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INTER AND INTRASPECIES COMPARISONS OF THE SUSCEPTIBILITY OF SALMONIDS TO *MYXOBOLUS CEREBRALIS* THE CAUSATIVE AGENT OF WHIRLING DISEASE

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Abstract: Laboratory challenges of trout with known concentrations of waterborne triactinomyxons (TAMs), the infectious stages of *Myxobolus cerebralis*, demonstrated a range of susceptibility among five species of salmonids tested. Two levels of TAMs were employed for each test species of salmonid and their response was compared to a parallel exposure of rainbow trout control groups. These experimental studies indicate that the Deschutes strain of rainbow trout, two strains of cutthroat trout and bull trout are susceptible to *M. cerebralis*. In contrast, Arctic grayling appear to have an innate resistance to the parasite. Size differences between different species of fish precluded direct comparisons of their relative sensitivity. However, by comparing each test species to the standard rainbow trout strain used as a control some generalizations can be proposed. In this regard, the Deschutes River strain of rainbow has similar susceptibility to the control rainbow trout strain. Both cutthroat trout species tested seem to be slightly less susceptible than the rainbow trout test strain. Bull trout, while showing large numbers of trophozoites in vertebrae early in infection, appeared to eliminate many parasites by the final sampling at 5 mo post exposure. These results support the hypothesis that a spectrum of susceptibility exists among salmonid fish that is not easily predicted on a generic basis. Evidence from our and other host range studies suggest that multiple host factors must be influencing the parasite throughout its development and by virute of their action or inaction provide the spectrum of resistance to *M. cerebralis* observed in salmonid fish.

Introduction

Several species of salmonid fish have been reported as being susceptible to *Myxobolus cerebralis* (Halliday 1976; O'Grodnick 1975, El-Matbouli et al. 1992). This information has come principally from observations of epizootics in captive populations or from experimental live-box exposure studies. As a result, the conditions governing the severity of the disease following exposure were seldom controlled. Having populations of infected oligochaetes producing rather constant numbers of the infective stage or triactinomyxons (TAMs) in our laboratory has allowed us to conduct controlled exposures of several salmonid species. With each species tested, a control population of rainbow trout (Mt. Lassen strain), matched as closely as possible in degree days of age, was exposed simultaneously to the same known doses of triactinomyxons. Fish were then held under the same conditions and observed for the onset of clinical signs and sequentially sampled to assess development of microscopic lesions. After 5 mo, the fish were terminated such that both microscopic lesions and spore counts could be evaluated.

Methods

Fish - Eyed eggs or fry of the salmonid species tested and rainbow trout controls were received from hatcheries in Montana or California. Eggs were incubated in well water ranging from 10 to 12°C depending on the species being reared. Mt. Lassen strain rainbow trout, which served as a standard control stock for experimental trials, were obtained as eggs directly from a photoperiod controlled broodstock population. They were matched as closely as possible with the age in degree-days of the salmonids tested for susceptibility to *M. cerebralis*. In the trial with the Deschutes River rainbow trout, the Hot Creek strain of rainbow trout was used as the control rather than the Mt. Lassen stock. The source, age and, size at exposure for all of the salmonids tested is presented in Table 1.

Parasite source – Triactinomyxons (Figure 1), the infectious stages of *M. cerebralis* for trout, were propagated in the laboratory by seeding known susceptible oligochaete populations as described by McDowell et al. (1997). Briefly, 10^7 spores (Figure 2) extracted by the plankton centrifuge method (O'Grodnick 1975) from heads of an infected hatchery rainbow trout population (California Department of Fish and Game, Mt. Whitney, California) were seeded onto approximately 20 g of oligochaetes placed in a sterile sand substrate in a 10 L aquaria. After 1 hour, well water was added to aquarium which was placed in a 15°C incubator. The aquarium water was aerated with an air pump and day/night cycles (14 h/10 h) were controlled with a lamp on a timer. Up to ½ of the

water in the aquarium was changed three times each week. After 14 wk, all of the water was changed three times a week and triactinomyxons present in the water were separated by filtration with a 20 μm screen, resuspended in a known volume and counted using a petri dish with a marked grid. Triactinomyxons were held on ice in water until used for fish exposures, generally within 1 - 2 h after collection.

Fish exposures - Testing of each salmonid species was conducted with an experimental design that employed two replicate groups of 25 fish for each of the following treatments: low dose of exposure (100 - 200 triactinomyxons/fish), a high dose (1000 - 2000 triactinomyxons/fish) and a control unexposed group. Fish were placed in 20 L aquaria receiving 15°C well water at a rate of 2 L/min. After fish had been held for 1 week in the test aquaria, exposures were conducted. For exposure, the incoming water to the aquaria was turned off and triactinomyxons were added to two replicate tanks for the low and high dosage treatments. After a 2 h static exposure with aeration, water flow was resumed to the aquaria. Control aquaria received the same treatment but no addition of triactinomyxons (TAMs).

Evaluation of susceptibility - The progress of *M. cerebralis* infections after exposures to TAMs was evaluated by examining fish at 2 h and then at 2, 5, 8, 20 wk. Since mortality was not an anticipated sequale to infection, fish were evaluated by the presence of parasites and accompanying microscopic pathoglogy. At each sampling period 5 experimental and 5 control fish were euthanized with 500 ppm benzocaine. At the final

sampling period (20 wk) 10 experimental and 10 control fish were weighed. The heads from 10 experimental fish were cut in half along a midsaggital plane and prepared for microscopic pathology and enumeration of spores. Five control fish were prepared in the same manner. Spore counts for individual ½ heads were enumerated following pepsin trypsin digests (Markiw and Wolf 1974) with modifications (T. Baldwin, Washington State University and L. Chittum, Colorado Department of Wildlife, personal communications). After digestion was completed, 1 ml of bovine calf serum was added to each tube to saturate trypsin activity. The solution was then passed through a calculi filter (Gerson Heathcare, Middleboro, MA) to remove larger debris. The filter was rinsed twice with 10 ml aliquots of water and the solution and rinses passing the filter were centrifuged for 10 min at 1300 x g to pellet spores. Spores were resuspended in 1 ml of 10% neutral buffered formalin mixed and then aliquots placed on both sides of a hemocytometer counting chamber. Only spores which displayed the appropriate morphology at a total magnification of 400x (e.g. 2 polar capsules and a sporoplasm, or a clearly visible suture line) and size (8 - 12 μm diameter) were counted. Total spores per head was calculated by the following equation:

$$\text{Spores per head} = \frac{\text{average of spores counted on 2 sides} \times 10^4 \times \text{ml of suspension}}{\text{Number of grids counted}} \times 2$$

Preparation of tissues for histology and scoring of microscopic pathology - Whole fish 1-3" in length were preserved (after opening fish ventrally along the abdomen) in Davidson's fixative for 48-72 hours after which they were transferred to 70% ethanol alcohol. Fish

were collected and processed as whole fish or just as the head, depending on size of the fish. After fixation, tissues were embedded in paraffin using standard histological procedures (Humason 1979). Two 4-6 μm thick sections from each fish were stained with hematoxylin and eosin or giemsa. Cartilaginous tissue in each section 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 graded on a scale of 1-5. Grade 1was a minimal infection and grade 5 was severe disease. Descriptions of each histological grade are as follows: (1) minimal: few parasites, few areas of cartilage infected, no inflammatory cells. Often difficult to detect infection, little to no cartilage degeneration, especially very early in infection; (2) mild: several areas of cartilage infected but no or minimal (few inflammatory cells) host response. No bone distortion or involvement of other tissues, very localized response; (3) moderate: several areas of cartilage infected in each section, cartilage degeneration and necrosis, associated inflammatory response (not localized), minimal or mild impact on surrounding tissues; (4) moderately severe: several to numerous areas of cartilage infected, associated inflammatory response extensive and moderate to severe involvement of surrounding tissues; (5) severe: all areas of cartilage examined infected, extensive inflammatory response and tissue involvement, including bone displacement into brain or spinal cord. The scores from five fish from each of the two replicates from high dose experimental groups and five control fish were averaged to provide a value from 0 – 5.

Results

With the exception of the Artic grayling, all salmonid species tested were susceptible to *M. cerebralis* infections (Table 2). Both clinical signs and microscopic lesions associated with the parasite were more severe in the high exposure groups. Clinical signs (Figure 3) were present in fish in the high dose control exposed rainbow trout in all trials except the Yellowstone cutthroat trout where larger fish were exposed. Clinical signs were observed in low dose exposed rainbow trout exposed in the Deschutes River rainbow trout and the Arctic grayling trials.

Mean spore counts per head followed a similar pattern to clinical signs. The greatest spore concentrations were found in the high exposure groups of the Deschutes River and parallel control rainbow trout and control rainbow trout in the bull trout trial. Spores were also detected, but in lesser numbers, in low exposure control rainbow trout in the grayling and Westslope cutthroat trials. No spores were found in the low dose exposed grayling, cutthroat or bull trout. In all trials, rainbow trout controls had higher numbers of spores than the parallel test group. Among the salmonid species tested, grayling appeared to be the most resistant to infection since no clinical signs, microscopic lesions or spores were observed. Both cutthroat trout strains were susceptible but clinical signs were only evident in Westslope cutthroat that were exposed to a high dose at three weeks of age, in contrast to the Yellowstone cutthroat trout that were 3 months of age, when first exposed to the parasite. Only two bull trout from the high dose group showed any evidence of clinical signs and spore recoveries were greatly diminished compared to the parallel

exposed rainbow trout. No spores or microscopic lesions were observed among test species and their rainbow trout controls that were not exposed to *M.* cerebralis examined at the end of the study (5 mo). A more detailed description of the results for each species follows.

Deschutes River rainbow trout

The Deschutes River rainbow trout which are resistant to the myxosporean *Ceratomyxa shasta* (R. Holt, Oregon Division of Fish and Wildlife, personal communication), were not resistant to whirling disease. Clinical signs of whirling disease were seen in both the high and low dose exposure groups at 2 and 5 mo post exposure, respectively. Microscopic lesions were evident in both the Deschutes River and control rainbow trout at both 2 and 5 mo post exposure with areas of caudal, vertebral, and fin cartilage being infected with the parasite in low and high exposure groups. Deschutes rainbow trout in the high exposure group showed extensive granulomatous inflammation associated with infection (Figure 4). Spore counts per head averaged 3.0×10^6 and 1.2×10^5 for the high and low exposed Deschutes rainbow trout, respectively. This compared to spore counts of 1.0×10^6, 1.1×10^6 for high and low exposed control rainbow trout, respectively.

Arctic grayling

The first Arctic grayling trial was terminated due to complications associated with bacterial coldwater disease caused by *Flavobacterium psychrophilum* which was presumably transmitted with the eggs. A second grayling susceptibility study was initiated

and suffered fewer ill effects due to bacterial coldwater disease. Grayling did not show gross or microscopic signs of whirling disease throughout the study. Histological examination at 35 d, 2 mo and 5 mo post exposure did not show infection with the parasite. Spores were not recovered from any grayling in the low or high exposure groups. Several control rainbow trout in the high exposure group showed blacktail and whirling at 2 and 5 mo post exposure. Only a few fish fish in the low exposure control rainbow trout showed signs and parasite infection. At 5 mo post exposure, moderate to moderately severe microscopic lesions associated with parasite infection and spore counts of 5.5 $x10^5$/head were found in high exposure control rainbow trout.

Westslope and Yellowstone Cutthroat trout

Black tails were seen in two Westslope cutthroat trout in the high dose group 6 wk post exposure. At the end of the study, 60% of the high dose group showed black tail and 20% were whirling. In contrast, black tail and whirling behavior were seen in 88% of the high dose rainbow controls. Histological examination of both rainbow and cutthroat trout 2 mo post exposure showed similar infection rates and microscopic lesions associated with parasite and these were rated moderate in cutthroat trout and moderately severe in rainbow trout. In the low dose groups, only one rainbow trout was infected and all cutthroat trout examined were negative for the parasite. By 5 mo post exposure, Westslope cutthroat trout showed mostly mild to moderately severe cranial lesions and few lesions in gill or jaw cartilage. In contrast, rainbow trout controls showed extensive cranial lesions including a moderately severe inflammatory response and involvement of

gill and jaw cartilage. Spore counts averaged 7.7 $x10^4$ per head for the high exposed Westslope cutthroat trout, compared to 3.6 $x10^5$ spores per head for the high exposed control rainbow trout. No spores were observed in the low exposed cutthroat and a reduced number were found in the lose dose exposed rainbow trout.

Yellowstone cutthroat trout were exposed at an older age (3 mo) and clinical signs of whirling disease were not observed. However, histological examination of these fish showed mild to moderate lesions in cranial cartilage with limited sporulation of the parasite (Figure 5). Spores were not recovered from the low dose control rainbow or Yellowstone cutthroat trout. High exposed spore counts averaged $9x10^3$ in the Yellowstone cutthroat and $1.2x10^5$ in the rainbow trout controls.

Bull trout

Bull trout were susceptible to infections as shown by limited external signs first appearing at 7 wks post exposure. There were no signs of whirling and only two bull trout in the high exposure group showed black tail at any time throughout the trial. Microscopic examinations at 35d post exposure showed parasite infections in vertebrae, fins and some crainial cartilage (Figure 6). By 2 mo, parasite infections and a mild to moderate inflammatory response were more common in vertebrae than in cranial cartilage compared to those in parallel high exposed rainbow trout which showed mild cranial lesions and no inflammation in all fish examined. Diminished lesions, tissue repair, and very few spores were evident in stained tissues sections of bull trout 5 mo post exposure (Figure 7). This was supported by low spore recovery of 1.1 x 10^4/head for bull trout in the high dose

compared to $1.4x10^6$/head in rainbow trout. Sections of rainbow trout 5 mo post exposure showed extensive granulomatous inflammation, abundant spores, numerous prespore stages of the parasite and no indication of tissue repair (Figure 8). No spores were found in either bull or rainbow trout in the low exposure groups.

Discussion

Water temperature, fish species, dose, and age of exposure are critical factors in the progression of infection and development of whirling disease (El-Matbouli et al. 1992). Our experimental studies showed a range of susceptibility to *M. cerebralis* from fish suffering full clinical signs (e.g. black tail and whirling behavior) to fish showing neither clinical signs, microscopic lesions nor development of spores. In addition, innate resistance to another myxosporean disease caused by the parasite *C. shasta* did not extend to protection from whirling disease.

The Deschutes River strain of rainbow trout, two strains of cutthroat trout and bull trout were all found susceptible to *M. cerebralis.* In contrast, Arctic grayling appear to have an innate resistance to the parasite. Although the sizes of the fish precluded a driect comparison of their relative sensitivity, indirect comparisons with their control rainbow trout strain counterparts allow some generalizations. In this regard, the Deschutes River strain of rainbow had similar susceptibility to the control trout strain. Both cutthroat trout species tested seem to be slightly less susceptible than the rainbow trout test strain. Bull trout that showed large numbers of trophozoites in vertebrae early in infection appeared to eliminate many parasites by the final sampling at 5 mo post exposure. These results

support the hypothesis of a spectrum of potential susceptibilities to *M. cerebralis* amond salmonid fish that is not easily predictable on a generic basis or by resistance to other myxosporeans. The results of this testing suggests that resistance is due to impairment of the parasites development at multiple steps throughout the infection cycle. Understanding the mechanisms associated with preventing development and eradicating or containing the parasite and how this might be exploited for management of whirling disease are research goals of our laboratory.

Acknowledgements

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Table 1. Species of salmonids, age, size and hatchery origin, compared for susceptibility to whirling disease following experimental exposures to *Myxobolus cerebralis*.

Common Name (Scientific Name)	**Age* (size) at Exposure**	**Hatchery Origin, Location**
Experimental species		
Deschutes River rainbow trout (*Oncorhynchus mykiss*)	3 wk/0.22 g	Oaksprings, Maupin, OR
Arctic grayling (*Thymallus thymallus*)	3 wk/0.02 g	Washoe, Alcoa, MT
Westslope cutthroat trout (*O. clarki*)	3 wk/0.16 g	Washoe, Alcoa, MT
Yellowstone cutthroat trout (*O. clarki*)	3 mo/1.26 g	Washoe, Alcoa, MT
Bull trout (*Salevlinus confluentus*)	4 wk/0.07 g	Washoe, Alcoa, MT
Control species		
Mt. Lassen rainbow trout	-+	Mt. Lassen Trout Farm, Anderson, CA
Hot Creek rainbow trout	6 wk/0.40 g	Hotcreek, Mammouth, CA

*Age of all fish is expressed as degree days based on a constant 12°C water temperature.
+Matched for age with test species compared.

Table 2. Presence of clinical signs, microscopic lesions and spore concentrations found among five species of salmonids exposed to the infectious stages of *M. cerebralis* at two doses.

Common Name	Clinical Signs *		Microscopic Lesions		Spores/head**	
	High	Low	High[+]	Low	High	Low
Deschutes rainbow trout	+	+	3.6	+	3.0×10^6	1.2×10^5
Control rainbow trout	+	+	3.4	++	1.0×10^6	1.1×10^6
Arctic grayling	-	-	0	-	-	-
Control rainbow trout	+	+	3.8	+	5.5×10^5	2.5×10^3
Westslope cutthroat trout	+	-	2.4	+	7.7×10^4	-
Control rainbow trout	+	-	3.6	+	3.6×10^5	2.4×10^3
Yellowstone cutthroat trout	-	-	2.9	-	1.9×10^4	-
Control rainbow trout	-	-	3.5	-	2.4×10^4	-
Bull trout	+	-	0.4	-	1.1×10^4	-
Control rainbow trout	+	-	4.2	-	1.4×10^6	-

*Appearance of black tail or whirling signs in the fish.
**Spores present is the mean for the heads of 10 fish.
[+]Score of severity of microscopic lesions from none (0) to severe (5).

Figures

Figure 1. Triactinomyxons (*Myxobolus cerebralis*) used in laboratory infection trials as harvested from experimentally-infected oligochaete populations. Phase contrast photomicrograph.

Figure 2. Spores of *Myxobolus cerebralis* as extracted from infected rainbow trout. The spores measure 9 – 10 um in diameter as shown in this phase contrast photomicrograph.

Figure 3. Experimentally-infected and control rainbow trout after 5 mo. The fish on the left column showed typical signs of whirling and black tail and cranial and vertebral deformities are evident. Control uninfected fish (left column) are larger in size and were free of signs of *Myxobolus cerebralis*.

Figures 4 – 8. Hematoxylin and eosin stained tissue sections from salmonids experimentally infected with *Myxobolus cerebralis*.

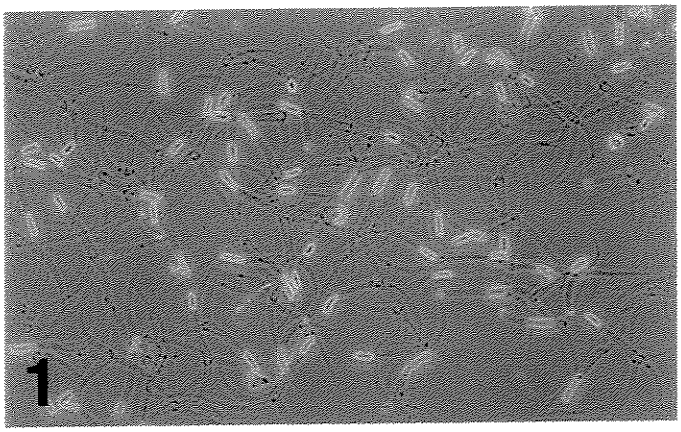
Figure 4. Severe granulomatous inflammatory response to developmental stages of *Myxobolus cerebralis* in the region of the gill arches of Deschutes River rainbow trout at 5 mo post challenge.

Figure 5. Limited sporulation of the parasite among Yellowstone cutthroat trout exposed as older fish (3 mo) 5 mo post challenge with *Myxobolus cerebralis*.

Figure 6. Trophozoites (arrows) of *Myxobolus cerebralis* as found in the spinal vertebrae of bull trout at 35 d post challenge.

Figure 7. Tissue repair in the spinal region of bull trout at 5 mo post challenge. Few parasites of any stage in development were present late in infection and the pathology was minimal.

Figure 8. Extensive granulation tissue and numerous developmental stages and spores of *Myxobolus cerebralis* as found among rainbow trout controls in the high exposure group of the bull trout trial at 5 mo post challenge.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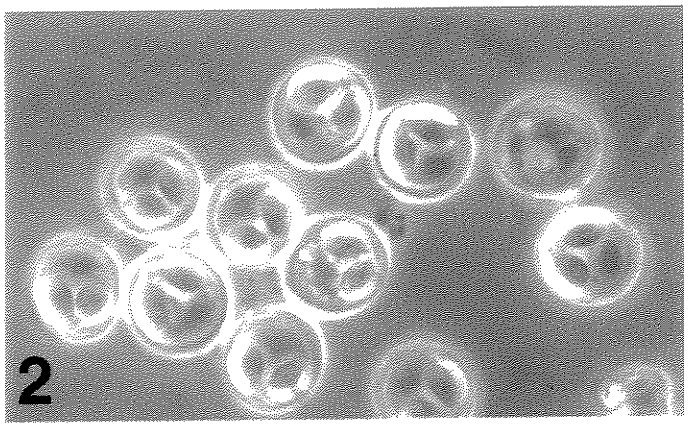
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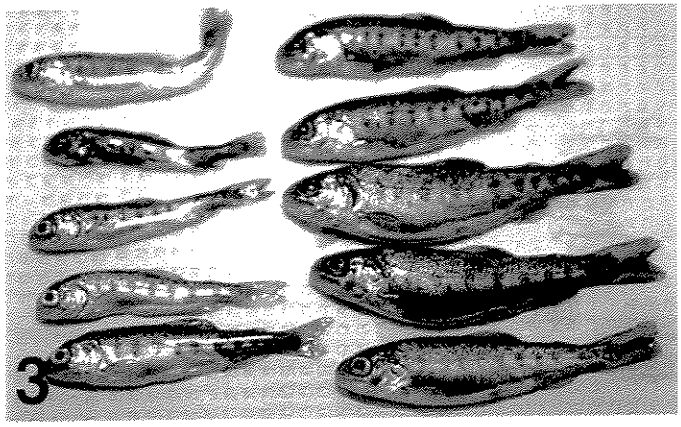
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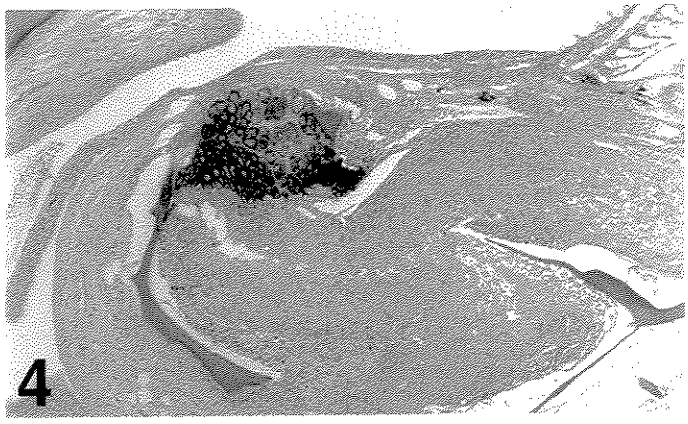
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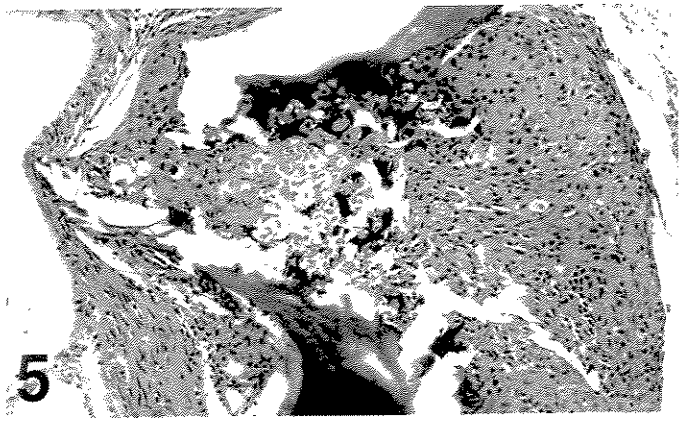
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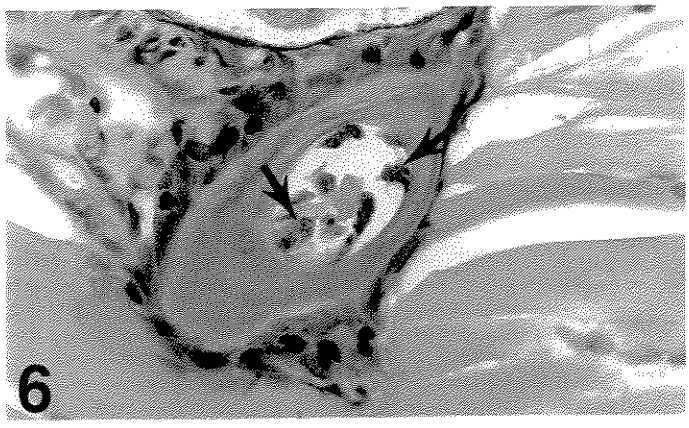
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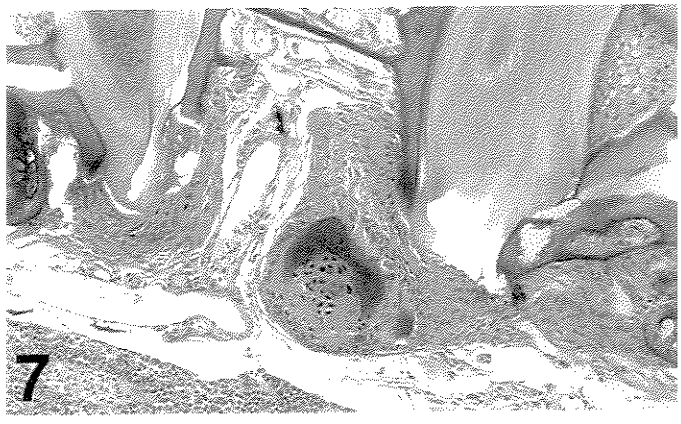
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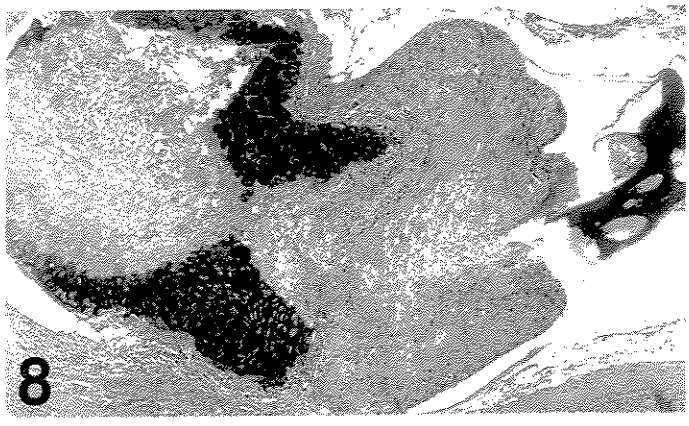
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