the exposure, neither the exposure dose ($F = 1.66$, $P = 0.1773$; Figure 4.14) nor the immunization dose ($F = 0.87$, $P = 0.4571$; Figure 4.15) significantly affected the level of anti-*M. cerebralis* antibodies present in the serum. Antibody levels recorded from the fish 2 hours after the exposure were not significantly greater than that from the negative controls. Similarly, in serum collected 1 month after the exposure, neither the exposure dose ($F = 0.58$, $P = 0.6314$; Figure 4.16) nor the immunization dose ($F = 0.75$, $P = 0.5281$; Figure 4.17) had affected the level of anti-*M. cerebralis* antibodies. Also, the level of anti-*M. cerebralis* antibodies present in the serum 1 month after the exposure was not significantly different from that of the negative controls.

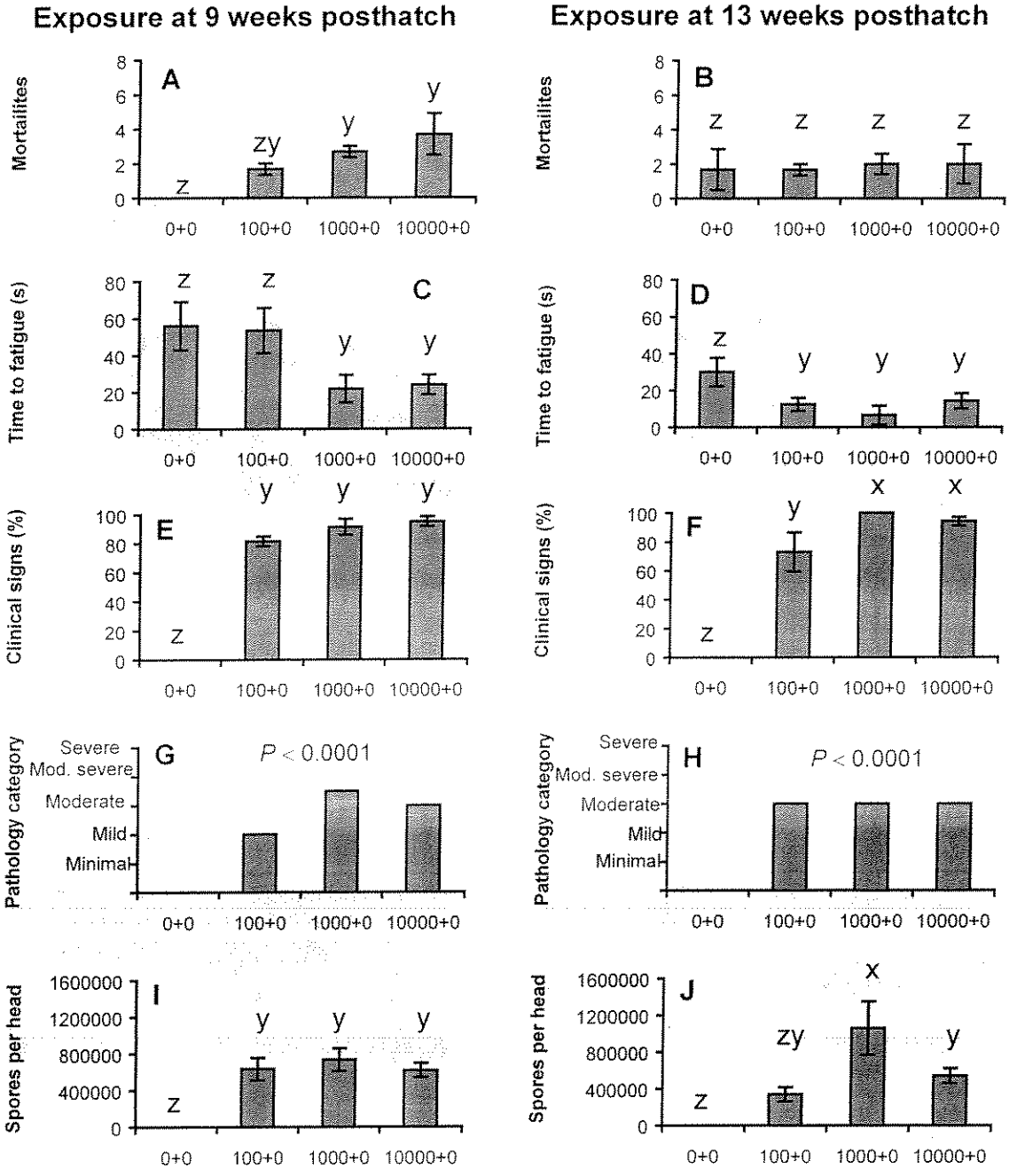


Figure 4.13A-J—Mean (±SE) value of disease indicator. X-axis is level of immunization and exposure (triactinomyxons per fish). Fish were immunized at 5 weeks posthatch to a range of parasite doses (0, 100, 1,000 or 10,000 triactinomyxons per fish) followed by a sham exposure (0 triactinomyxons per fish) at either 9 or 13 weeks posthatch. Different letters above bars indicate a significant difference (Bonferroni's multiple comparison procedure $P < 0.05$). P values on graphs G and H are results from the chi-square test of homogeneity, $P < 0.05$ indicates a significant difference in response within the group.

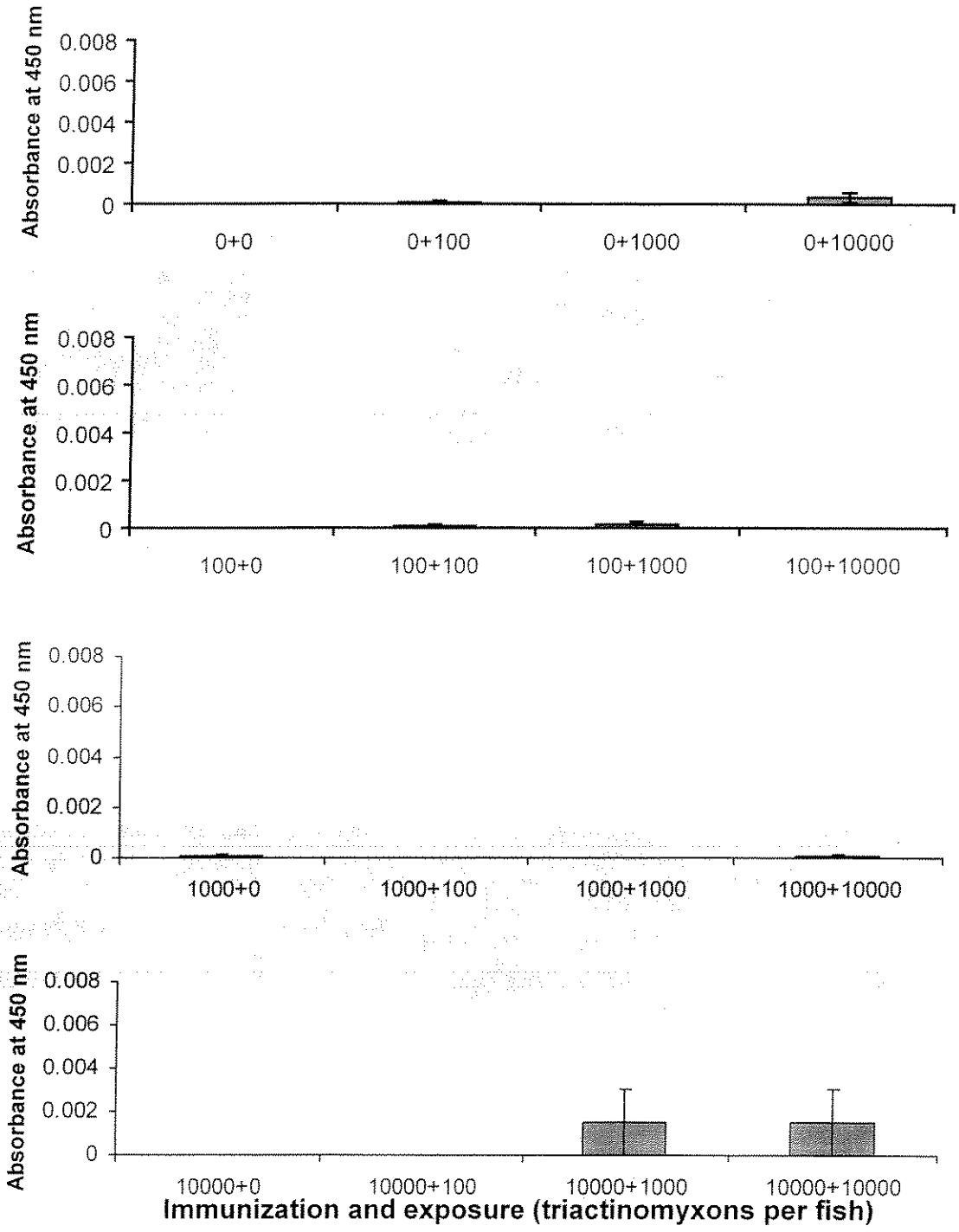


Figure 4.14—Level of serum anti-*Myxobolus cerebralis* antibodies measured as mean absorbance at 450 nm (±SE), 2 hours after the exposure. Fish were immunized at 5 weeks posthatch to a range of parasite doses (0, 100, 1,000, or 10,000 triactinomyxons per fish) followed by an exposure at 9 weeks posthatch to a range of parasite doses (0, 100, 1,000 or 10,000 triactinomyxons per fish).

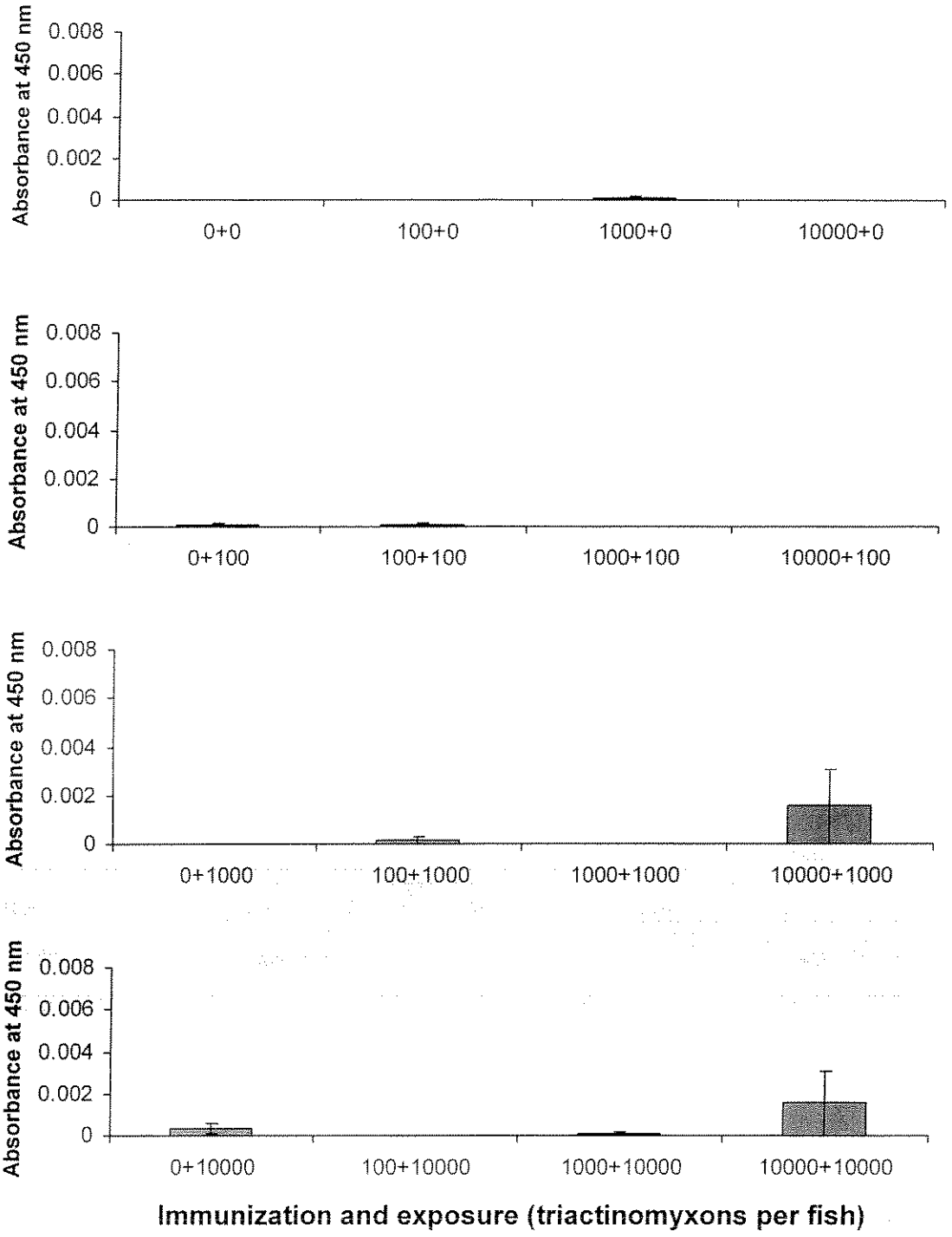


Figure 4.15—Level of serum anti-*Myxobolus cerebralis* antibodies measured as mean absorbance at 450 nm (±SE), 2 hours after the exposure. Fish were immunized at 5 weeks posthatch to a range of parasite doses (0, 100, 1,000, or 10,000 triactinomyxons per fish) followed by an exposure at 9 weeks posthatch to a range of parasite doses (0, 100, 1,000 or 10,000 triactinomyxons per fish).

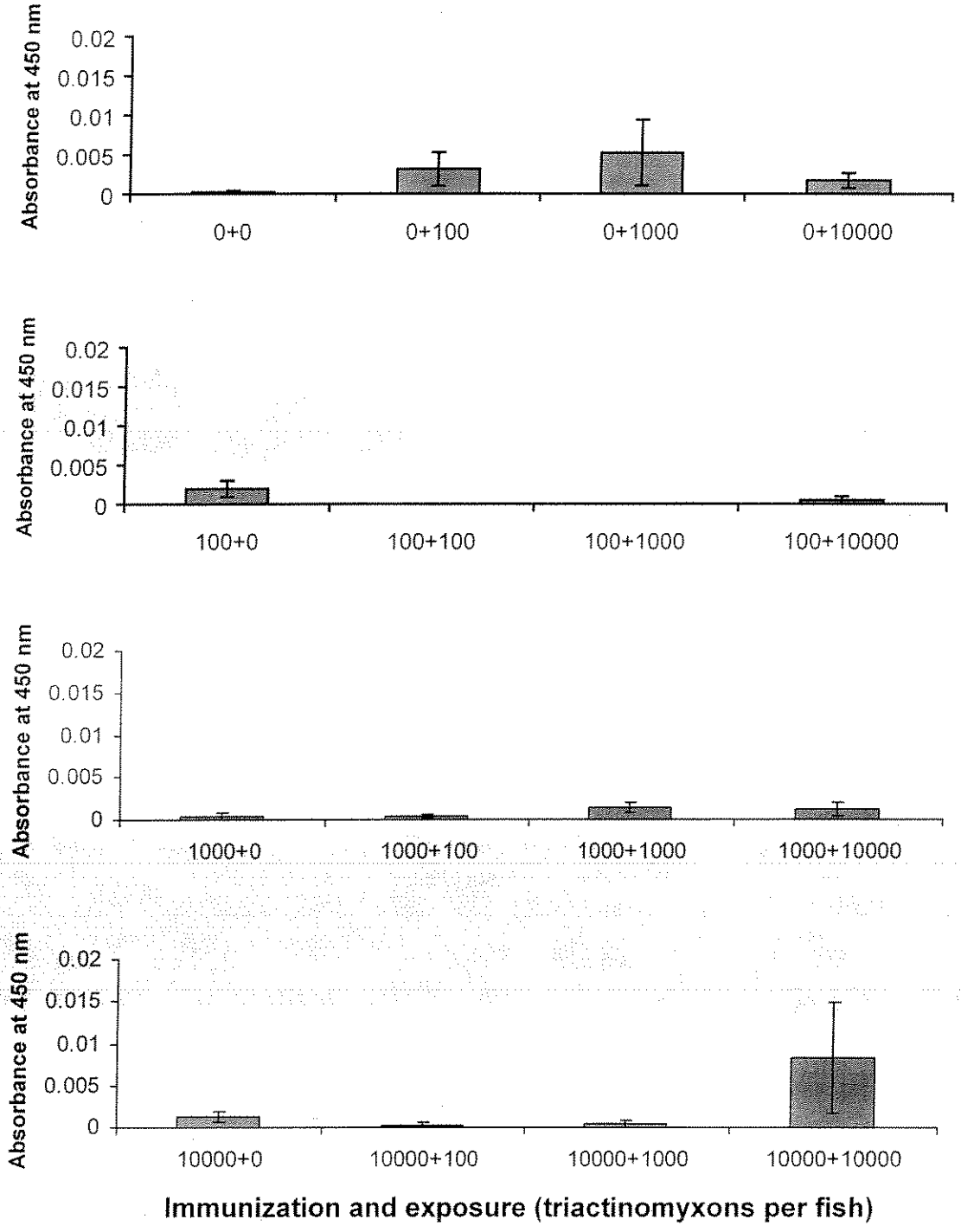


Figure 4.16—Level of serum anti-*Myxobolus cerebralis* antibodies measured as mean absorbance at 450 nm (±SE), 1 month after the exposure. Fish were immunized at 5 weeks posthatch to a range of parasite doses (0, 100, 1,000, or 10,000 triactinomyxons per fish) followed by an exposure at 9 weeks posthatch to a range of parasite doses (0, 100, 1,000 or 10,000 triactinomyxons per fish).

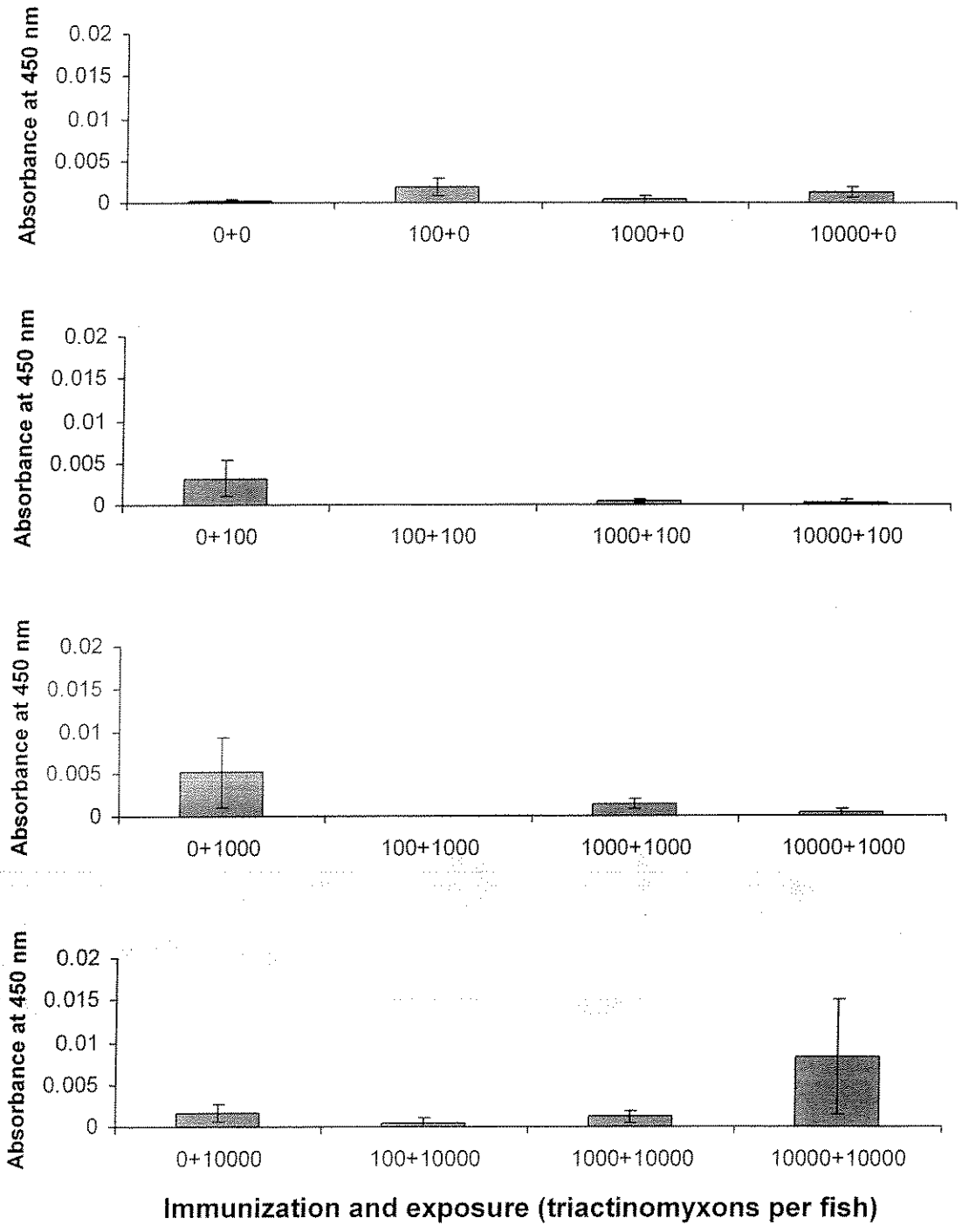


Figure 4.17—Level of serum anti-*Myxobolus cerebralis* antibodies measured as mean absorbance at 450 nm (±SE), 1 month after the exposure. Fish were immunized at 5 weeks posthatch to a range of parasite doses (0, 100, 1,000, or 10,000 triactinomyxons per fish) followed by an exposure at 9 weeks posthatch to a range of parasite doses (0, 100, 1,000 or 10,000 triactinomyxons per fish).

The exposure dose ($F = 3.77$, $P = 0.0212$; Figure 4.18) and the immunization dose ($F = 3.81$, $P = 0.0203$; Figure 4.19) did however influence the amount of anti-*M. cerebralis* antibodies present in the serum collected at 20 weeks after the immunization dose. The treatment groups, which received an exposure but no immunization, had significantly more anti-*M. cerebralis* antibodies than the negative controls (Figure 4.18). However, the exposure dose failed to influence the anti-*M. cerebralis* antibodies when the immunization dose was maintained constant at either 100 or 10,000 triactinomyxons per fish (Figure 4.18). Fish immunized with 1,000 triactinomyxons per fish and exposed with 1,000 triactinomyxons per fish had significantly lower levels of anti-*M. cerebralis* antibodies than the groups exposed to either 100 or 10,000 triactinomyxons per fish and immunized with 1,000 triactinomyxons per fih (Figure 4.18). However, the fish exposed and immunized to 1,000 triactinomyxons per fish had the same amount of anti-*M. cerebralis* antibodies as the fish immunized to 1,000 triactinomyxons per fish but not exposed, as did the groups exposed to either 100 or 1,000 triactinomyxons per fish (Figure 4.18).

All treatment groups immunized but not exposed had significantly higher levels of the antibody than the negative controls at 20 weeks after the immunization (Figure 4.19). However, treatment groups exposed to either 100 or 1,000 triactinomyxons per fish had no significant difference in anti-*M. cerebralis* antibody levels regardless of whether or not they were immunized (Figure 4.19). Fish exposed to 10,000 triactinomyxons per fish and immunized with 1,000 triactinomyxons per fish had significantly higher levels of anti-*M. cerebralis* antibodies than the corresponding group

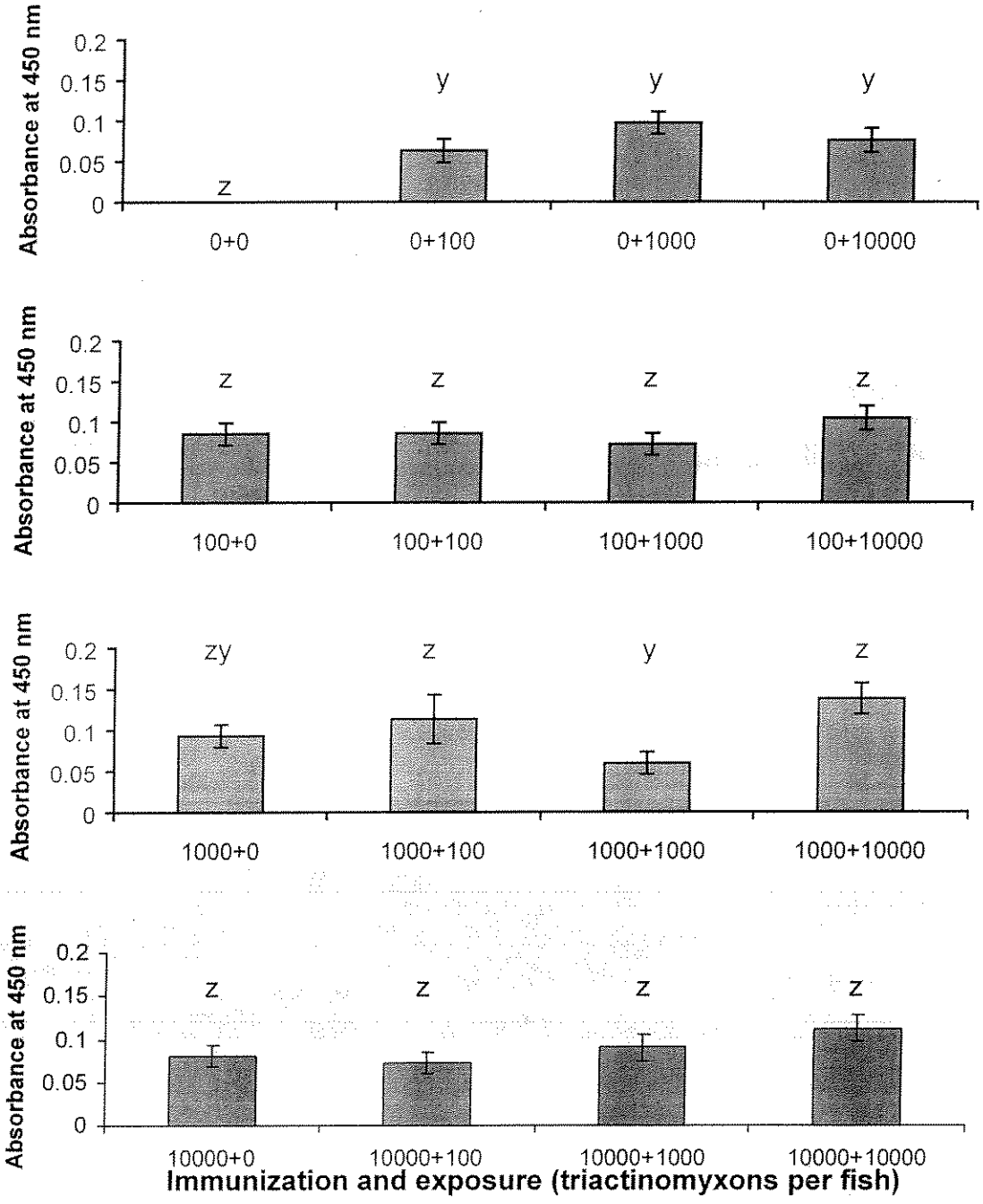


Figure 4.18—Level of serum anti-*Myxobolus cerebralis* antibodies measured as mean absorbance at 450 nm (±SE), 20 weeks after the immunization. Fish were immunized at 5 weeks posthatch to a range of parasite doses (0, 100, 1,000, or 10,000 triactinomyxons per fish) followed by an exposure at 9 weeks posthatch to a range of parasite doses (0, 100, 1,000 or 10,000 triactinomyxons per fish). Within each horizontal graph different letters indicate significant differences (Bonferroni's multiple comparisons procedure $P < 0.05$).

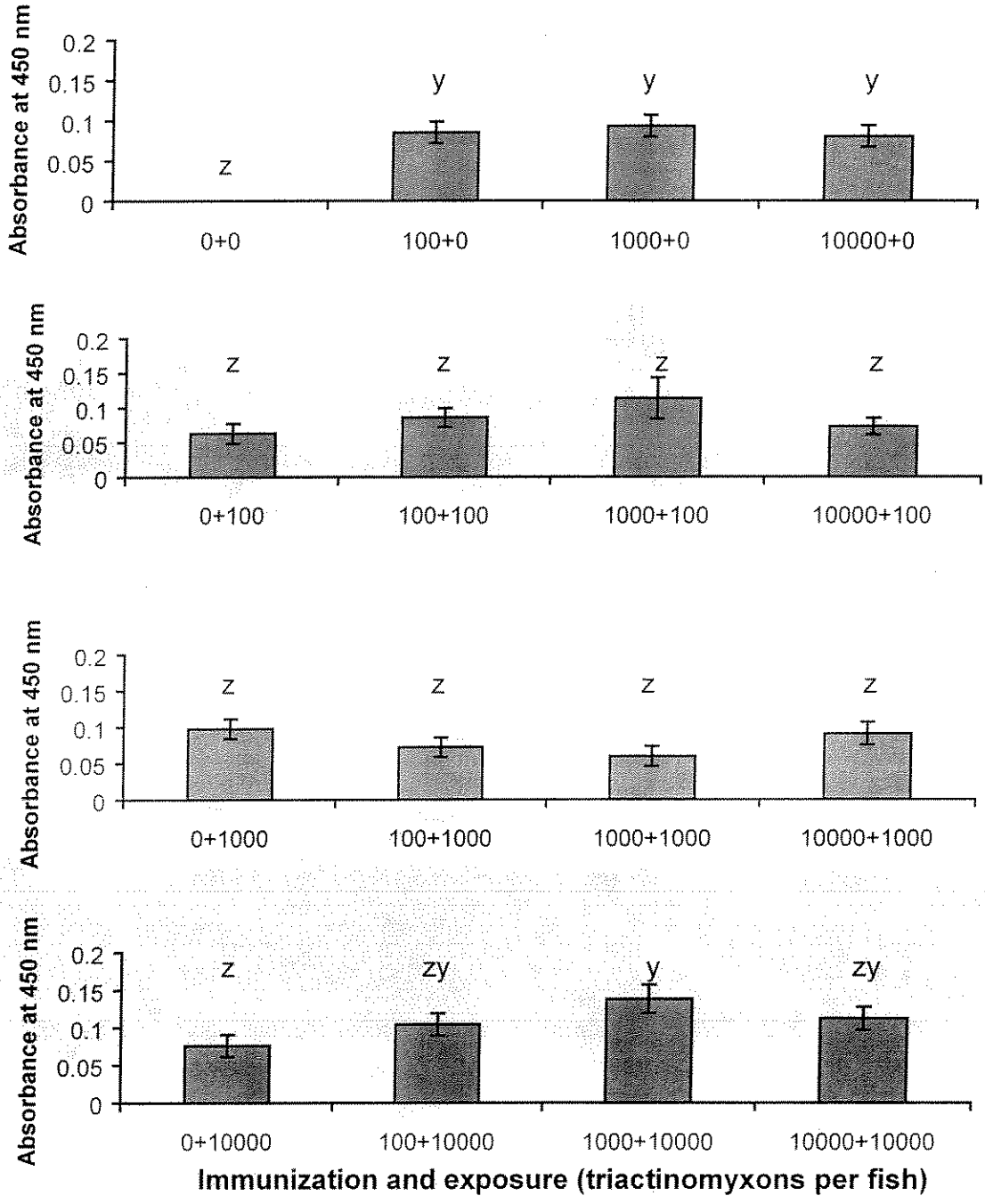


Figure 4.19—Level of serum anti-*Myxobolus cerebralis* antibodies measured as mean absorbance at 450 nm (±SE), 20 weeks after the immunization. Fish were immunized at 5 weeks posthatch to a range of parasite doses (0, 100, 1,000, or 10,000 triactinomyxons per fish) followed by an exposure at 9 weeks posthatch to a range of parasite doses (0, 100, 1,000 or 10,000 triactinomyxons per fish). Within each horizontal graph different letters indicate significant differences (Bonferroni's multiple comparison procedure $P < 0.05$).

which was not immunized (Figure 4.19); however, no significant differences were present among the groups exposed to 10,000 triactinomyxons per fish and immunized with either 100, 1,000 or 10,000 triactinomyxons per fish.

Serum antibody levels: 13-week posthatch exposure

Two hours after the exposure at 13 weeks posthatch, neither the exposure dose ($F = 0.37$, $P = 0.7733$; Figure 4.20) nor the immunization dose (F = 0.24, P 0.8684; Figure 4.21) significantly affected the level of anti-*M. cerebralis* antibodies. Antibody levels recorded from the fish 2 hours after the exposure were not significantly greater than that from the negative controls.

One month after the 13-weeks posthatch exposure the immunization dose did significantly affect the level of anti-*M. cerebralis* antibodies present in the fish ($F = 12.50$, $P < 0.0001$; Figure 4.22); however, the exposure dose continued to fail to affect the level of antibodies present (F = 0.21, P = 0.8877; Figure 4.23). The level of anti-*M. cerebralis* antibodies present in the serum 1 month after the exposure increased with increasing level of immunization dose (Figure 4.22).

Twenty weeks after the immunization both the exposure dose ($F = 2.99$, $P = 0.0309$, Figure 4.24) and the immunization dose ($F = 39.67$, $P < 0.0001$, Figure 4.25) had significantly affected the level of anti-*M. cerebralis* antibodies present in the fish serum amongst some of the treatment groups. However, the exposure dose failed to affect the amount of anti-*M. cerebralis* antibodies present unless the fish had first received an immunization dose; and only slight differences in antibody production existed between the fish that received an immunization and those that received both the immunization and

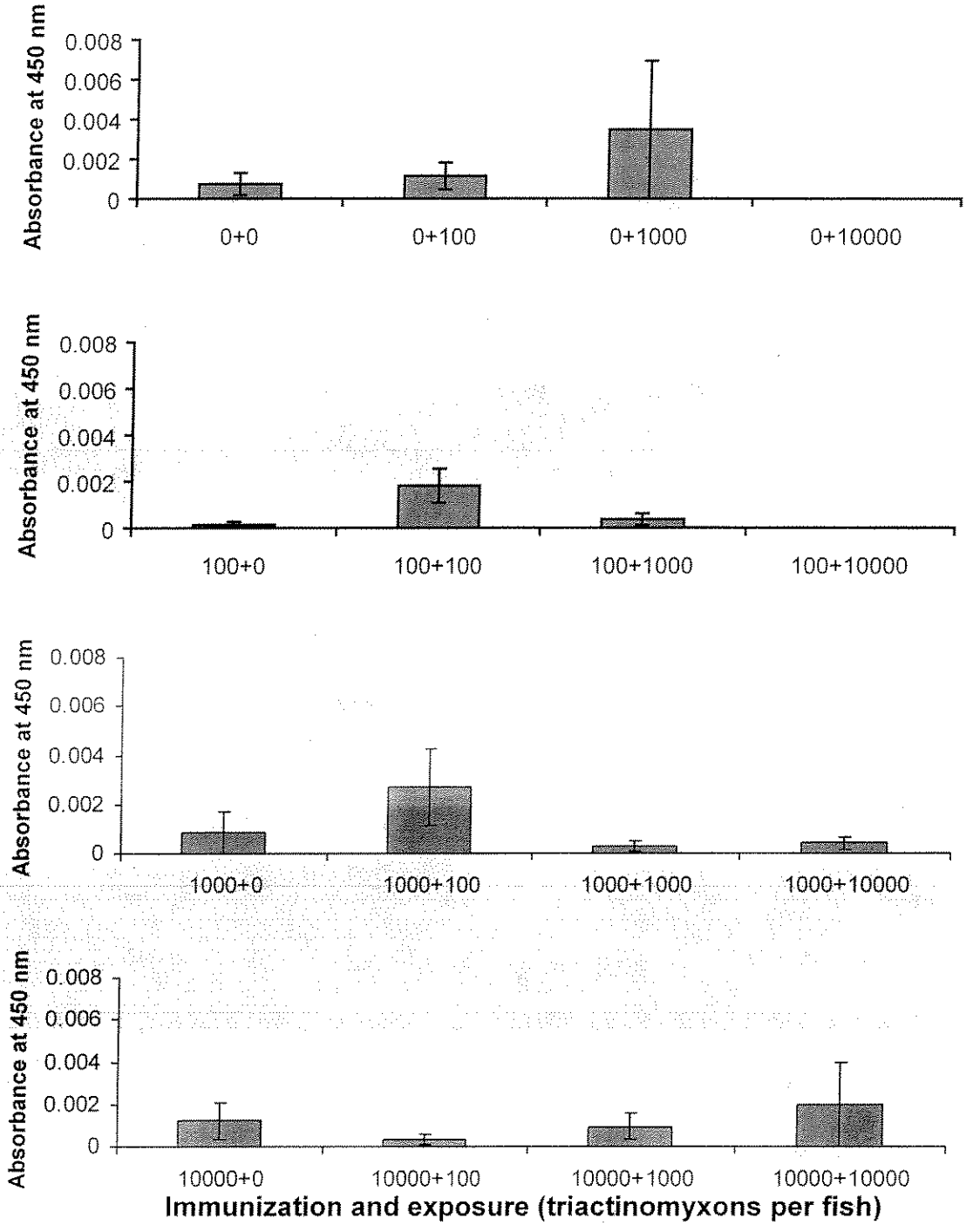


Figure 4.20—Level of serum anti-*Myxobolus cerebralis* antibodies measured as mean absorbance at 450 nm (±SE), 2 hours after the exposure. Fish were immunized at 5 weeks posthatch to a range of parasite doses (0, 100, 1,000, or 10,000 triactinomyxons per fish) followed by an exposure at 13 weeks posthatch to a range of parasite doses (0, 100, 1,000 or 10,000 triactinomyxons per fish).

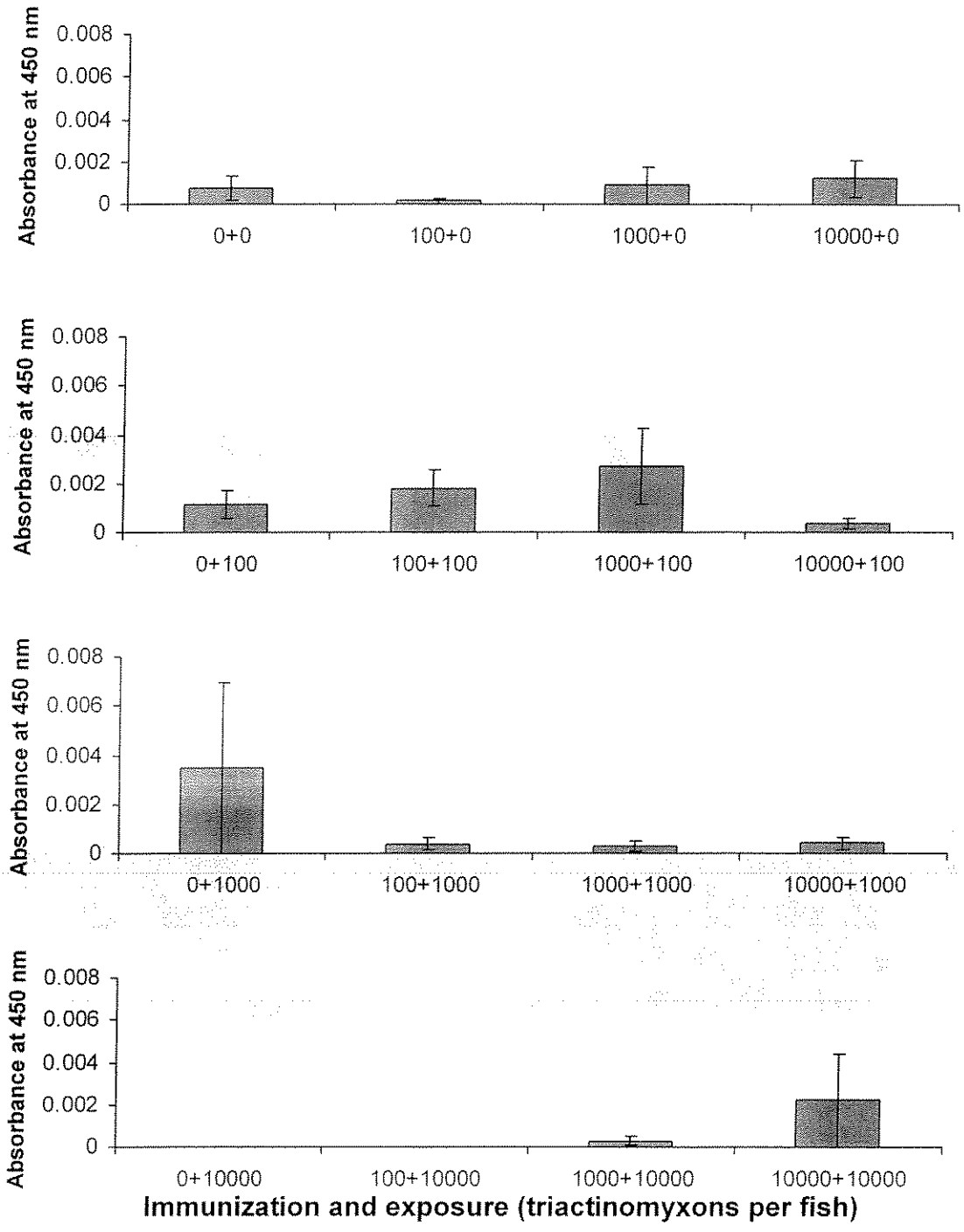


Figure 4.21—Level of serum anti-*Myxobolus cerebralis* antibodies measured as mean absorbance at 450 nm (±SE), 2 hours after the exposure. Fish were immunized at 5 weeks posthatch to a range of parasite doses (0, 100, 1,000, or 10,000 triactinomyxons per fish) followed by an exposure at 13 weeks posthatch to a range of parasite doses (0, 100, 1,000 or 10,000 triactinomyxons per fish).

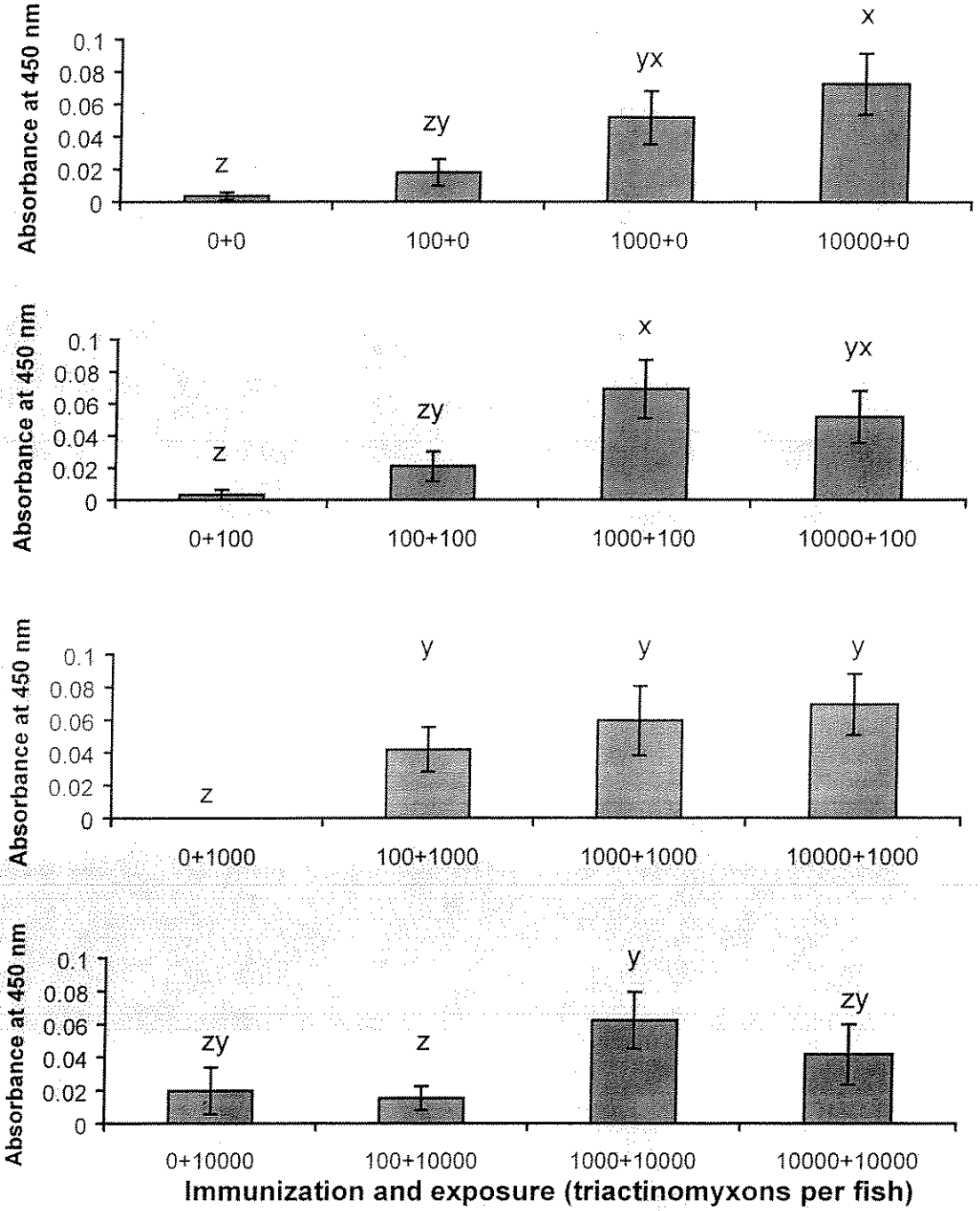


Figure 4.22—Level of serum anti-*Myxobolus cerebralis* antibodies measured as mean absorbance at 450 nm (±SE), 1 month after the exposure. Fish were immunized at 5 weeks posthatch to a range of parasite doses (0, 100, 1,000, or 10,000 triactinomyxons per fish) followed by an exposure at 13 weeks posthatch to a range of parasite doses (0, 100, 1,000 or 10,000 triactinomyxons per fish). Within each horizontal graph different letters indicated significant differences (Bonferroni's multiple comparison procedure $P < 0.05$).

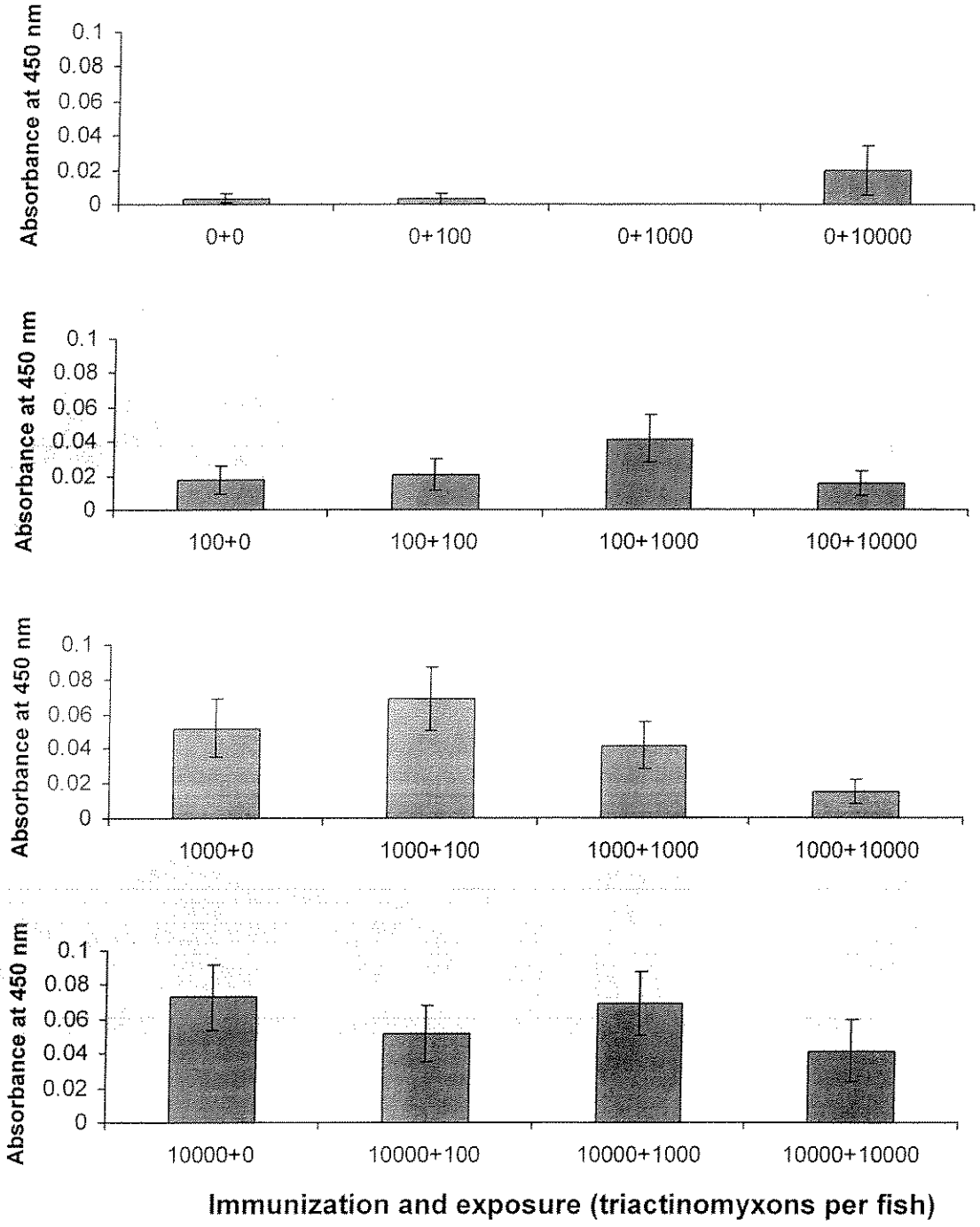


Figure 4.23—Level of serum anti-*Myxobolus cerebralis* antibodies measured as mean absorbance at 450 nm (±SE), 1 month after the exposure. Fish were immunized at 5 weeks posthatch to a range of parasite doses (0, 100, 1,000, or 10,000 triactinomyxons per fish) followed by an exposure at 13 weeks posthatch to a range of parasite doses (0, 100, 1,000 or 10,000 triactinomyxons per fish).

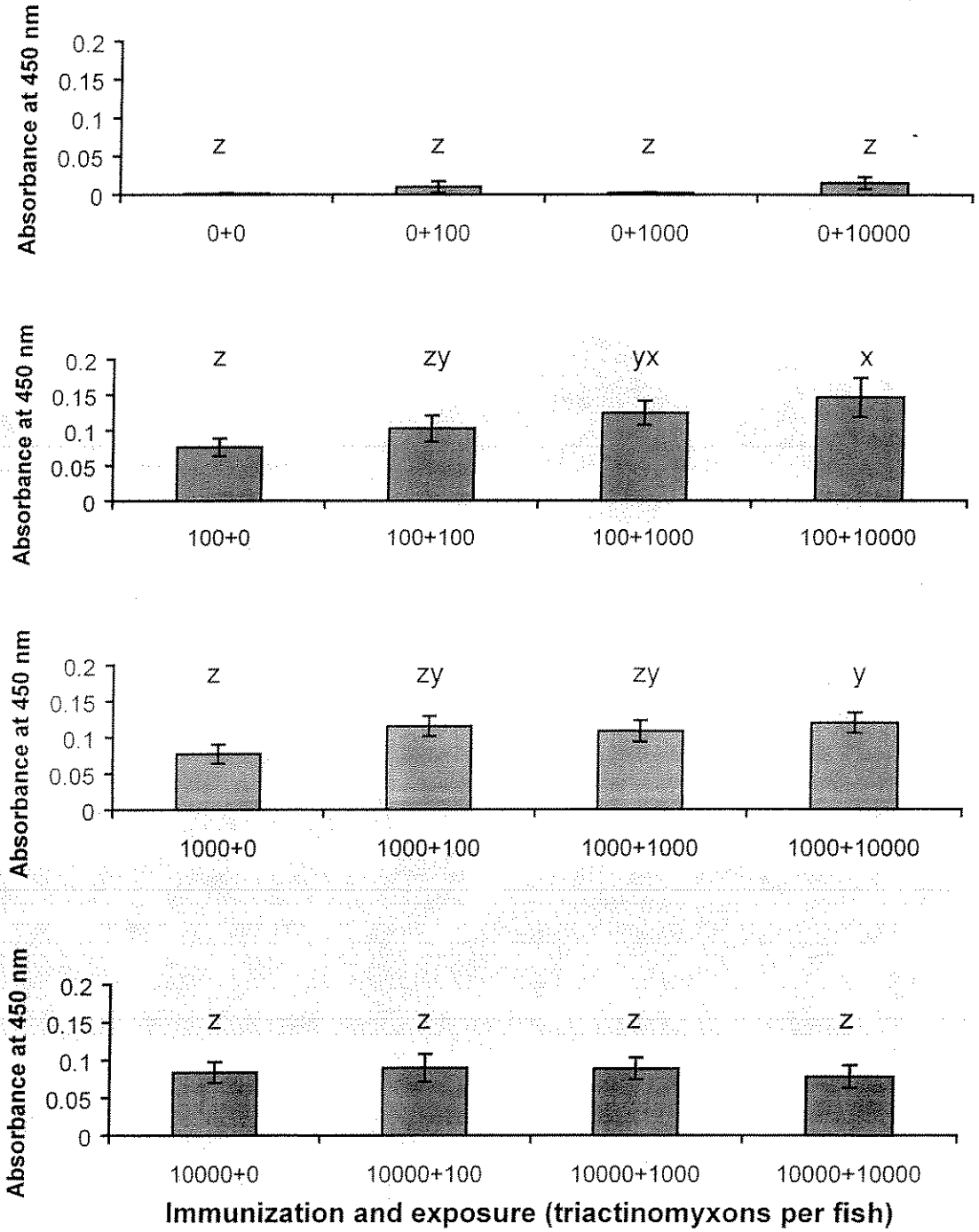


Figure 4.24—Level of serum anti-*Myxobolus cerebralis* antibodies measured as mean absorbance at 450 nm (±SE), 20 weeks after the immunization. Fish were immunized at 5 weeks posthatch to a range of parasite doses (0, 100, 1,000, or 10,000 triactinomyxons per fish) followed by an exposure at 13 weeks posthatch to a range of parasite doses (0, 100, 1,000 or 10,000 triactinomyxons per fish). Within each horizontal graph different letters indicate significant differences (Bonferron's multiple comparison procedure $P < 0.05$).

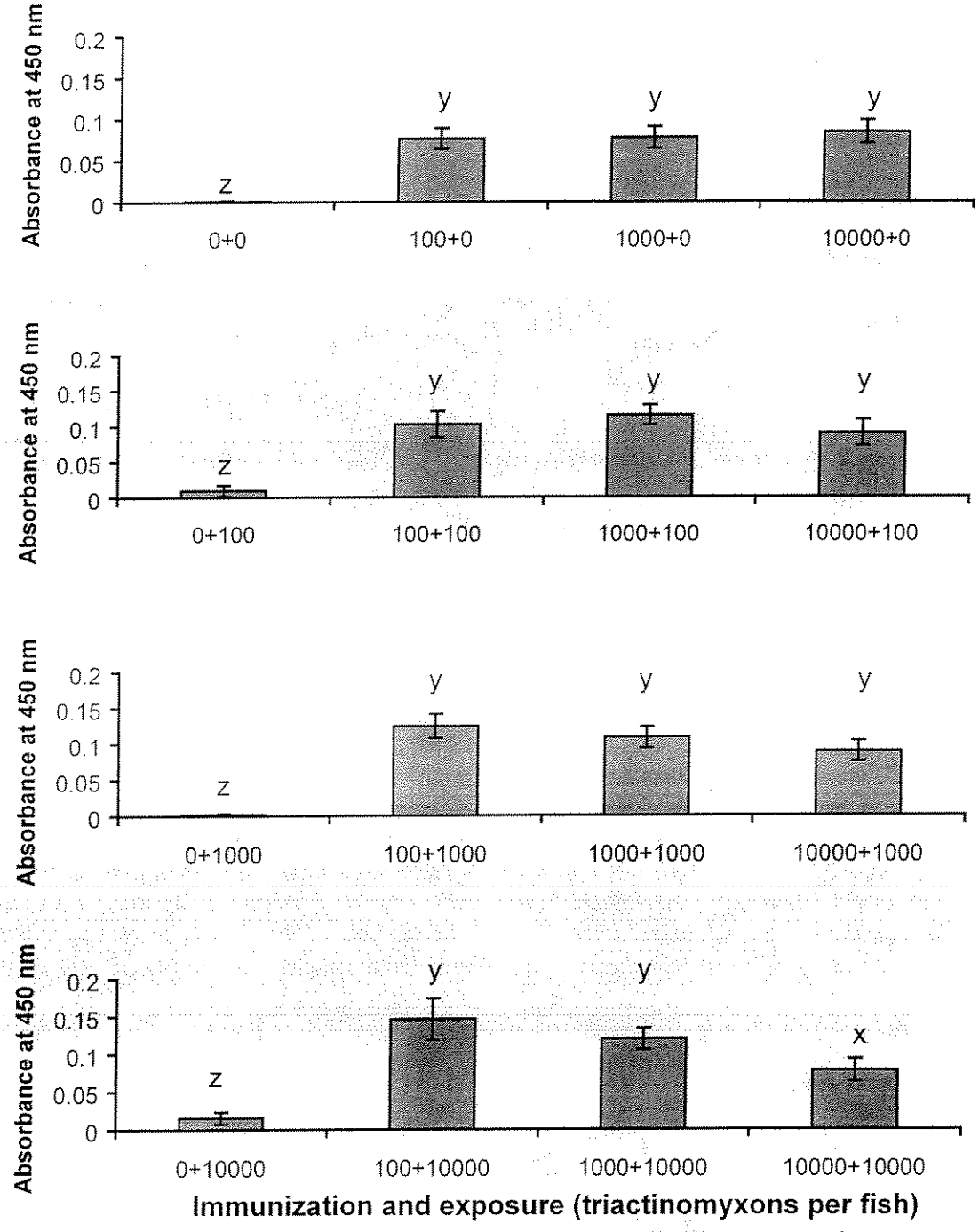


Figure 4.25—Level of serum anti-*Myxobolus cerebralis* antibodies measured as mean absorbance at 450 nm (±SE), 20 weeks after the immunization. Fish were immunized at 5 weeks posthatch to a range of parasite doses (0, 100, 1,000, or 10,000 triactinomyxons per fish) followed by an exposure at 13 weeks posthatch to a range of parasite doses (0, 100, 1,000 or 10,000 triactinomyxons per fish). Within each horizontal graph different letters indicate significant differences (Bonferroni's multiple comparison procedure $P < 0.05$).

the exposure (Figure 4.24). All treatment groups that received an immunization dose had significantly higher levels of anti-*M. cerebralis* present 20 weeks after the immunization than those that did not receive an immunization dose ($F = 12.50$, $P < 0.0001$; Figure 4.25). For the fish that either received no exposure dose or an exposure dose of either 100 or 1,000 triactinomyxons per fish, no significant difference existed in response amongst those immunized with either 100, 1,000 or 10,000 triactinomyxons per fish ($F = 12.50$, $P > 0.0001$; Figure 4.25). The fish immunized and exposed with 10,000 triactinomyxons per fish had significantly lower levels of anti-*M. cerebralis* antibodies than the fish that received the same exposure dose level but lower levels of immunization ($F = 12.50$, $P < 0.0001$; Figure 4.25).

Discussion

The first part of this objective was to determine if an immunization with *M. cerebralis* triactinomyxons provided rainbow trout with protection against subsequent exposures to *M. cerebralis*. Protection against an exposure at either 4 or 8 weeks after the immunization was evidenced; however, the immunization, which provided the fish with protection against the development of disease induced by a subsequent exposure, also induced disease in the fish. Therefore, the disease that the immunization induced in the fish far outweighed the benefits the fish received from the protection. The second part of this objective was to determine if rainbow trout develop a specific acquired immune response after exposure to triactinomyxons of *M. cerebralis*. Rainbow trout do develop an acquired immune response to *M. cerebralis*, as evidenced by the presence of

anti-*M. cerebralis* antibodies in serum from infected fish, confirming earlier indirect fluorescent antibody (Griffin and Davis 1978) and ELISA tests (Hedrick et al. 1998). The first incidence of anti-*M. cerebralis* antibodies was detected one month after the 13-week posthatch exposure. The detection of antibodies in rainbow trout at this time was assumed to be solely the result of their immunization at 5 weeks posthatch, because the exposure was found to not significantly influence the presence of antibodies. Therefore, no anti-*M. cerebralis* antibodies were detected until 12 weeks after the immunization. Hypothesis 3.1 and 3.2 of this dissertation were therefore rejected.

The protection provided by the immunization exposure did not correlate with the observed acquired immune response after exposure to *M. cerebralis*. Specific anti-*M. cerebralis* antibodies were not detected in the fish until 12 weeks after initial exposure; however, protection against the effects of an exposure was provided at 4 weeks after the immunization. Therefore, something other than the antibody response must have been responsible for the observed protection. One possibility is that the innate (or non-specific) immune response in the fish was activated by the first exposure and although it did not provide the fish with protection against the initial exposure it may have been able to defend the fish against the subsequent exposure. A similar situation is observed in human disease. In humans the non-specific immune system is stimulated by infection. The number and functionality of phagocytes increase as an immediate consequence of infection. In addition, there is a general increase in the number and activity of the other fractions of the non-specific immune system (Relman and Falkow 1995). Similarly, the non-specific immune system of newborn pigs is rapidly activated after the pigs first encounter with pathogens (Sinkora et al. 2002). After *M. cerebralis* infects rainbow trout

it is susceptible to the effects of the non-specific immune system of the fish for a relatively short time (4 days) before it enters the nervous system of the fish where it is protected against the immune responses of the fish (El-Matbouli et al. 1995). Therefore, after the initial infection by the immunization exposure any stimulation of the non-specific immune system may not be effective quick enough to influence the effects of the immunization; however, the immune system may be stimulated long enough to reduce the impacts of any later exposures.

The fact that specific anti-*M. cerebralis* antibodies were not detected until 12 weeks after the first infection is explained by the life history of the pathogen and the host response to it. Generation of an antigen-specific antibody response first requires activation from the host cellular immune response (Janeway et al. 1999; Goldsby et al. 2002). The parasite is protected from the host immune system while it harbors in the host nervous system (Hoffmann et al. 1991, cited in El-Matbouli et al. 1995), and does not induce a significant cellular immune response in the host until after significant damage has been caused to the host cartilage, which occurs at least 2 months after infection (Hedrick et al. 1998), resulting in the specific acquired immune response being mounted and antigen specific antibodies being produced.

The immune response increased, as measured by an increase in serum anti-*M. cerebralis* antibodies, when the level of the first parasite dose the fish received increased. I demonstrated in Chapter 2 that whirling disease severity in susceptible rainbow trout increases with increasing levels of parasite dose. Therefore, as the severity of whirling disease increases, including an increase in cellular host immune response, a concomitant increase in the specific humoral (antibody) immune response occurs. Microscopic

pathology in rainbow trout caused by *M. cerebralis* characteristically contains remnants of cartilage, parasites, and a diffuse to focal granulomatous infiltrate of host macrophages and lymphocytes (Hedrick et al. 1998). The combined pressure of the parasites and host inflammatory cells on the brain stem and spinal cord is thought to give rise to some of the whirling disease clinical signs, namely blacktail and the whirling swimming behavior (Rose 2000). Therefore, severity of disease symptoms increases as the immune response increases with increasing level of parasite dose.

Fish exposed at 13 weeks posthatch with no prior immunization exhibited no difference in the presence of anti-*M. cerebralis* antibodies when compared to that of the control fish, whereas fish exposed at 9 weeks posthatch with no prior immunization showed a significant increase in the presence of anti-*M. cerebralis* antibodies when compared to the controls. This can be explained by the age of the fish at exposure to *M. cerebralis* triactinomyxons. I demonstrated in Chapter 2 that resistance to the development of whirling disease in rainbow trout occurs 9 weeks after hatch. Therefore, the fish exposed to *M. cerebralis* triactinomyxons for the first time at 13 weeks posthatch did not produce an acquired immune response to *M. cerebralis* because they were resistant to the development of the disease. Because the fish exposed at 13 weeks posthatch were resistant to the development of the disease they produced no cellular host immune response and resulting antigen specific immune response, which would only be induced by damage caused by the parasite to the cartilage.

Protection provided to rainbow trout from an acquired immune response does not determine whether or not the fish will develop whirling disease. By the time the humoral immune response developed, the fish were physiologically resistant to the development

of whirling disease because of their age (see Chapter 2). I demonstrated in this chapter that the parasite dose level the fish received during their first exposure to *M. cerebralis* triactinomyxons was the most critical factor for determining the severity of whirling disease. I demonstrated in Chapter 2 and 3 that rainbow trout develop a resistance to whirling disease at 9 weeks posthatch or 756 degree-days of development. Therefore, the level of *M. cerebralis* triactinomyxon dose that the rainbow trout receive during this critical period will determine the severity of whirling disease the fish will develop, regardless of subsequent exposures after this critical period. Although the immunization exposure provided the fish with protection against subsequent exposures it failed to prevent them from developing the disease. Therefore, control of the pathogen and the disease it manifests can only be achieved by maintaining rainbow trout in *M. cerebralis*-free waters during the period that they are susceptible to the development of the disease. Several recommendations were made in Chapter 2 to achieve this in hatcheries and in the wild.

The development of a vaccination for use as a management tool in hatcheries could be developed in future investigations. I demonstrated that protection was provided 4 weeks after an immunization exposure; therefore, a vaccine, which does not induce disease in the fish, may be able to provide them with protection against the parasite at least 4 weeks after they are vaccinated. Any vaccination would have to be administered before the fish are stocked into positive waters with enough time being allowed between vaccination and exposure for the immune response to the parasite to develop. An attenuated (non pathogenic) or dead form of the pathogen may be able to induce an immune response in the fish without causing the development of the disease. A bath or

oral vaccination would be the most efficient means of administering the vaccine to large numbers of fish; however, the best route of vaccination to induce an appropriate immune response against the parasite would need to be determined. If injections are determined to be the only effective way to administer an *M. cerebralis* vaccine, then vaccination may not be feasible because of extra handling and economic costs. If rainbow trout can be vaccinated against the pathogen they could be stocked or reared in *M. cerebralis*-positive waters during their first 9 weeks (756 degree-days) after hatch without the risk of developing the disease. Vaccinations are most likely only to be a useful management tool in hatchery systems.

Several examples exist in human medicine that can be compared to the immune response of rainbow trout to *M. cerebralis*, where the disease is caused not by the pathogen itself but by the immune response to the pathogen. For example, much of the disease caused by *Mycobacterium tuberculosis*, the causative agent of tuberculosis, results from the immune response that the pathogen induces. The inhaled bacilli are ingested by alveolar macrophages where they can survive and multiply. When the macrophages lyse large numbers of bacilli are released resulting in activation of T cells which induce the infiltration of large numbers of activated macrophages into the infected area. These cells wall off the organism inside a granulomatous lesion called a tubercle. The massive activation of macrophages that occurs within tubercles often results in the concentrated release of lytic enzymes. These enzymes destroy nearby healthy cells, resulting in areas of necrotic tissue which eventually becomes calcified. In the majority of people infected with *Mycobacterium tuberculosis* the T-cell mediated immune response controls the infection and later protects against infection (Adams 1976;

Dannenberg 1989). Therefore, similarly to the infections caused by *Myxobolus cerebralis* the immune response results in disease symptoms; however, protection is provided against later re-infections. The difference with whirling disease is that the protection provided against re-infection is irrelevant because the damage caused by the parasite before the protection is induced is so extensive that the fish are severely compromised by the infection.

Lyme disease, caused by the bacteria *Borrelia burgdorferi*, is an example of a human disease in which the immune response causes the disease and fails to provide the patient with protection. Specific antibodies are produced against *B. burgdorferi*; however, the antibodies fail to confer protection and instead are the cause of the pathogenesis of the disease. Immune complexes consisting of the bacteria antigens and antibody are thought to result in a hypersensitive reaction. The antigen-antibody complexes can activate the complement system, resulting in direct lytic damage to the joints or vasculature. Alternatively, complement (a group of serum proteins that participate in enzymatic cascade, resulting in the production of a cytolytic attack complex) products can induce neutrophil chemotaxis and activation. Some tissue damage can then result from lytic enzymes released by the activated neutrophils (Evans 1998). Therefore, the whirling disease pathogen is not alone in inducing an immune response that contributes to the pathogenesis of the disease.

An example of a fish disease with a similar immune response to that caused by the whirling disease pathogen is bacterial coldwater disease caused by *Flavobacterium psychrophilum*. Clinical signs of bacterial coldwater disease can be similar to that of whirling disease, including skeletal deformities, blacktail and a whirling swimming

behavior. The cause of these symptoms is related to an inflammatory response as part of the immune response mounted by the host in order to control the pathogen (Meyers 1989). The inflammatory response induced by the bacteria helps control the spread of the bacterium but it is unclear if the response provides the fish with protection against subsequent exposures.

The immune response of vertebrate hosts has evolved to protect the host against attack by parasites. Parasites have in turn, evolved a wide array of mechanisms to counter the immune response. *Myxobolus cerebrals* avoids the immune system of its host by using the nervous system to travel to areas of cartilage. If the parasite were to use some other means, such as the circulatory system, to travel to areas of cartilage the host cellular immune response would be induced sooner and the parasite could be destroyed before reaching the cartilage. Brown trout show a greater resistance to the development of whirling disease than rainbow trout and will only become infected when exposed to high triactinomyxon densities (Hedrick et al. 1999a). The granulomatous inflammation often associated with the parasite is less effective at destroying or restricting the parasite in rainbow trout than in brown trout (Hedrick et al. 1999a). Other host immune responses in brown trout may also be responsible for retarding or eliminating the parasites at each stage of development as they move through the three principal tissue compartments of skin, nerves, and cartilage in the fish. Perhaps fewer parasites attach, invade and replicate in the early stages of infection in the epidermis of brown trout compared to rainbow trout. Brown trout are thought to have co-evolved with *M. cerebralis* in Eurasia (Hoffman 1970), whereas rainbow trout have been isolated from *M. cerebralis*. Rainbow trout, over time, develop an immune response that could provide the

fish with greater protection against the parasite. A population of rainbow trout with an apparent resistance to whirling disease has been identified in Germany (Kent et al. 2001). These "resistant" rainbow trout from Germany, although originally from North America, have been raised in Germany in *M. cerebralis*-positive waters for up to 110 years. The mechanism for this resistance is unknown; however, the resistant population may have had sufficient time to evolve defenses to the pathogen and develop an immune response similar to brown trout providing the fish with protection against development of the disease. Given time, rainbow trout in North America may also develop resistance to the pathogen; however, before this is achieved many more populations are likely to suffer from the results of whirling disease. If we want to preserve the fisheries resource as it is now and limit the effects of the disease we need to intervene and use the information we have on the relationships between host and pathogen to better control the parasite and the disease it manifests.

One major problem with the design of this experiment was that disease severity was measured at 20 weeks after exposure. This equated to 11 weeks after exposure for the fish exposed at 9 weeks posthatch, and 7 weeks after exposure for the fish exposed at 13 weeks posthatch. These times after exposure may not have been sufficiently long enough for all of the whirling disease symptoms to develop fully. However, it still stands that specific antibodies were not produced until 12 weeks after exposure by which time fish were resistant to the development of the disease because of their age. Therefore, it can still be concluded that the most important factor in terms of the development of resistance to whirling disease in rainbow trout is the age at which they are first exposed to the parasite.

CHAPTER 5

CONCLUSIONS, MANAGEMENT IMPLICATIONS AND FUTURE INVESVESTIGATIONS

I showed in Chapter 2 that the development and severity of whirling disease pathology in rainbow trout is dependent on the age of fish when first exposed to the triactinomyxon stage of *Myxobolus cerebralis*, and that the effects of whirling disease on rainbow trout are substantially reduced when they are exposed to the parasite for the first time at 9 weeks posthatch or older, as compared to fish exposed at younger ages. In Chapter 3 I showed that both the age of fish at first exposure and the size of fish at first exposure are important for the development of whirling disease; therefore, age may not be the best measurement of when young trout become resistant to the effects of the disease. I demonstrated in Chapter 4 that although rainbow trout are protected against subsequent exposures after an immunization exposure, and although rainbow trout do develop an acquired immune response after exposure to *M. cerebralis*, the parasite dose level the fish receives during their first exposure to *M. cerebralis* triactinomyxons is the most critical factor for determining the severity of whirling disease. Therefore, my primary recommendation for reducing the effects of whirling disease on rainbow trout, which incorporates the information gained from the three objectives of this study is: rainbow trout reared in *M. cerebralis*-free waters for 756 degree-days of development or until they are 40 mm in length, whether in the wild or in a hatchery situation, should exhibit enhanced survival and swimming performance, and reduced prevalence of clinical

signs, spore counts and severity of microscopic pathology, in comparison to fish first exposed to the parasite at an earlier stage of development.

Understanding the factors that affect the pathogenesis of any pathogen is crucial to its control. The information gained from my dissertation work can be used to develop management strategies useful for use in hatcheries and in the management of wild trout fisheries. In hatcheries, whether the rainbow trout being reared are for stocking into infected waters or are for food production, if the fish are maintained in *M. cerebralis*-free water for the first 756 degree-days of development (9 weeks at 12 °C) after hatch the production and survival of the fish will be greatly increased.

Whereas the situation is simple in hatcheries, controlling the pathogen and the disease it manifests in the wild is more complicated. When and where rainbow trout spawn, when the fry leave the redd, where the fry rear, and spatial and temporal triactinomyxon abundances, are all essential to any management strategy (Downing et al. 2002). Habitats known to be *M. cerebralis*-free, and where rainbow trout spawn and rear, should be maintained and conserved to encourage the fish to rear in these areas, especially if later in their life history they are known to travel into areas where the parasite is present. Flushing flows, such as an increased release from a dam, may be used to dilute triactinomyxons, and reduce the likelihood of infection and disease during periods when high triactinomyxon abundances are known to coincide with the rearing of susceptible juvenile salmonids. Early spawning strains may also be stocked in areas where emergence and rearing of susceptible juveniles currently coincides with times of high triactinomyxon abundance. Introducing early spawners into the system will result in fry hatching earlier and theoretically rearing and developing to an age that provides them

with protection against the development of the disease before triactinomyxon abundances are at their highest. Alternatively, eggs could be collected and fertilized from adult rainbow trout returning to the spawning areas earlier than the average spawners in the population. The fry from these collections could subsequently be reared in a hatchery and stocked back into the system to increase the numbers of early spawners within the population. The fry of early spawning fish have a greater chance of survival; therefore, early spawning fish may also become dominant within the population through the process of natural selection.

The information gained from this work can be applied to the management and control of whirling disease in wild and cultured rainbow trout populations. However, it may be less applicable to the management of other salmonid species and perhaps also to other strains of rainbow trout. The rainbow trout is the principal salmonid host for the parasite and is therefore the species most commonly studied in whirling disease research. Most other salmonid species are susceptible to the effects of the disease also, but to varying degrees (MacConnell and Vincent 2002). The relationship of increasing age or size at exposure and decreasing risk of development of disease is likely also present amongst the other whirling-disease susceptible salmonid species, but the threshold level when the fish become resistant to the development of the disease is likely to be different. The general principles demonstrated in my work (increasing age or size and decreasing levels of parasite dose result in a decrease in the effects of whirling disease) can be applied loosely to the management of any salmonid species in *M. cerebralis*-positive areas. However, managing a mixed salmonid population or a population other than

rainbow trout in a positive area may require more specific information as to when the particular species being managed becomes resistant to the development of the disease.

Several areas of future investigation arose from this work which would be beneficial to the management of the disease in both the wild and in hatcheries. Future investigations involving exposures of fish to a wider range of parasite doses than used in this experiment could be very beneficial to the management and understanding of whirling disease. Increasing parasite dose at exposure produced an increase in disease severity in rainbow trout exposed at 9 weeks posthatch or younger. An increase in parasite dose exposure increased the disease severity in rainbow trout, but this was only demonstrated in a very broad sense using triactinomyxon doses of different orders of magnitude. For the precise assessment of the effects of smaller differences in parasite dose we would need to know in more detail the effects of increasing parasite dose on the development of whirling disease severity. A parasite dose level likely exists beyond which increasing levels fail to increase whirling disease severity. Similarly, a minimum triactinomyxon dose below which whirling disease does not develop in the fish is likely to exist. Exact determination of these thresholds would be useful.

Further areas of investigation, which became apparent from this work, include refining the histological grading scale used for determining the severity of whirling disease. The most commonly used index of whirling disease severity, both in laboratory exposures and in sentinel fish exposures, is the histological pathology index of severity (as used in this experiment) (MacConnell-Baldwin scale, Hedrick et al. 1999b). Although this histology scale gives the investigator an index of microscopic pathology severity, it does not provide the investigator with absolute information on the dose of

triactinomyxons to which the fish were exposed. Only relative relations can be inferred. The MacConnell-Baldwin scale provides qualitative information on microscopic pathology severity. However, it does not provide quantitative information on how the different categories relate to disease severity. An additional common mistake made in whirling disease research is to treat the MacConnell-Baldwin grades as if they were quantitative continuous data, with grades commonly being reported as means. This is statistically incorrect because the scale is not technically continuous and the difference in pathology between the grades is not known to be equal in size. Therefore, a great need is present for this scale to be studied in more detail and to be calibrated so that the data can be treated properly using more powerful parametric techniques, instead of the current situation where the data can only be correctly used as categorical data with non-parametric statistical tests.

The development of a vaccination for use as a management tool in hatcheries could be developed in future investigations. I demonstrated that protection was provided 4 weeks after an immunization exposure; therefore, a vaccine, which does not induce disease in the fish, may be able to provide them with protection against the parasite at least 4 weeks after they are vaccinated. Any vaccination would have to be administered before the fish are stocked into positive waters with enough time being allowed between vaccination and exposure for the immune response to the parasite to develop. An attenuated (non pathogenic) or dead form of the pathogen may be able to induce an immune response in the fish without causing the development of the disease. A bath or oral vaccination would be the most efficient means of administering the vaccine to large numbers of fish; however, the best route of vaccination to induce an appropriate immune

response against the parasite would need to be determined. If injections are determined to be the only effective way to administer an *M. cerebralis* vaccine, then vaccination may not be feasible because of extra handling and economic costs. If rainbow trout can be vaccinated against the pathogen they would be able to be stocked or reared in *M. cerebralis*-positive waters during their first 9 weeks (756 degree-days) after hatch without the risk of developing the disease. Vaccinations are most likely only to be a useful management tool in hatchery systems.

At present, we know that rainbow trout reared in *M. cerebralis*-free waters for 756 degree-days of development or until they are 40 mm in length, whether in the wild or in a hatchery situation, should exhibit enhanced survival and swimming performance, and reduced prevalence of clinical signs, spore counts and severity of microscopic pathology, as compared to fish first exposed to the parasite at an earlier stage of development.

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APPENDICES

APPENDIX A

ANOVA TABLES FROM CHAPTER 2

Response: Cumulative Mortality at 17 weeks after exposure

Type 3 tests of Fixed Effects			
	DF	F	Pr > F
Age	8	49.53	< 0.0001
Dose	3	8.66	< 0.0001
Age*Dose	24	2.65	0.0006

Response: Cumulative Mortality at 33 weeks posthatch

Type 3 tests of Fixed Effects			
	DF	F	Pr > F
Age	8	27.55	< 0.0001
Dose	3	6.28	0.0007
Age*Dose	24	1.69	0.0442

Response: cumulative mortality at 17 weeks after exposure

Type 3 tests of Fixed Effects			
	DF	F	Pr > F
Age	8	49.53	< 0.0001
Dose	3	8.66	< 0.0001
Age*Dose	24	2.65	0.0006

Response: Swimming Performance at 17 weeks after exposure

Type 3 tests of Fixed Effects			
	DF	F	Pr > F
Age	8	1.65	0.1194
Dose	3	4.99	0.0029
Age*Dose	22	1.02	0.4451

Response: Swimming Performance at 33 weeks posthatch

Type 3 tests of Fixed Effects			
	DF	F	Pr > F
Age	8	1.30	0.2523
Dose	3	4.57	0.0045
Age*Dose	23	1.19	0.2688

Response: Spores at 33 weeks posthatch

Type 3 tests of Fixed Effects			
	DF	F	Pr > F
Age	10	35.05	< 0.0001
Dose	3	77.70	< 0.0001
Age*Dose	30	8.33	< 0.0001

Response: Aggregated Clinical Signs at 33 weeks posthatch

Type 3 tests of Fixed Effects			
	DF	F	Pr > F
Age	10	475.16	< 0.0001
Dose	3	415.33	< 0.0001
Age*Dose	30	73.97	< 0.0001

Response: Blacktail at 33 weeks posthatch

Type 3 tests of Fixed Effects			
	DF	F	Pr > F
Age	10	21.70	< 0.0001
Dose	3	24.72	< 0.0001
Age*Dose	30	8.84	< 0.0001

Response: Major Skeletal Deformities at 33 weeks posthatch

Type 3 tests of Fixed Effects			
	DF	F	Pr > F
Age	10	6.62	< 0.0001
Dose	3	6.74	0.0004
Age*Dose	30	3.75	< 0.0001

Response: Minor Skeletal Deformities at 33 weeks posthatch

Type 3 tests of Fixed Effects			
	DF	F	Pr > F
Age	10	34.88	< 0.0001
Dose	3	31.61	< 0.0001
Age*Dose	30	10.35	< 0.0001

Response: Whirling Behaviour at 33 weeks posthatch

Type 3 tests of Fixed Effects			
	DF	F	Pr > F
Age	10	3.28	0.0013
Dose	3	2.73	0.0496
Age*Dose	30	1.37	0.1352

APPENDIX B

ANOVA TABLES FROM CHAPTER 3

Response: Cumulative Mortalities

Type 3 tests of Fixed Effects			
	DF	F	Pr > F
Age	1	1.56	0.2236
Dose	1	3.34	0.0802
Size	3	0.59	0.6249
Age*Dose	1	1.91	0.1801
Age*Size	1	0.11	0.7452
Size*Dose	3	0.16	0.9247
Age*Dose*Size	1	1.56	0.2236

Response: Swimming Performance

Type 3 tests of Fixed Effects			
	DF	F	Pr > F
Age	1	5.20	0.0318
Dose	1	18.72	0.0002
Size	3	0.14	0.9338
Age*Dose	1	0.12	0.7293
Age*Size	1	0.80	0.3811
Size*Dose	3	0.42	0.7226
Age*Dose*Size	1	0.00	0.9618

Response: Clinical Signs

Type 3 tests of Fixed Effects			
	DF	F	Pr > F
Age	1	23.10	< 0.0001
Dose	1	405.96	< 0.0001
Size	3	4.41	0.0132
Age*Dose	1	21.10	< 0.0001
Age*Size	1	10.91	0.0030
Size*Dose	3	4.41	0.0132
Age*Dose*Size	1	10.91	0.0030

Response: Spores

Type 3 tests of Fixed Effects			
	DF	F	Pr > F
Age	1	7.46	0.0117
Dose	1	47.74	< 0.0001
Size	3	3.08	0.0466
Age*Dose	1	7.46	0.0117
Age*Size	1	0.08	0.7803
Size*Dose	3	3.08	0.0467
Age*Dose*Size	1	0.08	0.7790

Response: Cartilage

Type 3 tests of Fixed Effects			
	DF	F	Pr > F
Age	1	0.05	0.8287
Size	3	20.74	< 0.0001
Age*Size	1	3.23	0.0786

APPENDIX C

ANOVA TABLES FROM CHAPTER 4

Response: Antibodies 2 hours after exposure at 9 weeks posthatch

Type 3 tests of Fixed Effects			
	DF	F	Pr > F
Immunization	3	0.87	0.1773
Exposure	3	1.66	0.4571
Immunization*Exposure	3	1.09	0.3751

Response: Antibodies 1 month after exposure at 9 weeks posthatch

Type 3 tests of Fixed Effects			
	DF	F	Pr > F
Immunization	3	0.75	0.5281
Exposure	3	0.58	0.6314
Immunization*Exposure	3	0.97	0.4860

Response: Antibodies 20 weeks after immunization, exposure at 9 weeks posthatch

Type 3 tests of Fixed Effects			
	DF	F	Pr > F
Immunization	3	3.81	0.0203
Exposure	3	3.77	0.0212
Immunization*Exposure	3	2.12	0.0604

Response: Antibodies 2 hours after exposure at 13 weeks posthatch

Type 3 tests of Fixed Effects			
	DF	F	Pr > F
Immunization	3	0.24	0.8684
Exposure	3	0.37	0.7733
Immunization*Exposure	3	0.78	0.6346

Response: Antibodies 1 month after exposure at 13 weeks posthatch

Type 3 tests of Fixed Effects			
	DF	F	Pr > F
Immunization	3	12.50	< 0.0001
Exposure	3	0.21	0.8877
Immunization*Exposure	3	0.65	0.7444

Response: Antibodies 20 weeks after immunization, exposure at 13 weeks posthatch

Type 3 tests of Fixed Effects			
	DF	F	Pr > F
Immunization	3	2.99	0.0309
Exposure	3	39.67	< 0.0001
Immunization*Exposure	3	1.12	0.0464