AN ABSTRACT OF THE THESIS OF

Todd A. Sandell for the degree of Master of Science in Microbiology presented on June 16, 2000. Title: A Study of *Myxobolus cerebralis* in the Lostine River, Oregon: Epizootiology, Distribution and Implications for Resident and Anadromous Salmonids

Abstract approved: Redacted for privacy

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Myxobolus cerebralis, a myxozoan parasite that infects almost all species of wild and cultured salmonids, was first identified in northeastern Oregon in the Lostine River in 1986. Fish that are heavily infected with *M. cerebralis* develop whirling disease, which was determined to be the cause of catastrophic declines in rainbow trout populations in Montana and Colorado in 1996. There have been no reports of severe salmonid population declines attributed to whirling disease in Oregon, although many populations are at levels that warrant protective listing under the Endangered Species Act. This study was undertaken to 1) establish the distribution and seasonal prevalence of *M. cerebralis* infection in the Lostine River, 2) investigate the epizootiological factors influencing the occurrence of whirling disease in this river, 3) determine the potential impact of the parasite on chinook salmon, and 4) evaluate the suitability of a non-lethal tissue sampling protocol for the detection of *M. cerebralis* in juvenile and adult salmonids for use in monitoring the effects and distribution of the parasite in protected salmonid populations. Results of this study indicate that the parasite is present throughout the main stem of the Lostine River during all seasons, and that rainbow and anadromous steelhead trout fry emerging in the lower river are most likely to become infected and develop whirling disease. Chinook salmon were shown to be relatively resistant to *M. cerebralis* at low levels of exposure, but this effect was less evident when the parasite exposure increased. The non-lethal sampling protocol was successful in detecting *M. cerebralis* in juvenile salmonids but not in adults, where it is most needed. The information gathered during these studies was then correlated with the life histories of resident and anadromous

salmonids in the Lostine River to determine the potential impact of *M. cerebralis* on salmonid populations in this region.

A Study of *Myxobolus cerebralis* in the Lostine River, Oregon: Epizootiology, Distribution and Implications for Resident and Anadromous Salmonids

By

Todd A. Sandell

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Presented June 16, 2000
Commencement June 2001

Master of Science thesis of Todd A. Sandell presented on June 16, 2000

APPROVED:

Redacted for privacy

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Todd A. Sandell, Author

CONTRIBUTION OF AUTHORS

Dr. Jerri L. Bartholomew was involved in the design, analysis and writing of each part of this project. Harriet V. Lorz assisted with maintenance of study animals and data collection. Donald G. Stevens assisted with sample collection and invertebrate identification. Sarah H. Sollid assisted with the enumeration of myxospores.

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INTRODUCTION

Whirling Disease: Distribution And Impacts

Whirling disease is the result of severe infection by the myxozoan parasite *Myxobolus cerebralis,* and affects wild and cultured salmonids. The parasite was originally described in Germany in 1903 (Hofer) and has since been spread, presumably by the transfer of live or frozen fish, throughout Europe, the former Soviet Union, South Africa, Japan, New Zealand and into at least 22 of the United States (Elson, 1969; Wolf, 1985; Hoffman, 1970, 1990; Bergersen and Anderson, 1997). Although observations of "myxosomiasis" were reported in South America, more recent studies repudiate that claim (Margolis et al., 1996).

Infection by *M. cerebralis* has been confirmed in the following salmonid species: rainbow (*Oncorhynchus mykiss*), steelhead (*O. mykiss mykiss),* cutthroat (*O. clarki* and *henshawi*), brook (*Salvelinus fontinalis*), bull (*Salvelinus confluentus*) and brown trout (*Salmo trutta*); chinook (*O. tshawytscha*), coho (*O. kisutch*), sockeye (*O. nerka*), chum (*O. keta*), pink (*O. gorbuscha*), Atlantic (*Salmo salar*), kokanee (*O. nerka*, lacustrine), masou (*O. masou*), Koundja (*Salvelinus leucomaenis*), Danube (*Hucho hucho*) and malma salmon (*Salvelinus malma*) and mountain whitefish (*Prosopium williamsoni*) (Hoffman et al., 1969; Lom, 1971; Halliday, 1976; El-Matbouli et al., 1992; Baldwin et al., 1998; Markiw, 1992; MacConnell et al., 2000). Although the relative susceptibility of many of these species has not been studied, it is apparent that variation exists between species (O'Grodnick 1979; Hedrick et al., 1999b). A recent study by Thompson et al. (1999) suggests that susceptibility to *M. cerebralis* may also vary between strains of the same species; these researchers reported variations in the susceptibility of four strains of simultaneously exposed cutthroat trout (*O. clarki: bouvieri, stomias, virginalis,*

pleuriticas). Coho salmon and brown and bull trout appear resistant to the disease but are still able to be infected at high parasite doses (O'Grodnick, 1979; Hedrick et al., 1999a,1999b), and lake trout (*Salvelinus namaycush*) and perhaps arctic grayling (*Thymallus* spp.) are refractory to *M. cerebralis* infection, although reports vary (O'Grodnick 1979; Hedrick et al., 1999b; Blazer et al., 2000).

Fish that are heavily infected with *M. cerebralis* may show signs that include blackened tails, deformed spines, blunted or misshapen heads, opercula, and jaws, and the characteristic whirling behavior. However, not all infected fish show these signs (Markiw, 1992), and there are several other salmonid pathogens or nutritional deficiencies which can produce similar signs, complicating diagnosis (Halliday, 1973; Margolis et al., 1996). Several studies have also documented physiological impacts in rainbow trout fry exposed to *M. cerebralis*, including decreased swimming performance with increasing parasite exposure (Ryce et al., 1999b) and a decrease in weight, total body length and reduced numbers of lymphocytes in comparison with age-matched, uninfected fish (Hoffman, 1974; Densmore et al., 1999).

When first identified in fish hatcheries of the Eastern United States during the 1950's, *M. cerebralis* was believed to be a nuisance parasite only of cultured salmonids. The response was to alter the rearing conditions at the facilities where the parasite was present and to stock larger fish, which are more resistant to the disease (Halliday, 1974; Wolf, 1991). Decades later, however, problems began to emerge among wild rainbow trout populations in the Rocky Mountain States, where trout populations are intensively monitored. Researchers noted a decline in recruitment among young-of-the-year (YOY) rainbow trout in the Madison River (MT) in 1991, and by 1994, rainbow trout numbers had declined 90% in much of the river, presumably as a result of *M. cerebralis* (spores were isolated from fish samples). Brown trout populations in these same areas remained stable (Vincent, 1996). In the upper Colorado River (CO), clinical signs of whirling disease were also observed among wild rainbow, brook and brown trout in the summer of 1994. Four successive years of low YOY recruitment resulted in a massive population decline among rainbow trout (Nehring and Walker, 1996). Although the disease appears to have had lesser impacts in the coastal states (Nehring and Walker, 1996; Modin,

1998), the economic impacts on the sport fishing industry and public outcry sparked intensive research efforts (Duffield et al., 1999).

Myxobolus cerebralis: Description

Myxobolus cerebralis is one of the approximately 1250 species comprising the phylum Myxozoa (Lom and Dykova, 1995). Originally classified as Protists, myxosporeans are now considered either to be in a separate kingdom (due to their characteristic multicellular infectious stages) or in the phylum Animalia. A close relationship to parasitic cnidarians (Animalia) is suggested based on their dimorphic life cycles and morphological and genetic similarities (Lom, 1987; Siddall et al., 1995).

The spores of *M. cerebralis* consist of two "broadly lenticular shell valves, two faintly resolvable [by electron microscopy] compressed nuclei surrounded by a sporoplasm, and two dense, ovoid polar capsules containing filamentous profiles" (Lom, 1992). Staining with India ink reveals a mucous coat which is heaviest on the posterior end, near the valve junction, although some authors failed to see any mucous coat by using scanning electron microscopy (Lunger et al., 1975; Lom and Hoffman, 1971). Spores average 8.7 μm in length, 8.2 μm in width, and 6.3 μm in thickness, and the polar capsules measure 5.1 μm by 3.2 μm. In comparison to other species, *M. cerebralis* spores are characterized by the presence of a deep ridge running parallel to the suture line of the shell valves (Lom and Hoffman, 1971).

Life Cycle

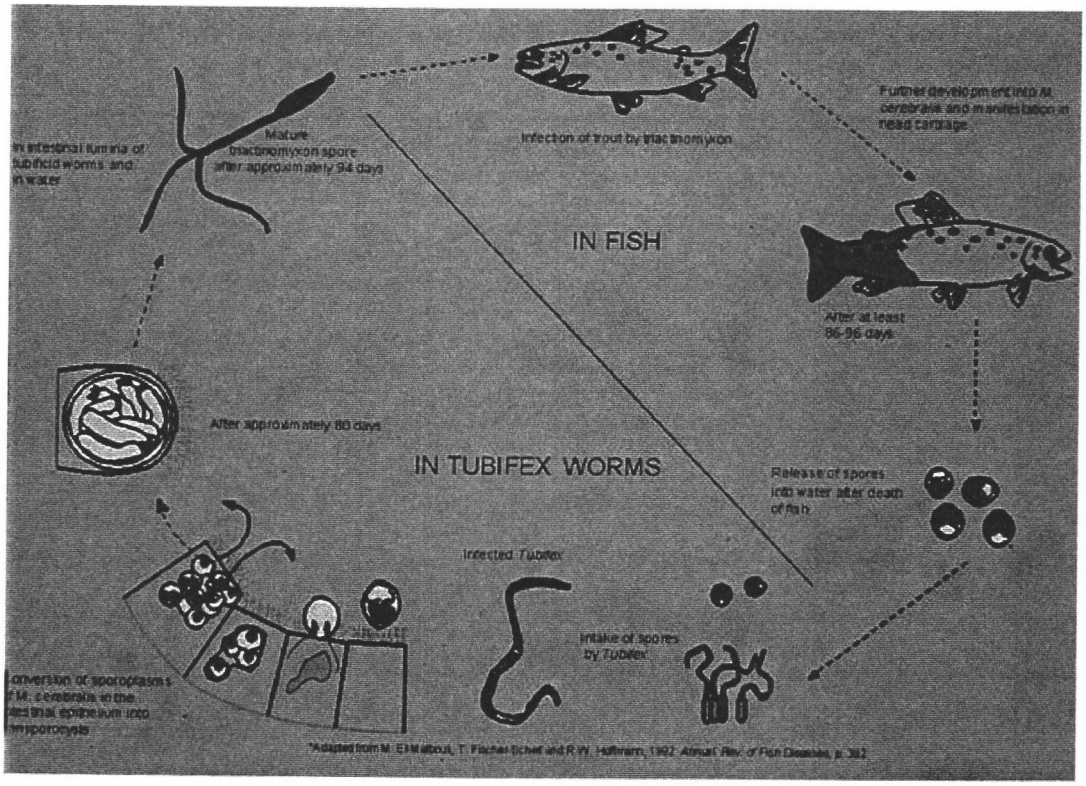
For several decades, researchers noted that salmonids exposed directly to the myxozoan spore stage of *M. cerebralis* did not develop whirling disease, but that leaving such spores in the mud of aquatic holding facilities ("aging") would result in new infections in fish several months later. In 1984, Wolf and Markiw proposed a radically

new explanation for the life cycle and infectivity of *Myxobolus cerebralis*. It had long been known that aquatic oligochaetes (annelid worms) were the hosts for a number of actinosporean parasites, and that several species of these oligochaetes were common in the fine sediment of rivers and fish hatcheries. In their attempts to elucidate the life cycle of *Myxobolus cerebralis*, Wolf and Markiw discovered that they were unable to infect salmonid fry with "aged" mature *M. cerebralis* spores, even after intramuscular or intraperitoneal injection. However, spores released into silt containing a variety of aquatic annelids would eventually produce new cases of whirling disease in previously uninfected fish held in the same tank. The worms were shown to release an actinosporean of the genus *Triactinomyxon*, which was unable to re-infect other worms but resulted in the development of whirling disease symptoms and spores in the cranial cartilage tissue of exposed rainbow trout. Trout fed the infected worms also developed the disease. Thus, the previously unconnected parasites known as myxosporean and actinosporean were shown to be different life stages of the same organism, and both hosts, the oligochaete worm and the salmonid, were required for the successful continuation of the life cycle (Figure 0.1).

The essential oligochaete in the *M. cerebralis* life cycle was shown to be *Tubifex tubifex*, a common aquatic worm that prospers in areas of high organic nutrient loading and/or areas of low dissolved oxygen (Brinkhurst and Kennedy, 1965; Aston, 1973; Kaster, 1980; Wolf et al., 1986; Thorpe and Covich, 1992; Gustafson, 1997). *Tubifex tubifex* is, to date, the only known oligochaete host for *M. cerebralis*, and is also the host for other Myxosporeans, including *Myxobolus carassii*, *Myxobolus cotti, Myxobolus pavlovskii*, and *Myxidium giardi* (family Myxididae) (Kent and Margolis, 1994; El-Matbouli et al., 1992; El-Matbouli et al., 1995). *Tubifex tubifex* is also a host for other triactinomyxons for which the corresponding myxozoan stage is unknown (reviewed in El-Matbouli et al., 1995; Lowers et al., 2000). The findings of Wolf and Markiw were confirmed by El-Matbouli and Hoffman (1989; El-Matbouli et al., 1998), and the two life stages were shown to cross-react in a direct fluorescent antibody test (DFAT) with rabbit antisera, further validating their common genesis (Markiw, 1989). Recently, comparison of small subunit ribosomal RNA sequences (18s rRNA) has provided additional evidence

that the actinosporean and myxosporean stages in question are indeed the same organism (Andree et al., 1997).

Figure 1.1 The life cycle of *Myxobolus cerebralis*



Developmental Stages

The development of the triactinomyxon spore (TAM) stage after attachment to the fish host is a complex one. Triactinomyxons are free-floating microscopic forms which resemble grappling hooks; each caudal process (typically there are three) measures roughly 175-200 μm in length. The central style or "epispore" measures 140 μm and contains 64 sporozoites (infectious nuclei), surrounded by a sporoplasm cell. Triactinomyxons attach to the epidermis of the host's fins (especially caudal), gills, or

buccal cavity, where they are anchored by the extruded polar filaments. The sporoplasm cell, containing the sporozoites, is then injected into the host's epidermis (Antonio et al., 1998; El-Matbouli et al., 1999). Polar capsule extrusion occurs almost exclusively with members of the salmonid family, although some cyprinids also trigger polar capsule extrusion but do not develop disease. The triggering of the polar capsules appears to be dependent upon mechanical and chemical stimulation, as mucus alone or non-living salmonid tissue do not activate the triactinomyxons (El-Matbouli et al., 1999). The initial infection is evident as aggregates of sporozoites between the host's epidermal cells 2-4 hours post-infection, but after 24 hours only single sporozoites are found, scattered intracellularly in the deep endothelial layers. Subsequently, sporozoites are found in the subcutis for up to four days, after which they can be detected in the host's central nervous system, especially in the dorsal spinal cord, where they migrate towards the brain by an unknown homing mechanism. By day 24 post-infection, multicellular stages can be detected in the host's cranium near the cartilage. A zone of lytic activity can be seen in areas where the plasmodia are phagocytosing host chondrocytes. By day 40 post-infection, mature myxosporean spores can be found either in or around the fish's cartilage, where they remain trapped by ossifying cells. Mature myxospores are released by the death and degradation of the host or digestion by a predator (vectors of transmission are discussed below) (El-Matbouli et al., 1995).

Once released into the environment, the myxospores settle into the substrate, where they are ingested by feeding *T. tubifex*. In the gut lumen of the worm, the spore extrudes its polar filaments, opens along the suture line, and inserts the spore nuclei into the intercellular space between cells of the intestinal epithelium (El-Matbouli and Hoffmann, 1989). Empty spore shell valves may be microscopically visible in the gut for up to 25 days post-infection. The binucleate sporoplasm multiplies by schizogony, infecting up to 10 adjacent segments of the worm, and one-cell stages divide and develop into multicellular stages. Forty days post-infection, pansporocyst formation is evident, and by fifty days the number of gametocytes increases and they begin to fuse, resulting in sexual fertilization and the production of zygotes. Each zygote divides to yield capsule and valve progenitor cells, plus a central generative cell which divides by mitosis to yield roughly 64 infectious germ cells (sporozoites). From day ninety onward, pansporocysts

are visible, each containing eight folded triactinomyxons; the entire process occurs between the cells of the gut epithelium. The mature triactinomyxons are then released by egestion or death of the worm, or consumption by a predator, including salmonids (El-Matbouli and Hoffmann, 1989). Interestingly, the process is asynchronous, and different stages of the developing sporoplasm may revert to earlier stages, resulting in continuous infection and release of mature triactinomyxons from the annelid host for up to two years (Markiw, 1986; El-Matbouli and Hoffmann, 1998; Gilbert and Granath, 2000). The actinosporean developmental process occurs only in the segments of the worm posterior to segment eleven, and heavily infected worms may be macroscopically identified by a whitish, opaque swelling of the middle segments and a failure to curl up when handled, instead lying straight. The development of gametes, as evident by microscopy and haploid/diploid DNA quantification, has been demonstrated in the oligochaete worm but not in the salmonid, identifying *T. tubifex* as the definitive host for *Myxobolus cerebralis* (El-Matbouli and Hoffmann, 1998; El-Matbouli et al., 1998).

Infection

It has been shown that eggs from fish infected with *M. cerebralis* do not show signs of infection or develop whirling disease after hatching (O'Grodnick, 1975). A study by Markiw (1991) showed that rainbow trout fry younger than two days old are also refractory to infection by *M. cerebralis*. Interestingly, these fry were shown to have anchored triactinomyxons and intracellular aggregates of sporozoites present soon after exposure, but examination at four months showed that the fish were free of spores, suggesting that they do not physiologically support sporozoite survival. However, fry between the ages of two days and two weeks were the most susceptible to infection and sustained the highest mortality. Rainbow trout fry experimentally exposed to 10 or 100 triactinomyxons/fish in this age group had heavy infections at four months, and fry exposed to 1000 triactinomyxons/fish sustained 100% mortality within twelve days. Ryce et al. (1999a) reported similar results in rainbow trout, and demonstrated that signs

of whirling disease were reduced when fish were exposed after nine weeks of age, and absent in fish exposed at greater than thirteen weeks.

The declining susceptibility of salmonids with increasing age and size is believed to occur as a result of ossification of cartilage (Schaperclaus, 1986). Two month old fry exposed to one or ten triactinomyxons/fish showed no signs of spores following enzymatic digestion at five months. In two month old fry receiving 100 triactinomyxons/fish, significantly fewer spores were recovered at six months post-infection than among members of the same group sampled at five months post-infection. This suggests that either their immune systems partially cleared the infection or that the recovery of spores was hampered by increased ossification of the skeleton. In adult fish (1-3.5 years of age) continuously exposed to triactinomyxons for three and a half months, sampling performed seven months later demonstrated that the incidence of infection increased linearly when the dose of triactinomyxons increased from 100 to 10,000/fish, and plateaued at levels above 10,000 triactinomyxons/fish. In the adult fish, mortality levels were no higher than among control fish despite heavy levels of infection, and most of the fish exhibited no signs of the disease (Markiw, 1992).

Experiments designed to investigate the durability of the triactinomyxon spore (Markiw 1992) suggested that this stage is viable for only a short time. However, recent studies (El-Matbouli et al., 1999) have shown that triactinomyxons held in water at 15 °C or less may have 60% viability after 15 days. In contrast, the myxosporean spore is extremely durable. Published and unpublished reports suggest that these spores are still viable and infectious after years of dehydration in the soil of dried, dormant trout rearing ponds, and that they retain viability even after prolonged freezing at –20° or –80 °C (Hoffman and Putz, 1969; El-Matbouli and Hoffmann, 1991). The myxozoan spores will also survive prolonged exposures to chlorine at 5 ppm, which is a common treatment at fish rearing facilities to prevent the release of viable pathogens (El-Matbouli and Hoffmann, 1991). It has also been shown that the spores will retain viability following ingestion and passage through the digestive tracts of several predators, including great blue herons (*Ardea herodias;* Meyers et al., 1970) and mallard ducks (*Anas platyrhynchos*), as well as Northern pike (*Esox lucius*; El-Matbouli and Hoffmann, 1991).

The implication of avian vectors may account for the rapid spread of the disease once introduced to a watershed.

Epizootiological Studies

A growing body of research has focused on the epidemiological factors influencing the impacts of whirling disease in wild salmonid populations. This work is an attempt to understand why *M. cerebralis* has had catastrophic impacts in the inter-mountain West (Nehring and Walker, 1996; Vincent, 1996) while remaining seemingly benign in other regions of the United States (Modin, 1998; Schachte et al., 1998, 2000). As is the case with any pathogen, the severity of disease, if any, is determined by the complex interactions of variables relating to the host, the pathogen and the environment (Hedrick, 1998). The current understanding of these variables as they relate to *M. cerebralis* infection and whirling disease is outlined below.

a) The pathogen. A possible explanation for the difference in impacts among wild fish populations is variation in the virulence of different strains of *M. cerebralis*. However, because *M. cerebralis* is believed to have been recently introduced to the United States from Europe (Hoffman, 1990) and quickly spread by human intervention, it is unlikely that significant genetic variation has evolved which might be expected to generate virulence differences. Indeed, recent molecular analysis of five isolates taken from Germany and the United States (California, Montana, West Virginia, Colorado) indicate that, to date, little variation exists in the genetic makeup of the parasite (Andree et al., 1999). Thus, apparent differences in the impacts of *M. cerebralis* are most likely attributed to either variations in the host or to environmental factors.

b) The host(s). Comparative susceptibility studies have demonstrated that variation in susceptibility does exist among salmonid species and perhaps among strains of the same species, as previously described. However, the translocation of salmonid species by humans has generated a global reservoir of susceptible fish hosts such that most, if not all, areas contain susceptible fish in areas where the environment is favorable for the parasite. The opposite is true of the oligochaete host, *T. tubifex,* which has a

broad natural distribution. Tubificids comprise a natural fraction of the benthic macroinvertebrates found in most bodies of water in temperate climates. Analysis of the conserved 18srDNA gene in geographically isolated samples showed that variation exists between these isolates, indicating that *T. tubifex* has been naturally distributed long enough for these genetic differences to have arisen. Given this information, it is reasonable to assume that variation in susceptibility to *M. cerebralis* may have arisen. Analysis of geographically distinct variants by randomly amplified polymorphic DNA (RAPD) revealed that the species previously considered to be *T. tubifex* based on morphology alone is actually composed of at least two species or subspecies (Beauchamp et al. 1998, 1999; Rasmussen et al. 1998, 1999, 2000). Experiments designed to investigate whether these genetic differences affect the susceptibility of the oligochaete host have shown that geographic variants differ in triactinomyxon production when exposed to the same number of *M. cerebralis* spores (Stevens et al., 1999). These researchers also found that increasing myxospore exposure resulted in a decrease in both *T. tubifex* biomass and numbers, indicating that *M. cerebralis* infection may select against susceptible strains (Stevens et al., 2000).

c) The environment. Although environmental factors may increase the impact of a pathogen on the host through physiological stress, the effects of a single variable are difficult to quantify (Hedrick, 1998). There is evidence that temperature is important in the epidemiology of whirling disease, in both the fish and oligochaete hosts. Vincent (1998; 1999) noted that the levels of *M. cerebralis* infection among rainbow trout correlated with mean daily water temperature; infection was highest when water temperatures warmed or cooled to between 9 and 13 °C (Spring and Fall), although fish did become infected at levels below 9 °C. Temperature also appears to affect the survival of the parasite inside the oligochaete host, and influences the rate of triactinomyxon development. Triactinomyxons develop most rapidly within *T. tubifex* at 15 °C, but the parasite continues to develop at sub-optimal temperatures. When infected *T. tubifex* cultures were held at 25 °C or higher for 15 days, the developing stages of *M. cerebralis* appeared to degenerate, but growth resumed when the host was returned to 15 °C. In addition, triactinomyxons survived for up to 15 days after release from the oligochaete at water temperatures of 20 °C, and triactinomyxon viability increased at lower

temperatures (El-Matbouli et al., 1999). The prolonged survival of triactinomyxons at these lower temperatures may explain the seasonal variation in the severity of infection reported by Vincent (1998; 1999).

The influence of elevation has also been investigated in an effort to determine if factors common to high altitude habitats (besides temperature) limit the establishment of *M. cerebralis*. Studies performed in Colorado determined that both salmonids and *T. tubifex* collected from high elevation lakes and streams are found to harbor the parasite, eliminating the possibility that these areas might provide a refuge from *M. cerebralis* (McAfee, 1998; Schisler and Bergersen, 1999).

Attempts have also been made to discern which ecological factors encourage the establishment of large *T. tubifex* populations in enzootic areas. Although *T. tubifex* is widespread in the northern United States, preferring cooler temperatures, it becomes the dominant species only after disturbance has occurred, or, paradoxically, in areas where natural springs are found (Gustafson, 1997). Disturbances such as cattle grazing, fire, road building, dams, timber harvest, housing development and others have been shown to influence the composition of benthic macroinvertebrate communities (McWilliams et al., 1999; Smith et al., 1999). In particular, *T. tubifex* appears to thrive in areas with large amounts of fine sediment deposition and organic loading, in conjunction with low dissolved oxygen levels (Brinkhust and Kennedy, 1965; Aston, 1973; Kaster, 1980; Thorpe and Covich, 1992; Gustafson, 1997; Modin, 1998; Kerans et al., 1999; Smith et al., 1999), which is one of the common impacts of many of the disturbances listed above. However, *T. tubifex* has also been found at lower densities in areas with rapid currents and larger riverbed substrates (Allen and Bergersen, 1999; personal observations), indicating that this is not a limiting factor in the establishment of the oligochaete host.

Teleost Immunity And The Host Response To *Myxobolus cerebralis*

Both the innate and acquired immune systems appear increasingly similar in mammalian and teleost immune systems, although the different evolutionary pressures teleosts have faced seem to have generated unique aspects of immunity. The most significant

of these may be temperature, as teleosts are poikiothermic and exist within one degree of the temperature of the surrounding environment, with implications for the time needed to generate an acquired immune response (discussed below). The greatest differences between the two taxa are the absence of lymph nodes, the ontogeny of leukocytes and the presence of only one class of immunoglobulin (antibody) in teleosts. Bone marrow does not serve a hematopoietic function in teleosts; leukocytes are generated in the thymus and the kidney, which is divided in its function. The head kidney, or pronephros, is the site of the most hematopoietic capacity, and has limited renal function. Secondary lymphoid organs include the spleen and gut-associated lymphoid tissue (GALT). Immune competence, as indicated by the presence of antibodies, is achieved by four days post-hatch, although innate immune competence appears twelve days pre-hatch (reviewed by Zapata et al., 1996). In addition, teleosts have only one class of antibody, an IgM-like tetramer. This antibody can be found in the plasma or on leukocyte membranes, as in mammals, but it is also found in mucus, bile, and various other body fluids (Graham and Secombes, 1990). Most importantly, teleost antibodies differ from mammalian immunoglobulins in that there is little evidence of isotype switching or affinity maturation, and the rate of antibody production is limited by temperature (Bly and Clem, 1992; Kaattari and Piganelli, 1996.)

The immunological response of the host salmonid to *M. cerebralis* is an area which has received little attention thus far in the study of whirling disease. While brown trout show an increased resistance to *M. cerebralis* in comparison with rainbow trout, they have been shown to undergo the early stages of epidermal infection by sporoplasms following triactinomyxon exposure and will develop the disease if exposed to high numbers of triactinomyxons (Schisler and Bergersen, 1998; Hedrick et al., 1999a). This implies that the nature of their resistance is not mediated by a difference in their mucous coat or skin. Earlier researchers proposed that the myxosporean stages do not initiate a host immune response due to their propensity for migrating through nervous tissue (an immune-privileged site in many vertebrates) instead of the blood (El-Matbouli et al., 1995). However, more recent evidence suggests that both cellular and humoral immune defenses are eventually triggered when cartilage digestion begins (Hedrick et al., 1998).

Initial investigations into the salmonid immune response to *M. cerebralis* focused on the generation of protective antibodies. Griffin (1978) detected circulating antibodies

against spore antigens of *M. cerebralis* in infected adult rainbow trout via indirect fluorescent antibody labeling (IFAT). Adkison et al. (1997) reported poor anti-TAM antigen antibody responses in bath-exposed fish, but did find reactivity to TAM antigens at 25 days post-exposure in rainbow trout injected interperitoneally with triactinomyxons; this response was heightened at day 52 post-injection. No antibody reactivity was demonstrated in the mucus of exposed fish at any time. Interestingly, analysis by Western blotting of serum from chronically infected brown trout showed little or no antibody to TAM antigens in comparison with serum from chronically infected rainbow trout (Adkison et al., 1997). The absence of an antibody response may lead to a reduction in host tissue damage caused by the host's own immune response (immunopathology), which could explain why this species shows less severe clinical signs of whirling disease. The neurological effects of the parasite on the host, including blackened tails and whirling behavior, were thought to occur as a result of increasing pressure on the nerves of the vestibular organ (Schaperclaus, 1986; Hoffman, 1992), perhaps as a result immunopathology. A more recent proposal suggests that the disruption of swimming in infected fish may be due to the constriction of nerves in the brainstem and spinal cord caused by developing parasites; mechanical damage to the vestibular organ did not produce whirling behavior (Rose et al., 1998).

More recent work on the apparent resistance of brown trout to *M. cerebralis* has focused on the cellular immune response. Mammalian eosinophils express Fc receptors on their surface, and recognize antigen-bound antibodies via the Fc tails found on immunoglobulins (antibody-mediated cellular cytotoxicity). Although pressure on the vestibular organ may not be the cause of whirling behavior and blackened tails, cysts or a granulomatous (eosinophilic) chronic inflammatory response have been observed in the cranial histological sections of *M. cerebralis* infected fish (Baldwin et al., 1998; Hedrick et al., 1998,1999a). These are most commonly seen in sections of the cranium in advanced cases of infection, where trophozoites are digesting host cartilage. Hedrick et al. (1999a) reported finding eosinophils in the cranial nerve ganglia and roots of *M. cerebralis* infected brown trout but not among infected rainbow trout. In addition, microscopic examination of histological lesions revealed that, among infected rainbow trout, severe lesions were common in the cranium, while among infected brown trout

these lesions were more frequent in the vertebrae, fin rays, ribs and gill arches (Hedrick et al., 1999a). This suggests that the eosinophil response of brown trout is more robust and may occur earlier in the course of infection, before the parasite has migrated to the cranium. To date, this is the best explanation for the difference in susceptibility to *M. cerebralis* in these species.

Methods Of Detection

Positive confirmation of *M. cerebralis* commonly relies upon histological examination of infected fish heads and/or skeletons, as the parasite is known to attack the host's cartilage. However, this method is labor intensive, especially for larger fish, and bone and cartilage tissues are difficult to section. As a result, methods were developed to isolate the myxozoan spore stage by means of enzymatic disruption of cartilage and bone samples using a pepsin and trypsin digestion (Markiw et al., 1974). Although histological examination of cranial tissue is still an accepted method of confirming *M. cerebralis* infection, newer molecular techniques are becoming more prevalent, especially in research. Primary among these is the use of the nested polymerase chain reaction (PCR) to amplify a portion of the 18S rRNA gene of *M. cerebralis*. This method has been shown to be specific for *M. cerebralis*, with no resulting amplification of host genomic DNA or genomic DNA from four closely related species, including *M. arcticus, M. insidiosus, M. neurobius,* and *M. squamalis* (Andree et al., 1998; personal observations). Other researchers have developed single-round primers or used different primer sequences for amplification of *M. cerebralis* DNA, although a comparison of the sensitivity of these assays has not been described (Ellis et al., 1998; Epp and Wood, 1998; Walker et al., 1998; Schill, 1999). The PCR technique is more sensitive than methods relying on spore isolation or histological sectioning for visual confirmation, and allows detection of all developmental stages in both hosts. It also removes the risk of visual misidentification of *M. cerebralis* spores, which can closely resemble those of other species (Andree et al., 1998). This is particularly important in light of the fact that a single fish may harbor multiple species of myxobolid parasites (Hedrick et al., 1991).

Other detection techniques include *in situ* hybridization of infected tissue (Antonio et al., 1998) and the use of specific antisera for fluorescent antibody labeling. However, the former technique is time consuming and expensive and the latter technique has proven problematic due to the cross-reactive nature of some immunoglobulins and the auto-fluorescence of labeled spores (Markiw and Wolf, 1974; Pauley, 1974; Griffin, 1978; Markiw and Wolf, 1978; Hamilton and Canning, 1988; Markiw, 1989).

Management

Mitigation of the impacts of *M. cerebralis* in fish culture facilities was first accomplished in Europe by (1) raising fry to at least 6 cm in length in parasite-free water before introduction to enzootic areas, (2) the replacement of earthen ponds with concrete raceways, and (3) regularly disinfecting hatchery ponds with chemicals (calcium oxide, potassium hydroxide or chlorine) and drying (Hoffman and Hoffman, 1972; Halliday, 1974; Hoffman, 1990). After the parasite was identified in the United States in 1958 at a fish hatchery in Pennsylvania, various efforts were made to limit the impact and spread of *M. cerebralis*, including the destruction of infected stocks and the implementation of a state and federal transport permit system (Hnath, 1970).

The most effective means of preventing *M. cerebralis* infections in hatcheries is to supply the fish with specific pathogen-free well water for all or part of their development. A recent study in Idaho reported that both the prevalence and severity of infections was reduced when newly hatched chinook salmon were reared on well water until they reached 7 cm in size before being transferred to *M. cerebralis*-positive river water (Munson and Johnson, 2000). The sterilization of incoming water via filtration, ozone and ultraviolet light (UV) has also been investigated for use in situations where well water is unavailable (Hoffman, 1990). However, the small size of the organism and the potential toxicity of ozone-treated water often make the first two methods unsuitable. Flow rate and water clarity are important considerations when using UV sterilization, as suspended particles may impede light penetration (wavelength of 2537 Å). Prevention of whirling disease via inactivation of the water-borne triactinomyxon has been shown to

require a minimum dose of 43, 000 microwatts/sec/cm^2 if the water was unfiltered (Hoffman, 1974, 1975).

Several management options to mitigate the impacts of whirling disease in the wild are currently under investigation. One option is to exploit life history patterns to minimize the exposure of young fish to the parasite. A comparative study of rainbow trout populations in the Missouri and Madison rivers (MT) noted that fish in the Missouri river, which has been less severely effected by *M. cerebralis*, are more likely to spawn in tributaries, and also to spawn earlier in the year (Munro et al.,1998; McMahon, et al., 1999). Thus, salmonid strains and species could be selectively stocked which emerge either in areas of reduced infectivity (headwaters) or earlier in the year, when cooler water temperatures generally lead to less severe infections (Vincent, 1998, 1999).

Several salmonid hybrids have also been developed which exploit the inherent resistance of either brown or lake trout. Of these, splake (female lake trout X male brook trout) and reciprocal splake (male lake trout X female brook trout) appeared to survive best, and the prevalence of *M. cerebralis* infection in these hybrids was significantly less than among rainbow trout controls. However, the researchers noted that these hybrids "contributed to the creel" less than rainbow trout (Wagner et al., 2000), and the impacts of releasing hybrids into an ecosystem have not been investigated.

Intensive research efforts have also been made in the study of variation of *T. tubifex* susceptibility to *M. cerebralis*. Several research groups have identified genetic markers delineating races, strains, or subspecies of *T. tubifex* which show varying TAM production or susceptibility to the myxosporean stage (El-Matbouli et al., 1999; Beauchamp et al., 2000; Rasmussen et al., 2000; Sweeney et al., 2000). A strain of *T. tubifex* from Bozeman, MT was identified which releases fewer triactinomyxons after exposure to a low dose of myxospores in comparison with strains from either Mt. Whitney fish hatchery, CA or the Madison River, MT. Sweeney et al. (2000) attempted to generate resistant hybrids of *T. tubifex*, but only nonviable offspring were produced. Obviously, the generation of resistant strains that can compete with resident *T. tubifex* populations may offer an inroad to controlling the impacts of whirling disease in the wild. However, enthusiasm for the large-scale importation of competitive species of aquatic oligochaetes (or of resistant strains of *T. tubifex*) to biologically control *M. cerebralis*

infections in the wild (El-Matbouli et al., 1999) should be tempered by consideration of the effects on aquatic habitats and concern for the possible introduction of new parasites in non-enzootic areas, as indicated by the work of Lowers et al. (2000).

The treatment of infected fish with pharmaceuticals is still experimental, but there are two drugs which appear to inhibit the development of the disease. The first, Toltrazuril, is a triazonone drug which was shown "to have activity against all stages of *Myxobolus* sp. except the mature spores" in Bream (*Abramis brama*) experimentally infected with an unidentified species of *Myxobolus*. This drug could be efficacious against *Myxobolus cerebralis* (Schmahl et al., 1989; reviewed by Garden, 1992). The second, Fumagillin dicyclohexylamine (DCH), was used in a direct trial with rainbow trout. Two groups of fish were exposed to triactinomyxons for fourteen days and then fed medicated feed starting at day 14 or day 30 post-infection. In both cases, fish receiving medicated feed had milder infections (based on histological lesion scores) than control fish. Histological examination also revealed that the spores present in the fish receiving medicated feed were deformed, suggesting that Fumagillin DCH may alter spore viability (El-Matbouli and Hoffmann, 1991). Although both drugs are potentially beneficial, the long duration of treatment may be problematic; Hedrick (1988) showed that toxic effects were evident in long-term trials with chinook salmon and rainbow trout fed Fumagillin DCH to combat proliferative kidney disease (PKD). In addition, neither drug is currently licensed for the treatment of fish in wide-scale culture, and both may be prescribed by a veterinarian on an experimental basis only for cultured fish.

Myxobolus cerebralis In Oregon

The first detection of *M. cerebralis* in the Pacific Northwest of the United States occurred in Oregon in 1986. The parasite was first detected in clinically diseased fish at a private trout hatchery on the Lostine River (a tributary of the Wallowa and Grande Ronde Rivers), and was subsequently detected among wild populations of rainbow and brook trout and chinook salmon at sites in the Imnaha and Grande Ronde basins (Lorz et al., 1989). The Lostine River is typical of many tributaries of the Grande Ronde River; it

originates as a high-gradient stream in the Eagle Cap Wilderness Area and progresses to the valley floor, where it flows through an area of intensive agricultural and ranching activity. Although anadromous salmonid populations have been in decline in this region for numerous reasons, these declines can not be attributed to the presence of *M. cerebralis*. In addition, there is no evidence of the catastrophic declines in resident trout populations reported in Montana and Colorado (Nehring and Walker, 1996; Vincent, 1996). The reasons for this disparity are unclear, but the Lostine River differs from the aforementioned areas in that it contains anadromous salmonid species (steelhead trout and chinook salmon), as well as four resident species (rainbow, brook and bull trout and mountain whitefish) The impact of *M. cerebralis* in the inter-mountain West has generated concern about the parasite's potential impact in Oregon, but the protective listing of bull and steelhead trout and chinook salmon in this region (under the Endangered Species Act; ESA) has hampered efforts to monitor these populations.

Research Goals

The overall goals of this two year study were to evaluate the impact that *M. cerebralis* has on salmon and trout in the Lostine River, Oregon and to examine ecological factors which might explain why the parasite has had less of an effect on salmonid populations in Oregon versus the Rocky Mountain states. As previously mentioned, three of the salmonid species in the Lostine River are protectively listed under the ESA. This act, as applied to fish, precludes the sampling of any species in a manner which may impact the protected species, eliminating the use of electrofishing and other direct sampling methods to determine the distribution of *M. cerebralis* in this region. In accordance with this legislation, permission to perform this study was obtained from the National Marine Fisheries Service. The specific study questions and objectives are outlined below:

(1) Is the parasite present throughout the Lostine River, and does infection follow a seasonal pattern? *Approach:* Use sentinel fry to determine the relative abundance of the parasite at five sites on the Lostine River during the spring, summer and fall months.

(2) Is *T. tubifex* present at the sites where sentinel fish are exposed, and does the presence of the oligochaete host correlate with the percentage of infection among sentinel fish? *Approach:* Gather samples of benthic oligochaetes from each site at each sampling interval to establish the presence of *T. tubifex* over this time period, and determine if recovered *T. tubifex* are infected with *M.cerebralis* by monitoring for the release of triactinomyxons.

(3) Are environmental variables correlated with the relative abundance of the parasite and the presence of *T. tubifex*? *Approach:* Measure the temperature, dissolved oxygen, pH, conductivity and total dissolved solids in the river water at each sampling interval to determine if these data correlate with the presence of *T. tubifex* and percentage of infection among sentinel fry.

(4) Are chinook salmon populations in the Lostine River adversely affected by the presence of *M. cerebralis*? *Approach:* Compare the susceptibility of chinook salmon fry with rainbow trout fry in a natural exposure in the Lostine River when fry typically emerge and are most susceptible to infection. In addition, conduct controlled exposures to triactinomyxons in the laboratory using these two species to determine the relative susceptibility of chinook salmon.

(5) Can the current, lethal tissue sampling protocol for *M. cerebralis* detection by PCR be modified to allow for the non-lethal diagnosis of fish which may be protected under the ESA? *Approach:* Develop a non-lethal sampling protocol for PCR detection of *M. cerebralis* infection in rainbow trout fry receiving a controlled exposure and among naturally exposed adult steelhead trout recovered in the Deschutes River, OR.

THE DISTRIBUTION OF *MYXOBOLUS CEREBRALIS* IN THE LOSTINE RIVER, OREGON: IMPLICATIONS FOR RESIDENT AND ANADROMOUS SALMONIDS

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Introduction

The myxozoan parasite *Myxobolus cerebralis* was first identified among cultured salmonids in the United States in 1958. The parasite is believed to have been introduced from Europe by the transport of infected frozen fish, and to have then been spread to at least 23 of the United States (Hoffman, 1970, 1990; Bergersen and Anderson, 1997). This hypothesis was supported by Andree et al. (1999), who reported that no significant strain variation existed between five *M. cerebralis* isolates from Europe and North America when subjected to sequence analysis of the ribosomal DNA.

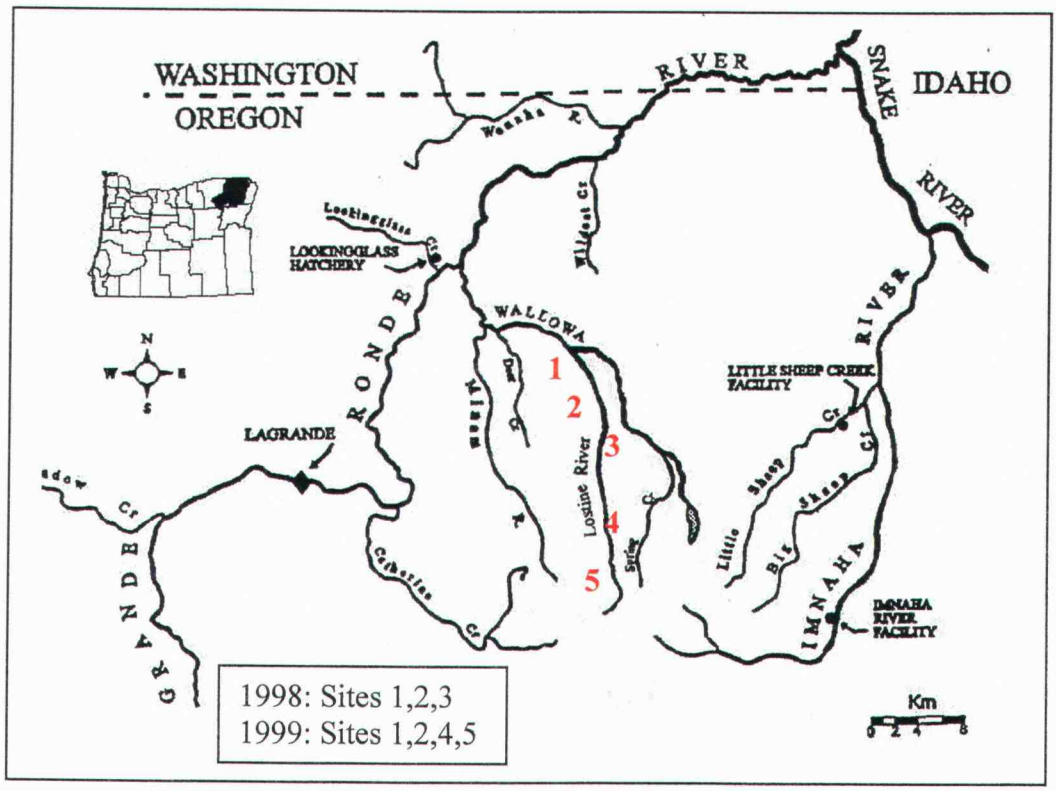
Myxobolus cerebralis has an obligate two-host life cycle, first described by Markiw and Wolf (1984), and many aspects of its biology have been well documented in both the salmonid and oligochaete (*Tubifex tubifex*) hosts (El-Matbouli et al., 1995, 1998). Infection of the fish host typically occurs when the sporoplasm of a water-borne triactinomyxon stage penetrates the epidermis, although fish ingesting infected oligochaetes or waterborne triactinomyxons may also become infected (El-Matbouli and Hoffmann, 1989; Markiw, 1989; El-Matbouli et al., 1999). Severe infection with *M. cerebralis* results in whirling disease, characterized by cranial, spinal and opercular deformities, blackened tails and whirling behavior in susceptible salmonid species (reviewed by Halliday, 1976; El-Matbouli, 1992; Hedrick et al., 1999a). Until the early 1990's, the disease was considered problematic only for cultured salmonids, when studies documented severe declines among rainbow trout populations in Montana and Colorado as a result of *M. cerebralis* infections (Nehring and Walker, 1996; Vincent, 1996). Impacts among salmonid populations outside of the inter-mountain West have been less severe, although there is little information currently available to explain this disparity.

Myxobolus cerebralis was first detected in Oregon in 1986 at a privately owned trout hatchery located on the Lostine River (Figure 2.1), a tributary of the Wallowa River in the Grande Ronde River basin (Lorz et al., 1989). Sampling surveys of natural fish populations and sentinel fish studies demonstrated that the parasite is enzootic in the Grande Ronde and Imnaha River basins, but there is no evidence that it has become established in tributaries of the lower Colombia River despite the migration of infected

anadromous salmonids into those rivers. There have been no reports documenting that declines in these populations can be directly attributed to this disease, although monitoring these species is now difficult due to their recent protective listing under the Endangered Species Act.

This study was undertaken to establish the distribution and seasonal prevalence of *M. cerebralis* infection in the Lostine River. This information was compared with the life histories of resident and anadromous salmonids in the river to assess the potential impact on these populations. Six species of salmonids are found in the Lostine River: rainbow (*Oncorhynchus mykiss*), steelhead (*O. mykiss mykiss)*, brook (*Salvelinus fontinalis*) and bull trout (*S. confluentus*), chinook salmon (*O. tshawytscha*) and mountain whitefish (*Prosopium williamsoni*). Information on *T. tubifex* abundance and water quality variables was also collected to determine if these factors are related to the incidence of *M. cerebralis* infection.

Figure 2.1 A map of the Lostine River, Oregon: Study Sample Sites



Methods

Information on spawning and emergence of Lostine River salmonids was obtained from the district biologist (Brad Smith, Oregon Department of Fish and Wildlife, personal communication; Table 2.1).

Table 2.1. Salmonid life histories in the Lostine River, Oregon

Species	Spawning Area (river mile)	Spawning Time	Emergence
Spring Chinook*	0-26	Aug-Sept	Mar-Apr
Steelhead Trout*	0-26, tributaries	Apr-early June	June-July
Rainbow Trout	0-26, tributaries	Apr-early June	June-July
Bull Trout*	15-26	Late Aug-Oct	March-May
Brook Trout	10-26, tributaries	Sept-Oct	March-May
Mtn. Whitefish	0-26	Sept-Nov	April-June

* indicates protective listing under the Endangered Species Act (ESA)

Sample Sites. In 1998, three sentinel sites were chosen to represent the diversity of habitats encountered on the Lostine River. Sites were sampled at approximately three-week intervals from July through late October, 1998. Site locations were: site 1 (river mile, RM 0.5), site 2 (RM 7) and site 3 (RM 9.5; Figure 2.1). Site 1 is an area of low gradient, with high sediment deposition and organic matter input from surrounding land use; site 2 is characterized by moderate gradient and moderate sediment deposition; and site 3 is an area of high gradient and low sediment deposition. Flow levels at both sites 1 and 2 were reduced in summer months by removal of water for irrigation; as a result, site 1 received supplementary water from the Wallowa river (also enzootic for *M. cerebralis*) via a canal in the summer months.

Based on data obtained in 1998, four sentinel sites were established in 1999, including two headwater sites where bull trout reside. These included sites 1 and 2 from 1998 and two new sites (site 4: RM 11.5; and site 5: RM 18) on the upper river. Both

sites on the upper river are characterized by moderate gradients and moderate sediment deposition.

Oligochaete Sampling. Seven sampling trips to collect oligochaetes were made in 1998, and six in 1999. At each sample period, benthic oligochaetes were gathered with a kick-net from the areas where sentinel cages were established. Three 1 meter^2 areas of sediment were sampled and combined from the area of the fry cage site; likely spawning redds were avoided. Samples of invertebrates collected with a kick net were kept live, on ice, until sorted in the laboratory. If oligochaetes were present, a sub-sample of presumptive *T. tubifex* (those specimens with pectinate and hair chaetae) were mounted on slides for positive identification (Brinkhurst, 1986). The remaining oligochaetes were maintained in culture at 15 °C and the culture water was screened through a 20 μm nylon mesh weekly for three months. The recovered material was examined microscopically (100x) for the presence of triactinomyxons (TAMs).

Water Quality Measurements. The following river water variables were measured at each site using a Corning M-90 Checkmate portable meter (Corning, NY): pH, water temperature, total dissolved solids, dissolved oxygen and conductivity. In 1999, remote temperature sensing units (Onset Computer Corporation, Pocasset, MA) were placed in the fry cages at sites 1, 2 and 4. Water temperature was recorded at four hour intervals for the duration of the 1999 sampling season.

Triactinomyxon Sampling. In 1998, 500 gallons of water were filtered at each of the three sites at each sampling interval. The water was filtered through a 20 μm nylon screen (Biodesign Inc., Carmel, NY) using either buckets, irrigation pipe, bilge pumps, or a combination of these methods. One half of the sample retained on the filter was preserved in 10% buffered formalin and examined microscopically (100x); the other half was concentrated by refiltering through 20 μm nylon mesh and suspended in 800 μl of lysis buffer (Buffer ATL, Qiagen; Valencia, CA) for the detection of *M. cerebralis* DNA by polymerase chain reaction (PCR).

Fish Exposure. Sentinel fish were obtained as eyed eggs requiring 126 daily temperature units to hatch from Mt. Lassen Trout Farm (Red Bluff, CA). Eggs were placed in two Whitlock-Vibert boxes (Federation of Fly Fishers, MT) which were filled with smooth gravel (12 mm diameter). The egg boxes were inserted into a fry cage made

from a 40.6 cm x 15.2 cm diameter PVC pipe. The entire fry cage was enclosed in 3 mm mesh. Hatching fry were able to swim out of the egg cages into the larger fry cage. The fry cage was filled halfway with larger gravel to submerge the cage and provide refuge for the emerging fish. Using this design, fry recovery varied but was usually between 25-50%.

In 1998, sentinel fry exposures began in July at the downstream site (site 1) and in August at the other sites. In 1999, sentinel fry exposures began in March at sites 1 and 2; exposures began in June at the other sites because they were inaccessible prior to this date due to snow. No eggs were placed in the river during May of either year due to high water flows.

Fish Sampling. Recovered fry were killed with an overdose of tricaine methanesulfonate (MS-222; Argent Laboratories, Redmond, WA) and netted onto a clean surface. Each fish was placed into a 2 ml microcentrifuge tube containing 400 ul of lysis buffer (Buffer ATL, Qiagen; Valencia, CA) for subsequent DNA extraction and analysis by PCR. Precautions were taken to prevent DNA contamination between fish. To determine the percentage of fry infected with *M. cerebralis*, 34 fry were randomly selected and processed for immediate analysis by PCR. If less than 34 fry were recovered, all were processed for PCR.

Following two exposure periods (April 9 and September 29, 1999), the fry remaining after samples were taken for PCR analysis were returned to the laboratory and placed in aquaria receiving specific pathogen-free water with aeration at 13 °C. The fish were treated for external parasites with malachite green (0.5 mg/ml) and observed for signs of whirling disease (black tails, spinal deformities, whirling behavior, etc.) for 5 months. Fish surviving to five months were killed with an overdose of MS-222; the heads were removed behind the operculum and split sagitally. To determine the severity of infection, half of each head was placed in 10% buffered formalin and processed for histology as described below; the other half was placed in a Stomacher bag (Fisher Scientific, Pittsburgh, PA) and a portion was processed for spore enumeration. The remainder of each spore enumeration sample was transferred to a microcentrifuge tube containing 400 ul of lysis buffer, boiled for 5 minutes, then processed for PCR.

PCR Analysis. In samples in which more than 34 fry were recovered from the egg cages, 34 fry were randomly selected and processed along with two unexposed control fry. Samples in lysis buffer were digested by the addition of 10 µl proteinase K and at least four hours on an orbital shaker at 37 °C. The samples were centrifuged to pellet undigested material and the supernatant was transferred to new microcentrifuge tubes. Ten microliters of pancreatic RNAse (5'-3'; Boulder, CO) were added to each tube and samples were incubated, with agitation, for one hour at 37 °C. DNA from the first three fry groups (1998 only) was extracted using a slightly different lysis buffer (Palenzuela et al., 1999) and a standard phenol/chloroform extraction procedure (Ellington and Pollard, 1994). Subsequent fry DNA was extracted using a Qiagen Tissue Kit (Qiagen; Valencia, CA), following the "Mouse Tail Protocol" provided by the manufacturer, modified for larger sample volumes. The extracted DNA was quantified by fluorometry (TKO 100, Hoefer Scientific Instruments, San Francisco, CA).

The presence of *M. cerebralis* DNA was determined using a nested PCR as described by Andree et al. (1998). The primers used were:

First round TR 3-16; 5'-GAATCGCCGAAACAATCATCGAGCTA-3'
TR 5-16; 5'-GCATTGGTTTACGCTGATGTAGCGA-3'

Second round TR 3-17; 5'-GGCACACTACTCCAACACTGAATTTG-3'
TR 5-17; 5'-GCCCTATTAACTAGTTGGTAGTATAGAAGC-3'.

The first round amplifications, using primers TR 3-16 and TR 5-16, were performed in 50 µl of reaction buffer using volumes of DNA template which provided between 200 and 250 ng of DNA. The second round amplifications, using primers TR 3-17 and TR 5-17, were performed in 25 ul reaction buffer, each containing one microliter of template from the first round reaction. All amplification reactions were preceded by a denaturation step of 5 minutes at 95 °C, followed by 35 cycles of 1 minute at 95 °C, 2.5 minutes at 65 °C and 1.5 minutes at 72 °C. The thermal cycler program (Amplitron II Thermocycler; Thermolyne, Inc., Dubuque, IA) was completed with a 10 minute elongation step at 72 °C, with a final dwell at 4 °C. Following the second amplification, samples were electrophoresed on a 1.5% agarose gel containing 0.01% ethidium bromide and photographed with UV illumination (Imagestore 7500, UVP Inc., Upland, CA). Positive samples were identified by the presence of a characteristic 415 bp band.

Histology. At 5 months post-exposure, a sub-sample of ten PCR-positive fry was taken from each experimental group. Half heads from sentinel fry were placed in tissue cassettes (Fisher scientific, Pittsburgh, PA) and stored in 10% buffered formalin. Samples were sent to the Washington Animal Disease Diagnostic Laboratory (Pullman, WA) for histological analysis. Infection was expressed as lesion severity using a scale from 0 (none) to 5 (severe). Descriptions of the histopathology represented by this scale are given in Hedrick et al. (1999a).

Spore Enumeration. Half heads from PCR-positive fry were disrupted in 2 ml of filter-sterilized water using a Stomacher tissue homogenizer run for 30 seconds per sample. Homogenized tissue was transferred to a 15 ml disposable centrifuge tube and the samples were digested using the pepsin and trypsin digest protocol (Thoesen, 1994). Recovered material was resuspended in 400 µl of sterile water and transferred to a microcentrifuge tube. To enumerate spores, 20 µl of the digest sample was transferred to a clean microcentrifuge tube and diluted 1:10 with 10% buffered formalin. Twelve microliters of this solution were then placed on a hemocytometer and four fields per sample were counted under magnification (200x).

Statistical Analysis. Attempts to detect significant correlations between water quality variables and the incidence of infection among sentinel fry and the presence of *T. tubifex* at the exposure sites were performed using a general linear regression model. The presence or absence of *T. tubifex* was treated as a binomial distribution. For the analysis of differences in the incidence of infection among sentinel fry analyzed immediately after exposure and at 5 months, a comparison of binomial proportions was made (Ramsey and Schafer, 1997). Significance was defined as $p < 0.05$. Statistical analyses of these data were conducted using the statistical software program S-PLUS (MathSoft, 2000).

Results

Oligochaete sampling. In 1998, 18 samples of benthic oligochaetes were collected and sorted from the three sentinel sites during six collection periods. *Tubifex tubifex* was identified in every sample collected at site 1 except on 6/24/99, when high water flows did not permit sampling, and in two of the six samples from site 3 (Table 2.2). Triactinomyxons were detected only from oligochaete cultures collected at site 1. During the second year of the study, 20 samples of benthic oligochaetes were collected and sorted from the four sentinel sites during 5 collection periods. *Tubifex tubifex* was identified in samples collected from sites 1, 2 and 4 in 1999, although only consistently at site 1 (Table 2.3). At site 2, *T. tubifex* was identified in one of the five samples, and in two of five samples collected at site 4. Of the samples returned to the lab and screened for three months, triactinomyxons were detected only from those cultures collected at site 1, on 4/9/99 and 7/28/99.

Water Measurements. Temperature, pH, total dissolved solids and conductivity measurements showed a tendency to increase with decreasing river mile in both years (Tables 2.2, 2.3), although there are several gaps in the data because the water meter was inoperable. Correlations between each of the water parameters measured and the incidence of infection among sentinel fry and presence of *T. tubifex* were examined using a general linear regression model. None of the measured variables correlated significantly with the presence of *T. tubifex*, but a significant correlation (one sided p-value <0.001) was found between conductivity and the incidence of infection among sentinel fry.

Water Filtration. All samples filtered in 1998 were negative for the presence of triactinomyxons by microscopic examination and PCR. Due to these results, water screening was discontinued in 1999.

Table 2.2. Incidence of *Myxobolus cerebralis* infection among sentinel fry, water measurements and presence of *Tubifex tubifex* at Lostine River Sites, 1998

Site/River Mile	Date [a] 1998	Analyzed/ recovered [b]	%Positive by PCR	Temp (°C)	pH	DO [c] (mg/ml)	TDS [d] (mg/l)	Cond. [e] (µS)	*T.tubifx* [f] found
Site 1	04/14	NA	NA	8.9	7.4	NA	59.5	119.8	Few
(RM 0.5)	06/24	NA	NA	8	NA	4.4	22.6	44.7	NA
	07/28	48/48	100	15.5	7.4	8	NA	NA	Several
	08/19	17/17	41.2	15.5	7.4	NA	121	244	Many
	09/10	34/49	65.3	15.7	NA	8.9	106	224	Few
	09/29	57/57	70	14.8	NA	8.6	104	206	Many
	10/23	34/63	62	10.1	8	5.8	108	216	Many
Site 2	04/14	NA	NA	7.5	7.2	NA	40.3	81.9	-
(RM 7)	06/24	NA	NA	8.5	NA	3.9	18.9	37.5	NA
	07/28	NA	NA	14	6.4	7	NA	NA	-
	08/19	34/44	29.4	13.4	6.7	NA	66.2	135.3	-
	09/10	34/62	8.8	14	NA	8.1	73.5	146	-
	09/29	34/64	50	13	NA	9.5	57.9	115.4	-
	10/23	34/95	14.7	7.5	7.2	8.3	70.8	70.8	-
Site 3	04/14	NA	NA	3.5	7	NA	31.8	58.6	-
(RM 9.5)	06/24	NA	NA	8	5.5	4.1	13.4	26.5	NA
	07/28	NA	NA	14	5.2	6.1	NA	NA	-
	08/19	34/41	0	14.6	7.1	NA	34.2	80.1	-
	09/10	34/86	34.4	12.3	NA	7.4	30.3	62.8	-
	09/29	34/66	0	10.3	NA	8.3	34.6	69.5	-
	10/23	34/105	8.8	7.1	6.3	6.3	34.2	41.4	-

[a] Date fry were recovered following a 14 day exposure

[b] Number of fish recovered/number of fish analyzed by PCR. NA= no fry exposed at this date.

[c] Dissolved oxygen, expressed in mg/ml. NA= meter inoperable.

[d] Total dissolved solids, expressed in mg/L. NA= meter inoperable.

[e] Conductivity, expressed in microsiemens (µS). NA= meter inoperable.

[f] *Tubifex tubifex* occurring in sample. Few:<10; Several=11-20; Many:>21. NA= no sample collected due to high water flows. (-)= none detected in sample.

Table 2.3. Incidence of *Myxobolus cerebralis* infection among sentinel fry, water measurements and presence of *Tubifex tubifex* at Lostine River Sites, 1999

Site/River Mile	Date [a] 1999	Analyzed/ recovered [b]	%Positive by PCR	Temp (°C)	pH	DO [c] (mg/ml)	TDS [d] (mg/l)	Cond. [e] (µS)	*T.tubifx* [f] found
Site 1	04/09	34/64	43	9.3	7.3	8.2	63.8	127.8	Yes
(RM 0.5)	06/20	0/0	NA	7	6.8	7.8	23.3	46.9	-
	07/28	2/2	50	13.4	7.5	9	42.9	86.2	Yes
	08/25	9/9	100	15.7	7.3	7.6	92.3	184.4	Yes
	09/29	26/26	69.2	9.3	7.7	10.64	118	239	Yes
	11/5	27/27	22.2	5.2	NA	NA	NA	NA	Yes
Site 2	04/09	34/73	30	7.1	7.2	6.7	54.6	109.1	-
(RM 7)	06/20	0/0	NA	7.2	5.8	8.6	15.3	32.6	-
	07/28	4/4	50	12.9	7.3	8	27.9	55.3	-
	08/25	34/37	8.8	15	7.7	6.9	47.7	95	-
	09/29	34/45	41.2	8.7	7.8	9.82	59.1	117.7	Yes
	11/5	34/38	6.3	5	NA	NA	NA	NA	-
Site 4	06/20	NA	NA	5.7	5.3	11.1	11.3	26	-
(RM 11.5)	07/28	34/36	8.8	12.3	6.7	7.2	14.8	28.2	Yes
	08/25	34/44	2.9	15.7	7.8	6.5	24.2	48.4	-
	09/29	0/0	NA	8.8	7.8	9.53	29.6	54.6	Yes
	11/5	0/0	NA	3.4	NA	NA	NA	NA	-
Site 5	06/20	NA	NA	NA	NA	NA	NA	NA	-
(RM 18)	07/28	34/56	3.1	12.2	6	7.6	6.81	13.7	-
	08/25	34/39	8.8	12.8	7.6	6.1	9.91	19.2	-
	09/29	26/26	11.5	6.4	7.7	4.88	10.3	20.4	-
	11/5	26/26	7.7	3.4	NA	NA	NA	NA	-

[a] Date fry were recovered following a 14 day exposure

[b] Number of fish recovered/number of fish analyzed by PCR. NA= no fry exposed at this date.

[c] Dissolved oxygen, expressed in mg/ml. NA= meter inoperable.

[d] Total dissolved solids, expressed in mg/L. NA= meter inoperable.

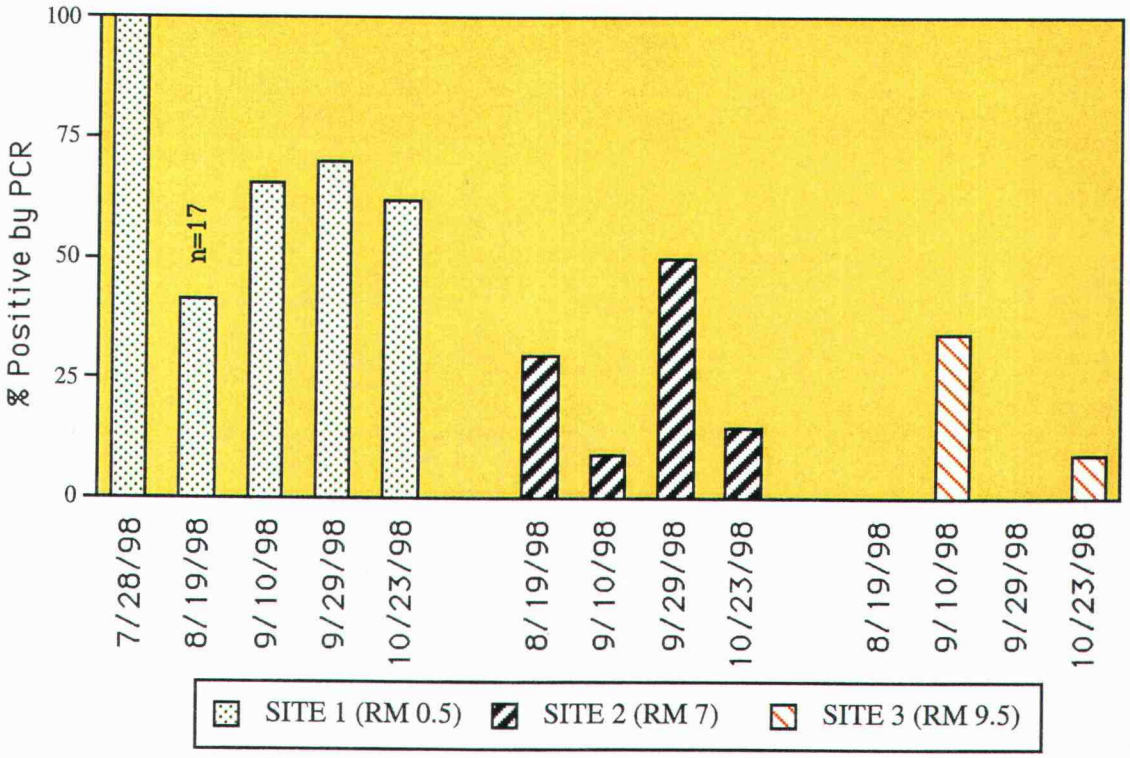
[e] Conductivity, expressed in microsiemens (µS). NA= meter inoperable.

[f] Presence or absence of *Tubifex tubifex* in sample. (-)= none detected in sample.

PCR analysis of sentinel fry. Thirteen groups of sentinel fry were recovered in 1998 following a 14 day exposure. At least 34 fish were analyzed by PCR from each group unless otherwise noted. *Myxobolus cerebralis* DNA was detected in fish exposed at all sample sites; however, the prevalence of infection varied. At site 1 (RM 0.5), 100% of the fry recovered on 7/28 were infected. The lowest percentage of infection at this site occurred in the subsequent exposure, with 42.2% of the fry positive on 8/19 (n=17). In

the three remaining samples, recovered on 9/10, 9/29 and on 10/23, the prevalence of infection was 65.3%, 70%, and 62%, respectively. At site 2 (RM 7) the incidence of infection ranged from 8.8% to 50%, with the greatest incidence of infection occurring in late September (9/29). The lowest incidence of *M. cerebralis* infection occurred at Site 3 (RM 9.5) and ranged from 0% percent on 8/19 and 9/10 to 34.4% on 9/29. An increase in the percentage of infected sentinel fry occurred as river mileage decreased for all exposure dates (Figure 2.2).

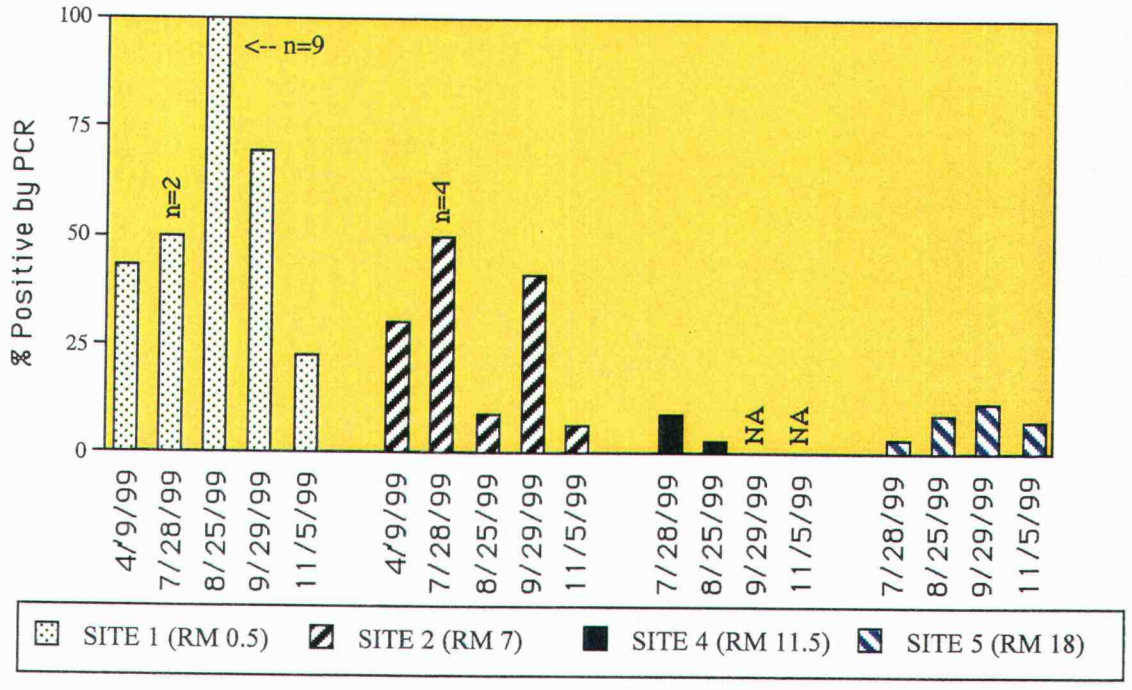
Figure 2.2 The incidence of *Myxobolus cerebralis* infection among sentinel fry exposed in the Lostine River, 1998



Nineteen groups of sentinel fry were recovered in 1999. At least 34 fish were analyzed by PCR from each recovered group of sentinel fish unless otherwise noted. At site 1 (RM 0.5) the incidence of infection among sentinel fish again fluctuated; the values obtained were: 43% (4/9), 50% (7/28; n=2), 100% (8/25; n=9), 69.2% (9/29), and 22.2%

(11/5). The percentages of sentinel fish recovered from site 2 (RM 7) that were positive by PCR were: 30% (4/9), 50% (7/28; n=4), 8.8% (8/25), 41.2% (9/29), and 6.3% (11/5; Figure 2.3). At site 4 (RM 11.5), only 2 groups of fry were recovered due to shallow water in the river channel at this site. These groups of sentinel fry were 8.8% and 2.9% positive on 7/28 and 8/25, respectively. At site 5 (RM 18), *M. cerebralis* infection was detected in all four exposure groups; the incidence of infection ranged from 3.1% to 11.5%, and was higher in the Fall/Winter exposures (11/5) than among those recovered in Summer (7/28; Figure 2.2). Samples with less than 9 fry recovered were used only as an indication that the parasite was present and were excluded from statistical analysis. A comparison of the data from 1998 and 1999 shows that the trend noted above was present during both years, with the percentage of infected sentinel fry increasing with decreasing river mileage.

Figure 2.3 The incidence of *Myxobolus cerebralis* infection among sentinel fry exposed in the Lostine River, 1999



Analysis of sentinel fry at 5 months post-exposure by spore enumeration, histology and PCR. Sentinel fry recovered at sites 1 (RM 0.5) and 2 (RM 7) on 4/9/99 and at sites 1, 2 and 5 (RM 18) on 9/29/99 were held in specific pathogen-free water for 5 months to determine the severity *of M. cerebralis* infection following a short natural exposure. The incidence of infection among fish recovered on 4/9 was 31.6% (n=19) at site 1 and 57.6% (n=33) at site 2 by PCR. No spores were detected in any of these fish, but cranial lesions were detected (mean lesion score 0.4) by histology in four of ten fry exposed at site 1 (Table 2.4). Of the fry recovered on 9/29/99, only those from sites 1 and 5 survived for 5 months; the fry from site 2 died of a fungal infection. The percent of infected fry from site 1 was 56% (n=25) by PCR at 5 months post-exposure, but none of the fry from site 5 were positive (n=34). Spores and cranial lesions were detected in most of the sampled fish exposed at site 1; the mean spore count was 540,000 per half head (8/10 positive) and the mean lesion score was 1.8 (9/10 positive). No spores or lesions were detected in the fish exposed at site 5. Signs of whirling disease (black tails) were evident in half of the rainbow trout fry recovered from site 1 on 9/29, but were absent in those fish recovered at site 5. No consistent patterns emerged when PCR data regarding the initial incidence of infection in each group was compared with the data at 5 months post-exposure (Figure 2.4).

Table 2.4. Results of histological analysis and spore counts for sentinel fish held for 5 months following a 14 day exposure in the Lostine River, Oregon

Site/River Mile	Date [a]	Mean spore count (No. positive) [b]	Spore count range	Mean lesion score (No. positive) [c]	No. with signs of WD [d]
Site 1 (RM 0.5)	4/9/99	0	-	0	0
Site 2 (RM 7)	4/9/99	0	-	0	0
Site 1 (RM 0.5)	9/29/99	$5.4x10^5$ (8)	0- $1.45x10^6$	1.8 (9/10)	5
Site 5 (RM 18)	9/29/99	0	-	0	0

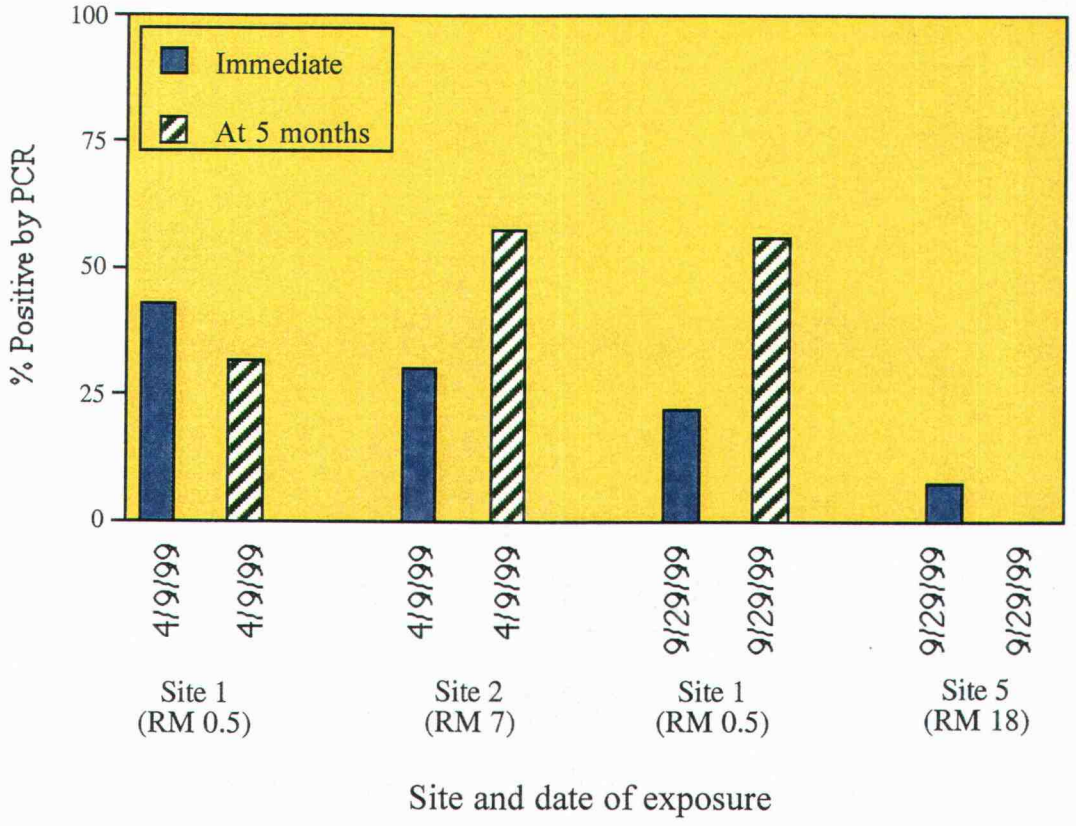
[a] Date fish were recovered following a 14 day exposure.

[b] Mean spore concentration per half head at 5 months post-exposure, and number of positive samples; n=10 for all groups.

[c] Lesions as determined by histological analysis on a scale from none (0) to severe (5) among 10 fish at 5 months post-exposure.

[d] Signs of whirling disease, including black tails and whirling behavior

Figure 2.4 The incidence of *Myxobolus cerebralis* infection immediately following and at five months post-exposure



Discussion

The importance of fish age and species on the susceptibility to infection by *M. cerebralis* (Markiw, 1991) prompted the use of rainbow trout fry of equal developmental stages in this study to establish where resident and anadromous salmonids are exposed to *M. cerebralis* in the Lostine River. The incidence of *M. cerebralis* infection in these sentinel fish was determined by PCR. Although this test is extremely sensitive, the assay only confirms the presence of parasite DNA and does not indicate the disease state of the host. To assess the severity of infection in exposed rainbow trout, selected groups of sentinel fry were returned to the laboratory and held for 5 months. These fish were

observed for developing signs of whirling disease and were analyzed by spore enumeration and histology to assess the potential impact of *M. cerebralis* infection.

In both years of this study, the incidence of infection varied among rainbow trout fry recovered at each site after a 14 day exposure, but infection occurred in fish recovered from all sites. The prevalence of infection among sentinel fry was highest at the lower river sites (sites 1 and 2; RM 0.5 and 7, respectively) in both years, particularly at site 1. *Tubifex tubifex* was found consistently only in those samples taken at site 1, and these were the only worms shown to be infected with *M. cerebralis* by the release of triactinomyxons. The river at site 1 contains many areas of heavy sediment deposition, and the river gradient is lowest at this site. Surrounding land use includes ranching and agricultural activity, resulting in increased input of organic matter to the riverbed. These conditions have been shown to encourage the establishment of large populations of *T. tubifex* and to decrease aquatic oligochaete species diversity (Brinkhust and Kennedy, 1965; Aston, 1973; Kaster, 1980; Thorpe and Covich, 1991; Modin, 1998), which may explain the higher incidence of *M. cerebralis* infection among sentinel fry exposed at this site. However, in the summer months, the Lostine River below site 2 is supplemented via an irrigation canal with water from the Wallowa River. The Wallowa River is enzootic for *M. cerebralis*, but this study did not examine the effect of this supplementation.

The strong correlation ($p<0.001$) between conductivity levels and the prevalence of infection among sentinel fry suggests that increased conductivity may lead to enhanced *M. cerebralis* infection. El-Matbouli et al. (1999) demonstrated that triactinomyxons have a chemotactic specificity for salmonids and that attachment only occurs on living tissue; it may be that the electrical field generated by living salmonids is a cue for the firing of the triactinomyxon polar filaments, and conductivity may be involved. However, a number of confounding variables are present in any natural exposure experiment; further research is warranted before conclusions can be drawn.

To assess whether the natural levels of *M. cerebralis* infection in the Lostine River can lead to disease, four groups of sentinel fry were held in the laboratory for five months after the 14 day exposure. Exposed fry were found to be positive for *M. cerebralis* both immediately after exposure and at five months, but no discernible trend was detected to indicate that sampling after 5 months increased the sensitivity of

detection by PCR. Spores were observed only in fish recovered from site 1 (RM 0.5) on 9/29/99 (mean spore count 540,000 per half head), and only these fish developed overt signs of whirling disease (black tails). In addition, only fish exposed at site 1 developed cranial lesions, with mean lesion scores of 0.4 (4/9/99) and 1.8 (9/29/99). These data suggest that salmonids residing in the lower river, where *T. tubifex* is more prevalent, would receive a chronic exposure to the parasite and are at risk of developing whirling disease, but more data are needed to clarify the relationship between river location, timing of exposure and severity of the resulting infection.

When information on the distribution and seasonal occurrence of *M. cerebralis* in the Lostine River is correlated with the life histories of resident and anadromous salmonids, it is evident that fry which emerge in the lower river during the period from June to September are most likely to become infected. This group includes resident rainbow and anadromous steelhead trout. Although several studies have documented differences in susceptibility between different salmonid species (Halliday, 1976; O'Grodnick 1979; Hedrick et al., 1999a; Thompson et al., 1999), there is consensus that rainbow trout, and presumably steelhead trout, are most likely to develop whirling disease following exposure at a young age. Chinook salmon have also been shown to be susceptible to *M. cerebralis*, but the comparative susceptibility of this species following a natural exposure at the time when they emerge (March to April) is unknown. However, because chinook salmon emerge in the lower Lostine river and typically reside in-river for a year before outmigrating, this species may also be at risk for infection and the development of whirling disease. The detection of *M. cerebralis* in the headwaters of the Lostine River also indicates that bull trout, which selectively reside in these areas, are being exposed to the parasite. However, a recent study by Hedrick et al. (1999a) showed that, while bull trout can be infected by *M. cerebralis*, this species appears to be considerably more resistant than rainbow trout to developing signs of whirling disease. Considering the low incidence of infection found at sites 4 (RM 11.5) and 5 (RM 18) in 1999, it is unlikely that bull trout are heavily impacted by *M. cerebralis*. Other species of salmonids emerging in the headwaters of the river would also be expected to receive a low exposure to *M. cerebralis*, but anadromous fry which emerge in the upper river must

pass through areas where infection levels are highest when they migrate as smolts and may become infected.

Although salmonid population dynamics in the Lostine River are no longer easily tracked due to restrictions on the sampling of protected fish populations, there are no data to suggest that *M. cerebralis* has had as severe an effect on salmonids in Oregon as in the inter-mountain West. The absence of severe population effects in the Lostine River may be the result of several factors, including: a) lower fish densities, which may inhibit transmission of the parasite via waterborne triactinomyxons, b) the limited occurrence of suitable habitat for the oligochaete host, *T. tubifex*, and c) the loss of myxozoan spore stages in infected anadromous hosts that migrate and do not return, which may reduce the prevalence of *M. cerebralis* infection in *T. tubifex* populations.

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THE COMPARATIVE SUSCEPTIBILITY OF SPRING CHINOOK SALMON (*ONCORHYNCHUS TSHAWYTSCHA*) AND RAINBOW TROUT FRY (*ONCORHYNCHUS MYKISS*) TO INFECTION BY *MYXOBOLUS CEREBRALIS* FOLLOWING NATURAL AND CONTROLLED EXPOSURES

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Introduction

Myxobolus cerebralis has been enzootic in northeastern Oregon since at least 1986 (Lorz et al., 1989), but the effects of the parasite on anadromous salmonid populations remain poorly understood. A number of anadromous species have been shown to be susceptible to infection by the parasite, including steelhead trout (*Oncorhynchus mykiss mykiss*), chinook (*O. tshawytscha*), coho (*O. kisutch*), sockeye (*O. nerka*), chum (*O. keta*), pink (*O. gorbuscha*), Atlantic (*Salmo salar*), masou (*O. masou*), Koundja (*Salvelinus leucomaenis*), Danube (*Hucho hucho*) and malma salmon (*Salvelinus malma*) (Hoffman et al., 1969; Lom, 1971; Halliday, 1976; Horsch, 1987; El-Matbouli et al., 1992; Baldwin et al., 1998). Susceptibility differences between species have been demonstrated (O'Grodnick 1979; Hedrick et al., 1999a, 1999b), although the relative susceptibility of many of these species has not been determined. A recent study by Thompson et al. (1999) suggests that susceptibility to *M. cerebralis* may also vary between strains of the same species; these researchers reported variations in the susceptibility of four strains of simultaneously exposed cutthroat trout (*O. clarki: bouvieri, stomias, virginalis, pleuriticas*).

Susceptible salmonids that are heavily infected with *M. cerebralis* develop whirling disease, characterized by spinal, cranial and opercular deformities, black tails and whirling behavior. Death may also result in severely infected juvenile fish; 100% mortality was reported by twelve days post-exposure in rainbow trout fry exposed to 1,000 triactinomyxons per fish (Markiw,1991). Sub-lethal infections by *M. cerebralis* have also been shown to decrease the swimming performance of juvenile rainbow trout, presumably impacting their ability to escape predation and compete for food and other resources (Eileen K. N. Ryce, Montana Cooperative Fishery Research Unit, Montana State University, personal communication).

A recent study by the authors (chapter 1 of this thesis) described the distribution of *M. cerebralis* in the Lostine River, a tributary of the Wallowa River. This third order stream has a high gradient in the upper reaches and a low gradient where it flows through the valley floor, and is typical of streams originating in the mountains of this region. The parasite was found to infect sentinel rainbow trout throughout the main stem of the river

(river miles 0-18) during the course of the study, even in areas where the oligochaete host *Tubifex tubifex* was not detected (Sandell et al., 2000). The findings of that study generated interest in the effects of *M. cerebralis* on anadromous species, whose populations have been in decline in this region for numerous reasons. To try to understand the potential effects of the parasite on populations of chinook salmon in this region and in the Lostine River in particular, chinook salmon fry of the Rapid River strain (upper Colombia River) were exposed as sentinel fish at a time when wild fry were emerging. In addition, these fry were exposed to two doses of *M. cerebralis* triactinomyxons under controlled conditions in the laboratory to ascertain the relative susceptibility of this strain of chinook salmon to the parasite.

Methods

Fish. Rainbow trout were obtained as eyed eggs (Mt. Lassen Trout Farm; Red Bluff, CA) and reared in specific pathogen-free (SPF) water at 13 °C. After absorption of the egg yolk, fry were fed a commercial trout food ad libitum. Two hundred and fifty chinook salmon were obtained from Rapid River Hatchery, Idaho, at six weeks of age. The two species were closely age-matched at the time of exposure, although the rainbow trout (average weight 0.74g) were slightly larger than the chinook salmon (average weight 0.59g) because of differences in growth rate.

Natural Exposure. Both species were transported to the exposure site on the Lostine River, OR in aerated, water-filled coolers. On March 26, 1999, one hundred chinook salmon and rainbow trout fry were placed in separate sentinel cages at river mile 0.5 on the Lostine River (OR) using methods previously shown to allow recovery of sentinel fish (Sandell et al., 2000). After the 14 day exposure, 34 rainbow trout fry were sampled to verify that the parasite was present at the time of exposure. The fish were killed with an overdose of tricaine methanesulfonate (MS-222; Argent Laboratories, Redmond, WA) and whole fry were placed in tissue lysis buffer (Qiagen; Valencia, CA) for PCR analysis, as described in Sandell et al. (2000). No chinook salmon fry were sampled at this time due to the limited number of survivors. The remaining fry of both

species were returned to the laboratory, treated for external parasites with malachite green (0.5 mg/ml) and maintained for five months in SPF water at 13 °C and fed a commercial trout diet. During this time the fish were observed daily and clinical signs of whirling disease were recorded. At the conclusion of the experiment, ten heads were processed for PCR, histology and spore enumeration as described in Sandell et al. (2000).

Triactinomyxon (TAM) production. Cultures of *T. tubifex* were fed *M. cerebralis* spores as described by Hedrick et al. (1999a). The worms were maintained in culture at 15 °C for eight weeks. Thereafter, the culture water was screened through 20 µm nylon screen (Biodesign Inc., Carmel, NY) weekly until TAMs were observed. The filtrate was resuspended in a known volume of water, 20 µl were stained with methylene blue, and triactinomyxons were enumerated on a hemocytometer (100x). Triactinomyxons were briefly held at 4 °C until used for exposures (within 15 minutes).

Controlled Exposures. Controlled laboratory exposures were performed in duplicate 20 liter tanks of 25 fish. The water level was reduced to 5 liters, maintained static with gentle aeration and triactinomyxons were added. The fish were exposed to doses of 200 and 2,000 triactinomyxons /fish for 2 hours. For each species, a control tank containing 20 fish was sham-exposed for 2 hours under identical conditions. Flow-through SPF water was then supplied at 13 °C and the fish were fed a commercial trout diet daily for five months until processed for PCR and spore enumeration as described in Sandell et al. (2000).

Assessment of infection and disease severity. At the conclusion of each exposure, a sub-sample of ten *M. cerebralis*-positive fry (by PCR) was taken from each group. Half heads were placed in tissue cassettes (Fisher scientific, Pittsburgh, PA) and stored in 10% buffered formalin. Samples were sent to the Washington Animal Disease Diagnostic Laboratory (Pullman, WA) for histological analysis. Infection severity was expressed as lesion severity using a scale from 0 (none) to 5 (severe). Descriptions of the histopathology represented by this scale are given by Hedrick et al. (1999b). The same ten samples were also examined for the presence of myxospores as described in Sandell et al (2000).

Statistical Analysis. Data regarding the presence or absence of *M. cerebralis* DNA by PCR were analyzed using a comparison of binomial proportions (Ramsey and Schafer,

1997). The significance of differences in the spore enumeration data collected was analyzed with a Student's t-test. Although the independence of the latter data could be questioned because the fish were exposed simultaneously in the same tank, it is common to assume independence among fish deliberately exposed under controlled conditions. Here, we report the level of significance calculated for the data when treated both independently and non-independently when significance was detected (significance was defined as $p < 0.05$).

Results

Natural Exposure. Analysis by PCR of those fish which survived to 5 months following a 14 day exposure in the Lostine River showed that 31.6% of the rainbow trout fry and 37.5% of the chinook salmon fry were positive for *M. cerebralis* DNA (Figure 3.1). The difference in the percentage of infection was not significant. No spores were detected from digested samples in either group and none of the fish developed signs of whirling disease. However, cranial lesions were detected among 9/10 rainbow trout fry examined; the mean lesion score was 1.8 (Table 3.1). No lesions were detected in the chinook salmon.

Controlled Exposures. Both rainbow trout and chinook salmon fry challenged with 200 and 2,000 triactinomyxons/fish were positive for the presence of *M. cerebralis* DNA by PCR. At 200 triactinomyxons/fish, the prevalence of infection in the replicate groups of rainbow trout fry was 91.6% and 79.2%; the prevalence of infection among replicate groups of chinook salmon fry at the same dose was 58.8% and 53.3%. *Myxobolus cerebralis* DNA was detected in almost all of the fry of both species following exposure at 2,000 triactinomyxons/fish (Figure 3.1). The difference between these groups at the low level of exposure was significant (two sided p-value= 0.0026), but no significant difference existed at the higher level of exposure.

The number of spores observed varied greatly between individual fish, but spores were observed most consistently in rainbow trout (Table 3.2). At an exposure level of 2,000 triactinomyxons/fish, spores were detected in 100% of the rainbow trout from both

replicate groups (mean spore counts of 156,000 and 63,000 per half head), and spores were observed in 50% of the fish exposed to 200 triactinomyxons/fish (mean spore counts of 26,666 and 15,000 per half head). Spores were detected in 80% of the chinook salmon in the first replicate group exposed to 2,000 triactinomyxons/fish (mean spore count of 75,500 per half head), but surprisingly, no spores were observed in the second replicate group, although infection was demonstrated by PCR.

Figure 3.1 The detection of *Myxobolus cerebralis* in rainbow trout and chinook salmon following natural and controlled exposures

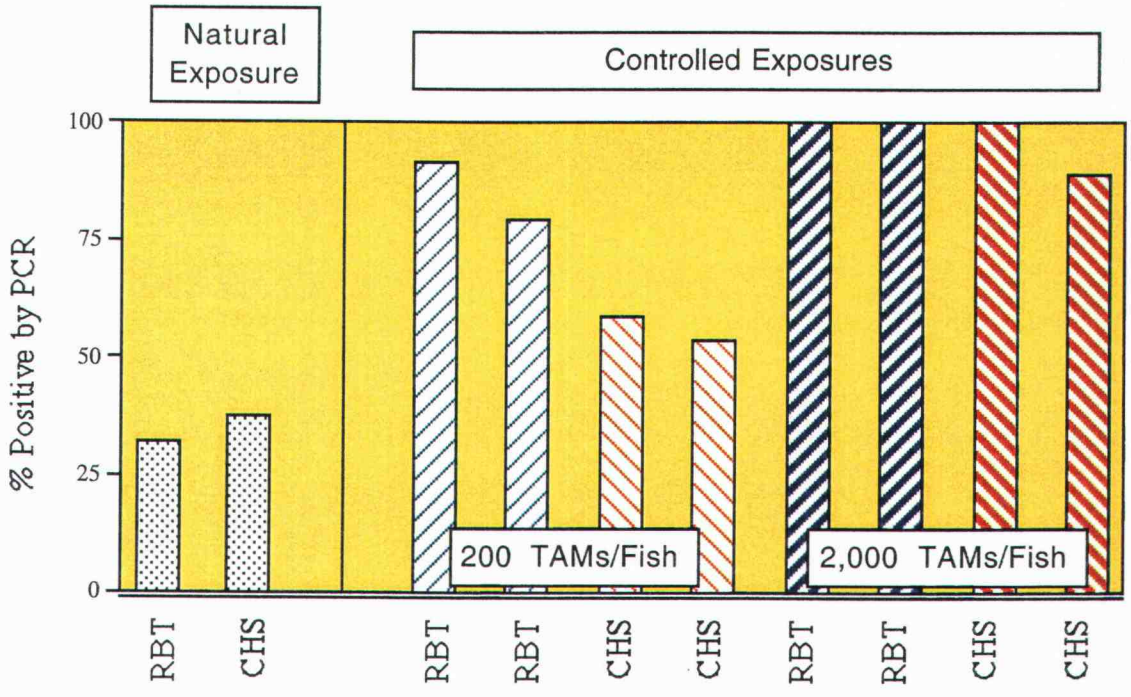

Table 3.1. Rainbow trout and chinook salmon fry exposed in the Lostine River, OR at river mile 0.5: Summary of spore counts and histology data at 5 months post-exposure to *Myxobolus cerebralis*

Species [a]	Date [b]	Mean spore count [c]	Mean lesion score (No. positive) [d]	No. with signs of WD
Rainbow trout	4/9/99	0	0.2 (1)	0
Chinook salmon	4/9/99	0	0.4 (1)	0

[a] Mt. Lassen rainbow trout and spring chinook salmon from Rapid River hatchery, ID.
[d] Date fish were recovered following a 14 day exposure at river mile 0.5.
[c] Average spore concentration per half head at 5 months post-exposure; n=10 for both groups.
[d] Lesions as determined by histological analysis on a scale from none (0) to severe (5) among 10 fish at 5 months post-exposure.

Cranial lesions among PCR-positive fish were more common in rainbow trout than in chinook salmon at both levels of exposure. Mean lesion scores for the rainbow trout replicate groups were 3.3 and 2.1 at 2,000 triactinomyxons/fish, and 9/10 had cranial lesions. Lesions were seen only in the first replicate group of chinook salmon exposed at this level (mean lesion score 1.5), of which 6/10 were positive. At the low level of exposure, lesions were detected only in the second replicate group of rainbow trout, and none were detected in either group of chinook salmon. Interestingly, the first replicate group of chinook salmon exposed to 2,000 triactinomyxons/fish contained the only fish to develop signs of whirling disease; three of twenty fish developed black tails and exhibited whirling behavior.

Table 3.2. Mean spore counts, ranges and mean histological lesion scores for replicate tanks of rainbow trout and chinook salmon fry sampled at 5 months after a controlled exposure to *Myxobolus cerebralis*

Species	TAM Dose	REP	Mean Spore Count	Mean Lesion Score (# positive)
Rainbow Trout	0		0	0
	200	1	26,666	0
		2	15,000	0.2 (2)
	2000	1	156,000	3.3 (10)
		2	63,000	2.1 (9)
Chinook Salmon	0		0	0
	200	1	0	0
		2	0	0
	2000	1	75,000	1.5 (6) **
		2	0	0

** Signs of whirling disease, including black tails and whirling behavior

Differences between the numbers of spores detected in the two species after exposure at 2,000 triactinomyxons/fish were not significant, even when independence was assumed for the data. To account for the tank effect seen in the second replicate of chinook salmon at 2,000 triactinomyxons/fish, pair-wise comparisons were made between each of the rainbow trout replicates groups and the first (positive) chinook salmon replicate group; no significance was detected. No spores were detected in any samples from the chinook salmon in either replicate group at the lower level of exposure. When spore count differences between rainbow trout and chinook salmon exposed to 200 triactinomyxons/fish were analyzed assuming independence, there was evidence of a significant difference (two-sided p-value= 0.0366). When the data were analyzed assuming non-independence (where n=2), the difference was not significant (two-sided p-value= 0.42). All unexposed control fish of both species were negative by PCR, spore enumeration and histology.

Discussion

Anadromous salmonid populations in the upper Colombia River and its tributaries have declined for numerous reasons, a discussion of which is beyond the scope of this report. However, *M. cerebralis* has been detected in all of the contiguous states in this region (Bergersen and Anderson, 1997), leading to concern over the potential role of the parasite in these declines. This study was undertaken to examine the potential effects of *M. cerebralis* on chinook salmon populations in the Lostine River, OR and throughout this region. To assess this, we exposed six week old chinook salmon fry of an upper Colombia River strain in the Lostine River at the time when chinook salmon in this river are naturally emerging. In addition, a controlled exposure was performed with chinook salmon fry to determine how severely they would be affected by infection with the parasite.

Five months after the natural, 14 day exposure, the incidence of infection among rainbow trout and chinook salmon fry by PCR was equal, but cranial lesions were observed only in the rainbow trout (mean lesion score1.8). This indicates that while chinook salmon exposed in the Lostine River are likely to become infected by the parasite, they may be resistant to the development of whirling disease at this level and duration of exposure.

Chinook salmon were also more resistant than rainbow trout to *M. cerebralis* infection and the development of whirling disease when exposed to low levels of the parasite in the laboratory, but this effect was not evident at the higher level of exposure. Five months after exposure to 200 triactinomyxons/fish, 56.3% of the chinook salmon were positive by PCR (combined average), while 85.4% of the rainbow trout were positive. Cranial lesions were only detected in the rainbow trout (mean lesion score 0.2).

At 2,000 triactinomyxons/fish, almost all of the rainbow trout and chinook salmon were positive for *M. cerebralis* DNA when analyzed by PCR after 5 months. Rainbow trout were more severely affected at this level of exposure, with higher mean spore counts (156,000 and 63,000) and a higher incidence (19/20) and severity of cranial lesions (mean lesion scores were 3.3 and 2.1 in the two replicates). Cranial lesions were also detected in 6/10 of the chinook salmon from the first replicate group (mean lesion

score of 1.5), and spores were detected in head digests from these fish (mean spore count of 75,500 per half head). The detection of cranial lesions and *M. cerebralis* DNA and spores indicates that these fish are capable of developing whirling disease when exposed to high levels of the parasite, but a tank effect was observed in the second chinook salmon replicate group. No spores or cranial lesions were detected in these fish, although a high incidence of infection was detected by PCR- evidence that they were exposed to *M. cerebralis*. It is possible that the triactinomyxons in this replicate tank were induced to extrude their polar filaments by mechanical agitation as a result of the tank aeration, which may have been more vigorous than in the other replicate tanks. This would have led to different average numbers of viable triactinomyxons/fish in the two tanks, and may explain these results.

The development of signs of whirling disease (black tails, whirling behavior) among the chinook salmon in only the first replicate tank (3/20) was also unexpected. Although these signs can arise from infection by other pathogens or dietary deficiencies (Margolis et al., 1996), all fish in the controlled exposure experiment were exposed and raised under similar pathogen-free conditions, making this explanation unlikely. It is also possible that, at this level of exposure, the impact of *M. cerebralis* on the host does not correlate well with the presence of myxospores in the cranium. In a report comparing the susceptibility of various salmonids following a natural exposure to *M. cerebralis* in Colorado, Thompson et al. (1999) reported that myxospore concentrations in cutthroat trout (*Oncorhynchus clarki*) strains did not correlate with survival, suggesting that this assay alone does not adequately predict the disease state of the host.

Overall, the data from the controlled exposures suggest that chinook salmon are relatively resistant to *M. cerebralis* infection and the development of disease when the exposure to triactinomyxons occurs at a low level. This resistance appeared to be negated when the triactinomyxon exposure level increased to 2,000 triactinomyxons per fish. The prevalence of *M. cerebralis* infection among chinook salmon following a brief natural exposure and the demonstrated capacity of this species to develop whirling disease when exposed to high triactinomyxon levels suggest that chinook salmon may be detrimentally impacted by the parasite. Chinook salmon are typically present in the river as juveniles for one year before smolting, and thus receive a chronic, low level exposure

to *M. cerebralis* which could result in disease. The effects on chinook salmon smolts may be even more pronounced, as they are subject to physiological stress as evidenced by elevated corticosteroid levels during the parr-smolt transformation and while adapting to salt water, although not all smolts migrate (Mazeaud et al., 1977; Hoar, 1988). A correlation between physiological stress and immune suppression has been well documented (Angelidas et al., 1987; Maule et al., 1987; Maule et al., 1989; Wang and Belosevic, 1995), but there have been no published studies on the effects of immune suppression on *M. cerebralis* infection in anadromous salmonids.

Future studies examining the effects of a long-term natural exposure in chinook salmon juveniles and smolts are needed to more accurately predict the impact of *M. cerebralis* on Lostine River chinook salmon and other populations of the upper Colombia River.

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AN EVALUATION OF A NON-LETHAL SAMPLING PROTOCOL FOR PCR-BASED DETECTION OF *MYXOBOLUS CEREBRALIS* DNA IN JUVENILE AND ADULT SALMONIDS

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Introduction

Efforts to identify and contain the spread of whirling disease have intensified since the mid-1990's, when the disease was shown to have catastrophic impacts on rainbow trout populations in the Rocky Mountain states (Nehring and Walker, 1996; Vincent, 1996). Diagnosis of the disease relies upon the identification of spores of the causative agent, *Myxobolus cerebralis,* in histological sections or in digests of cartilage tissue. Histology and the pepsin and trypsin digest protocol (Markiw et al., 1974; Thoesen, 1994) are still commonly used for the identification of *M. cerebralis*. However, visual identification of the spores may lead to misidentification due to their similarity to those of other species (Andree et al., 1998), and early stages of the parasite are often difficult to detect in histological sections and are destroyed by enzymatic digestion. The development of a *M. cerebralis*-specific nested polymerase chain reaction assay (PCR; Andree et al., 1998) has provided a sensitive method to detect all stages of this parasite in both salmonids and the invertebrate host, *T. tubifex*. However, sample collection for the *M. cerebralis*-specific PCR assay typically requires lethal sampling of cranial tissue. The decline of resident and anadromous salmonid populations in many areas of the United States and the resulting protective listing of several populations under the Endangered Species Act (ESA) has generated increased interest in the development of non-lethal detection techniques. In addition, lethal sampling of economically important strains of brood fish represents a substantial economic loss to managers of aquaculture facilities (Wooster et al., 1993).

Previous studies have demonstrated the feasibility of non-lethal sampling for monitoring physiological changes or for the detection of fish pathogens. Zuagg and McLain (1971) and Rodgers et al. (1987) used gill filament samples to study changes in gill ATPase activity in coho salmon (*Oncorhynchus kisutch*) during their adaptation to salt water, and reported minimal adverse effects on the host. More intrusive surgical sampling was performed by Wooster et al. (1993) to obtain tissue samples for the detection of several fish pathogens, while minimizing detrimental effects. The study described here was performed to evaluate the suitability of using samples which can be collected non-lethally for detection of *M. cerebralis* infection by PCR in juvenile rainbow

trout (*Oncorhynchus mykiss*) after a controlled exposure to *M. cerebralis*. Caudal fin, gill filament and opercular tissues were selected for sampling because they contain cartilage (the target tissue), are easily sampled and minimize injury to the fish. Based on the results of *M. cerebralis* detection by this method in rainbow trout, the sampling protocol was also tested on adult steelhead trout (*Oncorhynchus mykiss mykiss*) to determine the efficacy at detecting *M. cerebralis* DNA in larger fish which were naturally exposed at an unknown level.

Methods

Controlled Parasite Exposure. Triactinomyxons for the controlled exposures were obtained by screening laboratory cultures of *Tubifex tubifex* previously infected by seeding with *M. cerebralis* spores, as described in Andree et al. (1998). Two groups of 20 rainbow trout fry (obtained as eyed eggs from Mt. Lassen Trout Farm, Red Bluff, CA) were raised in 20 liter tanks receiving specific pathogen-free well water at 13 °C. The fry were exposed to *M. cerebralis* triactinomyxons at 3 weeks post-hatch as described in Sandell et al. (2000a). Group 1 was exposed at a concentration of 200 triactinomyxons/fish, and group 2 was exposed at 2,000 triactinomyxons/fish. A third group of 20 rainbow trout was sham exposed under identical conditions to provide a negative control. Following the exposures, flow-through well water was supplied. After 72 hours, the fish were fed a commercial trout diet daily. At five months post-exposure, the fish were killed with an overdose of tricaine methanesulfonate (MS-222; Argent Laboratories, Redmond, WA) and processed for analysis by polymerase chain reaction (PCR; described below).

Natural Parasite Exposures. To test the efficacy of this protocol in detecting *M. cerebralis* infections among adult fish, fifty adult stray steelhead returning to Round Butte Dam on the Deschutes River, OR were collected by Oregon Department of Fish and Wildlife (ODFW) personnel. These fish are presumed to have originated from tributaries of the upper Colombia River, where *M. cerebralis* is enzootic, because there is no longer a migratory population of steelhead below Round Butte Dam. Steelhead trout

were also killed with a lethal dose of MS-222, sampled and processed for analysis by PCR as described below.

Sample Collection. Caudal fin, gill filament and operculum tissues were sampled from the juvenile rainbow trout challenged with *M. cerebralis*. For the operculum and caudal fin samples, a standard size (7 mm opening) stainless steel paper hole punch was used to remove tissue samples of equal diameter; for the gill filament samples, the tips of 4-5 filaments were cut from the posterior gill arch with scissors and weighed to ensure they did not exceed 0.02 g. All samples were immediately transferred to individual microcentrifuge tubes containing 400 µl of tissue lysis buffer (Qiagen; Valencia, CA) and labeled so that they could be compared after analysis. Dissecting tools were decontaminated between each use in a 15% bleach solution for a minimum of 2 minutes, and then rinsed thoroughly. As a positive control, the head of each fish was split sagitally and half was processed for PCR.

Gill filaments and control cranial tissues were sampled from the naturally exposed adult steelhead trout. Gill filament samples were collected as described above, weighed (samples weighed <0.02 g), transferred to labeled microcentrifuge tubes (to allow later comparison) containing 400 µl of tissue lysis buffer and processed for PCR. Cranial tissue samples were taken using a core borer as described in Lorz et al. (1989), then digested using the pepsin and trypsin digest protocol (Thoesen, 1994). The spores were pelleted by centrifugation, resuspended in 400 µl of sterile water, and 100 µl was removed and processed for PCR. The remaining spores were enumerated as described below.

Spore Enumeration. Cranial samples from the adult steelhead trout were examined for the presence of spores. Twenty microliters of each spore sample were transferred to a microcentrifuge tube and diluted 1:10 with 10% buffered formalin. Twelve microliters of this solution were then placed on a hemocytometer and four fields per sample were counted under magnification (200x). In cases where no spores were detected, samples were reexamined at a 1:4 dilution.

PCR Analysis. Samples from both the juvenile rainbow and adult steelhead trout were boiled for 5 minutes and digested by the addition of 10 µl proteinase K (Qiagen; Valencia, CA) with at least four hours of agitation on an orbital shaker at 37 °C. The

samples were centrifuged to pellet undigested material and the supernatant was transferred to new microcentrifuge tubes. Ten microliters of pancreatic RNAse (5'-3'; Boulder, CO) were added to each tube and samples were incubated, with agitation, for one hour at 37 °C. DNA was extracted using a Qiagen Tissue Kit, with modifications as described in Sandell et al. (2000b). The extracted DNA was quantified by fluorometry (TKO 100, Hoefer Scientific Instruments, San Francisco, CA) and diluted to provide 200-250 ng of DNA for the initial reaction (Amplitron II thermocycler; Thermolyne, Inc., Dubuque, IA). Samples were assayed for *M. cerebralis* DNA using a nested PCR as described by Andree et al. (1998). Following the second amplification reaction, samples were electrophoresed on a 1.5% agarose gel containing 0.01% ethidium bromide and photographed with UV illumination (Imagestore 7500, UVP Inc., Upland, CA). Positive samples were identified by the presence of a characteristic 415 bp band, indicating the presence of *M. cerebralis* infection in these fish.

Determination of Sampling Injury. Because the collection of the control cranial tissue required lethal sampling, the sampling injury caused by each procedure was determined in a separate experiment. Four 20 liter tanks were established, each containing five unexposed rainbow trout which were age-matched with the trout from the controlled exposure experiment. For each tank, fish were anesthetized with MS-222 and sampled for one of three tissues (caudal fin, operculum, gill filament tips) using the method previously described. The fourth group of fish was anesthetized and returned to well water as a control. After a 72 hour recovery period, all four groups of fish were fed a commercial trout diet daily and observed for three weeks; mortalities were recorded.

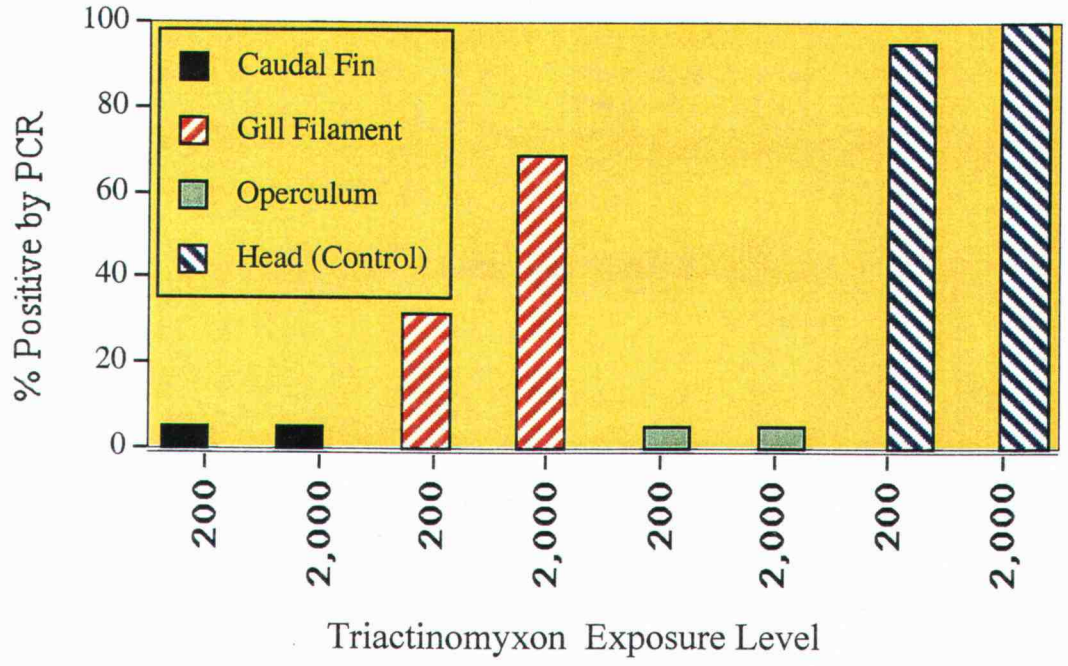
Statistical Analysis. PCR data indicating the presence or absence of *M. cerebralis* in the controlled parasite exposure experiment were compared using Fischer's exact test, as presented in Ramsey and Schafer (1997).

Results

Controlled Parasite Exposure. Detection of *M. cerebralis* infection among juvenile rainbow trout exposed to 200 triactinomyxons/fish and 2,000

triactinomyxons/fish was: caudal, 5.3% and 5.3%; gill filaments, 31.6% and 68.4%; operculum, 5.3% and 5.3%, respectively. At the lower exposure level, 94.7% of the controls (digested half-heads) were PCR-positive; 100% were positive at the high exposure (Figure 4.1). P-values generated by Fisher's exact test demonstrated significant differences between the gill filament samples and the operculum and caudal fin samples. The level of detection in the gill filament samples taken from fish exposed to 2,000 triactinomyxons/fish was significantly higher than those taken from fish exposed to 200 triactinomyxons/fish (one sided p-value= 0.023). Although the non-lethal gill filament samples detected the highest incidence of infection by PCR, the number of positive samples were significantly lower than among the positive control cranial tissue samples at both exposure levels (one-sided $p<0.001$ and $p=0.007$). All of the unexposed control fish were negative for *M. cerebralis* DNA by PCR, and none of the fish exhibited signs of whirling disease over the course of the experiment.

Figure 4.1 The detection of *Myxobolus cerebralis* in rainbow trout tissues sampled via a non-lethal sampling protocol



Determination of Sampling Injury. In the four groups, mortalities occurred among only the control fish (1/5) and the caudal fin-sampled fish (1/5). The small number of fish used in this trial (n=5 for each treatment) precluded statistical analysis. The cause of mortality in this trial is unknown, but may have occurred as a result of exposure to MS-222.

Natural Parasite Exposures. Among the cranial samples from adult steelhead trout, 24% were positive for *M. cerebralis* by spore enumeration, and 46% were positive by PCR analysis. All but one of the samples which were identified as positive by spore enumeration were positive by PCR. None of the adult steelhead trout gill filament tissue samples taken by the non-lethal sampling method were positive by PCR.

Discussion

Previous reports have shown that several variables, including fish age and level of parasite exposure, influence the development of *M. cerebralis* infections in salmonids (Halliday, 1976; El-Matbouli et al., 1992; Markiw 1992; Hedrick et al., 1999a,b; Thompson et al., 1999). In this study, we investigated the effects of fish age and parasite exposure level on the ability of a non-lethal, PCR-based protocol to detect the presence of *M. cerebralis* DNA. Results from the first study, using juvenile rainbow trout that had been challenged with a known parasite dose, indicated that gill filament tips provided the best detection of *M. cerebralis* DNA by PCR in comparison with lethally collected positive control cranial tissue samples. The level of detection was significantly higher at a parasite dose of 2,000 triactinomyxons/fish than at a lower dose of 200 triactinomyxons/fish. Detection of parasite DNA from caudal fin and operculum samples was low, with only one fish at each exposure level shown to be positive; these same fish were also positive when the gill filaments and cranial tissue samples were analyzed. When juvenile rainbow trout were sampled to determine the effects of sampling injury, those with caudal fin, gill filament or operculum tissue removed fared no worse than the control fish which did not have any tissue removed.

However, the gill filament sampling protocol that showed promise at detecting *M. cerebralis* in juvenile rainbow trout was not successful in detecting parasite DNA in adult steelhead trout. No positive samples were detected, although many of these fish were positive for *M. cerebralis* when cores of cranial tissues were examined by spore enumeration (24%) and PCR (46%). Failure of this protocol to detect the parasite in gill filaments of adult steelhead trout may have been caused by a difference in the distribution of the parasite in juvenile versus adult fish. The level at which these adult steelhead trout were naturally exposed while juveniles is unknown, but another study by the authors suggests that water-borne triactinomyxons occur at levels too low to be easily detected in some tributaries of the upper Colombia River (Sandell et al., 2000b).

These results suggest that there is potential for the development of a non-lethal, PCR-based detection technique for monitoring *M. cerebralis* infections, particularly in juvenile salmonids. However, the protocol as described here does not appear to be effective in detecting *M. cerebralis* in adult fish, for which the test is most needed. Further studies with adult fish that have received higher levels of exposure to *M. cerebralis* triactinomyxons, or studies utilizing different tissues for non-lethal sampling, are needed to determine if this protocol will be of use in monitoring protected or economically valuable adult salmonid populations.

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SUMMARY

Myxobolus cerebralis, the parasite causing whirling disease, was first detected in Oregon in 1986 (Lorz et al., 1989). Presently, the parasite is enzootic only in the northeastern corner of the state, in the Grande Ronde and Imnaha Rivers and their tributaries. Although this parasite was identified as the cause of catastrophic rainbow trout population declines in the Madison (Vincent, 1996) and Colorado (Nehring and Walker, 1996) Rivers, there have been no reports of detrimental impacts which can be directly attributed to *M. cerebralis* among salmonid populations in Oregon. However, the current protective listing of several salmonid populations in tributaries of the upper Colombia River under the Endangered Species Act (ESA) prevents extensive sampling for population studies, limiting our knowledge of the effects of the parasite on salmonid populations in this region.

The studies described here were performed to determine the distribution and seasonal occurrence of the parasite in the Lostine River, a typical tributary of the Wallowa River (both are tributaries of the Grande Ronde River). Information was gathered to analyze some of the ecological factors which may define the occurrence of *M. cerebralis* in this region, and to determine the effects of infection on resident and anadromous salmonids. In addition, a non-lethal tissue sampling protocol was developed for detection of the parasite by the polymerase chain reaction (PCR), although the protocol as described here was not sensitive enough at detecting the parasite in adult fish. If modified, this protocol could facilitate studies to determine the distribution and impacts of *M. cerebralis* on resident and anadromous populations, within the restrictions imposed by the ESA.

This research has established that *M. cerebralis* is present throughout the Lostine River from at least March to November, and that infections, as determined by the exposure of sentinel rainbow trout fry, occur in all areas of the river regardless of water temperature and the detection of the parasite's oligochaete host, *Tubifex tubifex,* at the exposure areas. The incidence of infection among sentinel rainbow trout fry was highest in the lower river, where *T. tubifex* was most commonly recovered. Sentinel rainbow

trout fry exposed in September and returned to the laboratory were shown to contain spores, cranial lesions and develop signs of whirling disease, including mild spinal, cranial and opercular deformities, black tails and whirling behavior. A trend was seen in the incidence of infection among sentinel fry as determined by PCR, with the highest percentage of infection occurring during the Summer and Fall months. When correlated with the life histories of Lostine River salmonids, this information indicates that rainbow and steelhead trout and chinook salmon, which emerge during these months, are most likely to be infected by *M. cerebralis*. In addition, the data suggest that protected bull trout populations, which selectively inhabit the headwaters of the Lostine River due to their preference for low water temperatures, are also potentially exposed to *M. cerebralis*.

To investigate the effects of *M. cerebralis* infection on chinook salmon fry, controlled exposures were conducted at 200 and 2,000 triactinomyxons/fish to determine the susceptibility of this species in comparison to that of rainbow trout. Relative to rainbow trout, chinook salmon had a lower incidence of infection at the lower level of exposure, but were equally susceptible when exposed to 2,000 triactinomyxons/fish. Cranial lesions and spores were not detected in chinook salmon at 200 triactinomyxons/fish, confirming the resistance of this species to *M. cerebralis* at this exposure level. The apparent resistance of chinook salmon was not present at the higher level of exposure; cranial lesions and spores were detected in these fish, indicating that chinook salmon can develop whirling disease if the parasite inoculum is high enough.

To determine whether chinook salmon fry would become infected at a location and time when they would naturally be emerging, rainbow trout and chinook salmon fry were placed in the Lostine River in late March and exposed for 14 days. When analyzed by PCR at 5 months after exposure, the incidence of infection in both species was equal, indicating that chinook fry can become infected following exposure in the Lostine River. When this information is combined with the cranial lesion and spore data discussed above, it would appear that chinook salmon are less likely to develop signs of whirling disease when the level of triactinomyxons is low and the exposure is brief. However, the effect of a prolonged exposure, even when triactinomyxon levels are low, is unknown. Spring chinook salmon fry are typically present in the river for one year before smolting,

and thus are potentially receiving an exposure of one year, part of which occurs at the times when the prevalence of infection was shown to be highest.

Although the evidence presented here indicates that *M. cerebralis* is present and infectious for sentinel rainbow trout and chinook salmon in the Lostine River, restrictions on the sampling of protectively listed species under the ESA have limited our knowledge of the distribution and effects of the parasite in salmonid populations in other tributaries of the Colombia River. In an attempt to address this problem, a non-lethal tissue sampling protocol for the detection of *M. cerebralis* by PCR was developed. Although this assay provides evidence only of the presence of *M. cerebralis* DNA and does not indicate the severity of infection or effect on the host, it is currently the most sensitive assay for detection of the parasite (Andree et al., 1998). This protocol was used to analyze caudal fin, opercular and gill filament tissues from rainbow trout exposed to two levels of triactinomyxons (200 and 2,000 triactinomyxons/fish). Of these tissues, only gill filaments provided a moderate level of detection (31.6% and 68.4% at 200 and 2,000 triactinomyxons/fish, respectively). However, the level of detection in gill filaments was significantly lower in both cases than the levels detected in the cranial samples. Analysis of the mortality due to tissue sampling showed that rainbow trout which had portions of the caudal fin, gill filaments or operculum removed fared as well or better than control fish which were not sampled, although this result needs to be confirmed in larger trials before the protocol is used to analyze protected salmonid populations.

This protocol was also used in an attempt to detect *M. cerebralis* infections in gill filament tissue from adult steelhead trout that had received a natural exposure. *Myxobolus cerebralis* DNA was not detected in any of these samples, although some of the fish were shown to be infected when analyzed by spore enumeration of cranial tissue, indicating that the protocol as currently defined may not be useful for the detection of the parasite in adult, anadromous salmonids. It may, however, be useful for detecting *M. cerebralis* infections among juvenile salmonids, and could provide information on the distribution and prevalence of infection among salmonids of this age class. In addition, the protocol may be of use in monitoring anadromous fish which stray into tributaries other than those of their origin.

Since the initial detection of *M. cerebralis* in the Lostine River in 1986 (Lorz et al., 1989), there have been no published reports on the distribution of the parasite or its effects on salmonid populations in Oregon. The absence of severe population effects in regions beyond the intermountain West, including Oregon, may be the result of several differences between these regions. In a review of *M. cerebralis* in California, Modin (1998) suggested that the impacts of the parasite have been mitigated due to the "high gradients and the flushing action of eastern Sierra and coastal streams due to heavy spring snowmelt and heavy winter rains preclude the buildup of large tubificid worm populations that are required for significant infections to occur." Similar conditions are found in the upper Lostine River, as evidenced by the low occurrence of *T. tubifex* in samples gathered at sentinel fish exposure sites above river mile 7. Other differences between the Lostine River and the Madison (MT) and upper Colorado Rivers include lower fish densities in the Lostine River, which may inhibit transmission of the parasite via waterborne triactinomyxons, and the loss of myxozoan parasite stages in infected anadromous hosts which migrate to the Pacific Ocean and fail to return.

It has already been suggested that infected, anadromous salmonids are potential vectors for the transmission of *M. cerebralis* into non-enzootic tributaries of the lower Colombia River. The detection of *M. cerebralis* in sentinel chinook salmon fry exposed in the Lostine River validates this concern. Horsch (1987) proposed that anadromous fish may have contributed to the spread of *M. cerebralis* in the Sacramento River system (CA), but a later review analyzing the sequential detection and distribution of *M. cerebralis* in California refuted this suggestion (Modin, 1998). However, *T. tubifex* have been identified in tributaries of the lower Colombia River (Harriet Lorz and Donald Stevens, co-authors, personal communication), indicating that this potential does exist. Concern generated by this information should be tempered by the knowledge that *M. cerebralis* has been present in tributaries of the upper Colombia River since at least 1986 (Lorz et al., 1989), yet there have been no reports of the parasite becoming established in resident fish populations of the lower Colombia River to date.

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