AN ABSTRACT OF THE THESIS OF

Sarah A. Sollid for the degree of Master of Science in Microbiology presented on September 27, 2002.
Title: Susceptibility of Select Salmonids to *Myxobolus cerebralis* and Effects of Exposure on Anadromous Salmonids in Oregon.

Abstract approved Redacted for privacy
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Myxobolus cerebralis, a myxozoan parasite of salmonids, is the causative agent of whirling disease. The parasite is considered widespread throughout northeastern Oregon in the Grande Ronde and Imnaha River basins where threatened and endangered salmonid populations exist. The work presented in this thesis comprises several studies that assess the effects of *M. cerebralis* on resident and anadromous salmonids in Oregon.

Laboratory challenges to determine the susceptibility of indigenous Deschutes River, Oregon, salmonids to *M. cerebralis* were conducted as part of a study to assess the risk of reintroducing anadromous salmon above a migration barrier on that river. This study was the first to assess the susceptibility of kokanee salmon *Oncorhynchus nerka* to *M. cerebralis*, and results contribute to the understanding of salmonid susceptibility as it relates to species and age. Further, this study demonstrates that the indigenous salmonids present in the river are susceptible to infection, but resident rainbow trout *Oncorhynchus mykiss* would be most at risk should introduction of *M. cerebralis* occur in this system.

The susceptibility of chinook salmon *Oncorhynchus tshawytscha* to *M. cerebralis* was assessed following laboratory challenges at different ages to different parasite levels. Results from this study indicate that chinook salmon are more resistant to *M. cerebralis* infection than susceptible rainbow trout; resistance to disease developed in chinook salmon exposed after 3 weeks of age. Sustained exposures to a low parasite dose were performed to model the conditions chinook salmon would encounter in a natural exposure. Following continuous exposure to low parasite densities, chinook salmon were very resistant to infection and disease.

Managed populations of chinook salmon and steelhead *Oncorhynchus mykiss* juveniles are held in acclimation facilities on their natal streams before release to commence their seaward migration. Rainbow trout fry were held at each of the acclimation facilities to detect the presence of *M. cerebralis* and to assess the prevalence of exposure during the acclimation period. Results from these exposures indicate that the Wallowa acclimation facility is a high-risk site for parasite exposure of steelhead juveniles. Further, detection was confirmed in several rivers and streams where the presence of *M. cerebralis* has been suggested.

Finally, a preliminary study on the effect of *M. cerebralis* on survival of steelhead juveniles upon transfer to saltwater was conducted. Results from this study indicate that *M. cerebralis* exposure and infection may increase mortality among juvenile steelhead during saltwater adaptation.

Susceptibility of Select Salmonids to *Myxobolus cerebralis* and Effects of Exposure on Anadromous Salmonids in Oregon

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CONTRIBUTION OF AUTHORS

Dr. Jerri L. Bartholomew was involved in experimental design, interpretation of results, and all phases of manuscript preparation. Harriet V. Lorz and Donald G. Stevens assisted with maintenance of study animals and data collection.

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CHAPTER 1: INTRODUCTION

WHIRLING DISEASE

Whirling disease is caused by the myxozoan parasite *Myxobolus cerebralis*, and signs of the disease are a result of its selective tropism for cartilaginous tissue. Heavy infection of rainbow trout *Oncorhynchus mykiss* by *M. cerebralis* can result in chronic disease characterized by caudal melanosis ("blacktail"), erratic swimming behavior ("whirling"), and cranial and spinal deformities (Höfer 1903; Uspenskaya 1957; Markiw and Wolf 1974, 1983; Markiw 1992a; Hedrick et al. 1998; MacConnell and Vincent 2002). Severely infected fish often have increased mortality rates and heightened susceptibility to predation (Markiw 1992a; MacConnell and Vincent 2002).

Clinical signs of disease are a result of parasite attack, as well as the inflammatory response its presence elicits (Hedrick et al. 1998; MacConnell and Vincent 2002). The infection of spinal cartilage and subsequent inflammation may result in pressure on the caudal nerves, leading to a loss of control over pigmentation in the tail region. The result is a darkened tail region, or "blacktail" (Halliday 1976; MacConnell and Vincent 2002). A recent study by Rose et al. (2000) found that compression of the brain stem and constriction of the spinal cord

by inflammation causes whirling behavior. Normal bone deposition can also be disrupted by the inflammatory response to the parasite, causing permanent spinal and cranial deformities in susceptible fish (MacConnell and Vincent 2002).

GEOGRAPHIC DISTRIBUTION OF *Myxobolus cerebralis*

Myxobolus cerebralis was first described in Germany in 1893 when the parasite was detected in rainbow trout culture facilities (Höfer 1903). The parasite spread to other fish culture facilities throughout Germany with the transfer of infected fish. *Myxobolus cerebralis* continued its spread to other European countries following WWII as rainbow trout culture expanded (Hoffman 1970). The parasite has since been confirmed in several countries worldwide, including Russia, New Zealand, and South Africa (Bartholomew and Reno 2002). A recent review by Bartholomew and Reno (2002) chronicles the spread of *M. cerebralis* and presents historical perspectives on the parasite.

The first report of *M. cerebralis* in the United States was in 1956 at a Pennsylvania state fish hatchery (Hoffman 1962). The parasite presumably spread to this continent with infected European imported for human consumption (Hoffman 1962; Bartholomew and Reno 2002). *Myxobolus cerebralis* was confined to the eastern United States until the 1960s when it was detected in hatcheries in Nevada and California (Yasutake and Wolf 1970). Transfer and stocking of fish with undetectable *M. cerebralis* infection furthered the spread of the parasite within the United States (Hoffman 1990). Although most movements and outbreaks of

whirling disease were initially restricted to hatcheries, *M. cerebralis* was implicated in devastating wild rainbow trout losses in Colorado and Montana in the1990s (Nehring and Walker 1996; Vincent 1996; Hedrick et al. 1998). Since the first outbreak of whirling disease in the United States, the parasite has been detected in 23 states (Bartholomew and Reno 2002).

Myxobolus cerebralis was first detected in Oregon in 1986 during an examination of fish from a private trout hatchery located on the Lostine River (Lorz et al. 1989) (Figure 1.1). Since1986, infected fish have been detected in sites located in Catherine Creek, Lostine River, Wallowa River, and Little Sheep Creek (Oregon Department of Fish and Wildlife databases) (Figure 1.1). The parasite was restricted to the Grande Ronde and Imnaha River basins of northeastern Oregon until December 2001 when *M. cerebralis* was detected at a private trout hatchery on Clear Creek, a tributary of the Clackamas River. The parasite was identified in rainbow trout samples collected as part of routine monitoring of private hatcheries by the Oregon Department of Fish and Wildlife (ODFW). Although not confirmed, stray anadromous adults from upriver tributaries are the likely source of *M. cerebralis* dissemination into the Clackamas River basin. Currently, the hatchery is investigating alternative water sources to continue rearing rainbow trout at the facility (Craig Banner, Oregon Department of Fish and Wildlife, personal communication).

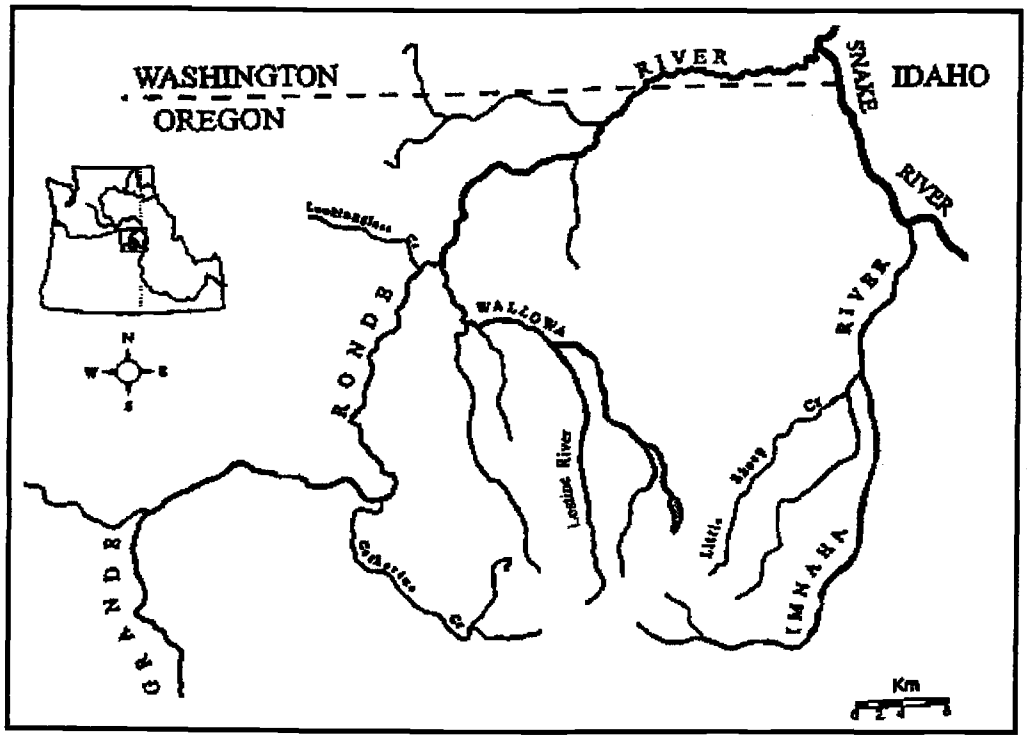

Figure 1.1. Map of Grande Ronde River and Imnaha River basins in northeastern Oregon.

THE LIFE CYCLE OF *Myxobolus cerebralis*

While *M. cerebralis* has been a known pathogen of salmonid fish since the late 1800s, the complete life cycle of the parasite was fully elucidated in 1983 (Markiw and Wolf 1983; Wolf and Markiw 1984). It is now accepted that *M. cerebralis* has a complex life cycle, alternating between an actinosporean and a myxosporean phase (Figure 1.2). It requires two hosts, the oligochaete worm, *Tubifex tubifex*, and one of the various salmonid species. Previously, the actinosporean stage was believed to be a separate organism (*Triactinomyxon spp.*)

and parasite of *T. tubifex* (El-Matbouli and Hoffman 1998). However, molecular comparison of 18s rDNA sequences of the triactinomyxon and myxosporean spore and thorough laboratory transmission studies confirmed that the two pathogens were instead one organism with alternating life forms and hosts (El-Matbouli and Hoffman 1989; Andree et al. 1997).

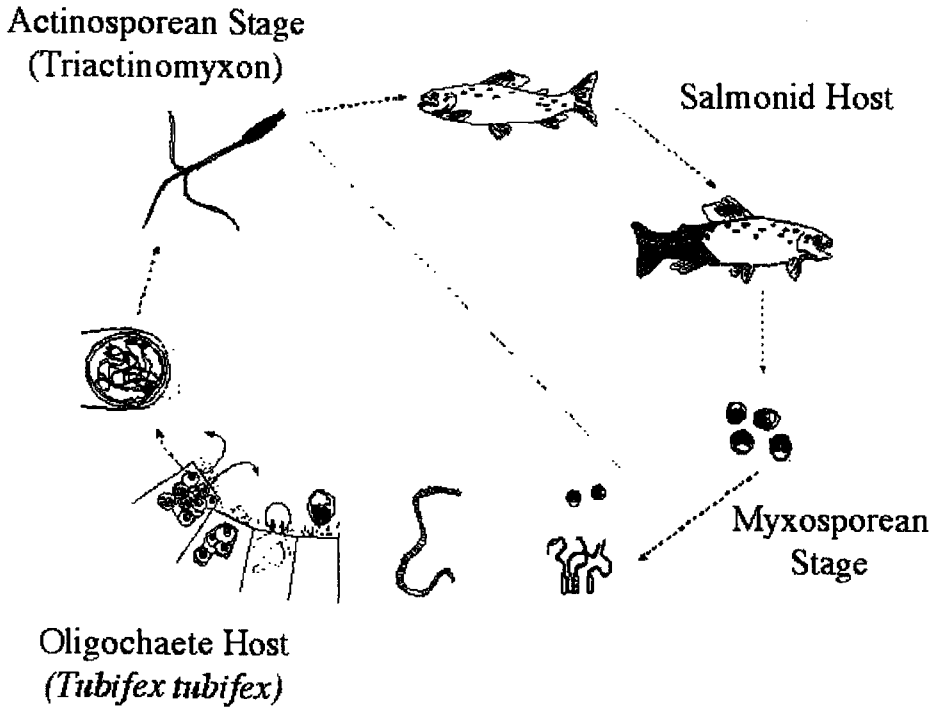

Figure 1.2. The life cycle of *Myxobolus cerebralis*. (Adapted from M. El-Matbouli, T. Fisher-Scherl, and R. W. Hoffman, 1992. Annual Review of Fish Diseases, p. 392).

The life cycle of *M. cerebralis* begins when an infected fish dies and myxospores are released from the cartilage (El-Matbouli et al. 1995). The lenticular spores are 8-10 μm in diameter, contain two polar capsules, and are very resistant

to environmental conditions (Lom and Hoffman 1970; El-Matbouli and Hoffman 1991). Studies have demonstrated that myxospore viability is maintained for at least 5 months at 13°C (El-Matbouli and Hoffman 1991) with some reports of spore survival of up to 30 years (Halliday 1976). Myxospores also remain viable after passage through the alimentary canals of piscivorous birds and fish, as well as surviving for 3 months at temperatures of –20°C (El-Matbouli and Hoffman 1991).

Upon settling to the sediments, myxospores are ingested by *T. tubifex*, the obligate intermediate host (Markiw and Wolf 1983; Wolf and Markiw 1984). In the intestinal lumen of the oligochaete, the spore extrudes its two polar filaments and anchors to the intestinal mucosa (El-Matbouli and Hoffman 1998; Hedrick and El-Matbouli 2002). Upon attachment, the germ cell enters the intercellular space between intestinal epithelial cells and undergoes sporogony (El-Matbouli and Hoffman 1998). Following a developmental period of about 90 d (at 15°C), the mature actinosporean triactinomyxon (named for its three grapple-hook appendages, measuring approximately 180 μm in length) is released from the worm into the water column. At water temperatures of 7-15°C, the triactinomyxon may remain viable for a period of 6-15 days (Markiw 1992b; El-Matbouli et al. 1999b).

A study by El-Matbouli et al. (1995) closely examined the development of the triactinomyxon after infection of the salmonid host at water temperatures of 13-15°C. Upon encounter of a susceptible fish host, the triactinomyxon preferentially attaches to the mucus cells of the fins, gills, and skin. The triactinomyxon sporoplasm penetrates the mucus cell pore and undergoes replication within the

epidermal or gill epithelial cells of the fish. By 4 days, parasitic cells are found within the peripheral nerves. The cells continue replication and migrate along the nerve bundles of the central nervous system. Upon reaching host cartilage, the parasitic cells continue to replicate and digest the cartilage matrix. The shift to sporogenesis occurs, and at 90 days postinfection (at 12-13°C), mature myxospores can be detected within the cartilage of the infected fish.

The developmental progress of both life stages of *M. cerebralis* within the respective host is temperature dependent (Halliday 1973; El-Matbouli et al. 1999b). Within the oligochaete host, lower temperatures (5-10°C) appear to delay the development and maturation of the triactinomyxons, while higher temperatures (15-20°C) seem to accelerate development. Temperatures between 10-15°C appear to provide optimal conditions for normal maturation and release of triactinomyxons (El-Matbouli et al. 1999b). The same pattern of temperature-dependent development is seen within the salmonid host. Maturation of myxospores occurs within 52 d at 16-17°C while a lower temperature (7°C) requires 120 d for formation of spores (Halliday 1973; Hedrick and El-Matbouli 2002). At elevated temperatures, the onset of disease is also earlier and intensity of infection is more severe. This is presumably a result of an increase in parasitic abundance due to an enhanced reproductive environment (Halliday 1973).

TECHNIQUES FOR THE DETECTION OF *Myxobolus cerebralis*

An accurate diagnosis of *M. cerebralis* is necessary to prevent the inadvertent transfer or stocking of infected fish. Currently, the approved method for diagnosis of *M. cerebralis* infection (in the salmonid host) is the detection of myxospores of the correct morphology and size within the target tissue (Thoesen 1994). This is accomplished through microscopic examination of spores that have been extracted and concentrated from skeletal elements. The diagnosis is usually confirmed through histological sectioning and microscopic verification of spore location (Thoesen 1994; Schisler et al. 2001).

Researchers utilize a variety of techniques to detect and quantify parasite abundance, ranging from visual observation of clinical signs to molecular techniques for the detection of an acute infection. This review will consider only those methods used in the studies reported in the following chapters. A current review by Andree et al. (2002) chronicles the development of several important assays and fully details the different techniques used in the detection of *M. cerebralis*.

Myxospore isolation

Mature *M. cerebralis* myxospores are localized in the cartilage and bone of infected fish, and the nature of these tissues requires mechanical and/or enzymatic separation to release the spores. Early techniques involved the maceration and sequential filtration of skeletal elements to release and purify spores (Plehn 1924;

Lucky 1970). The technique was further refined by Markiw and Wolf (1974) with the addition of enzymatic digestion. After the removal of soft tissues with forceps, skeletal elements are immersed in a solution of pepsin (0.5%) and incubated at 37°C with constant agitation. When the skeletal elements are reduced to small fragments, the sample is centrifuged to pellet the undigested material. A trypsin solution (0.5-0.05%) is added and the sample is agitated at room temperature for 30 minutes. Following the neutralization of the trypsin with an inhibitor such as fetal bovine serum, the sample is again centrifuged to pellet the undigested material. The pellet is then resuspended in a buffer and examined microscopically. The pepsin-trypsin digestion (PTD) has become a standard method for the purification and detection of *M. cerebralis* myxospores (Thoesen 1994).

Histological assessment

A histological examination of the cranial cartilage is often required to confirm a *M. cerebralis* infection. This ensures an accurate diagnosis since spores from other *Myxobolus* spp. can be present in cranial tissue and purified during the PTD (Thoesen 1994). To confirm a *M. cerebralis* infection, tissue samples fixed in 10% buffered formalin (or similar fixative) are processed using standard histological methods. Sections are cut and stained with hematoxylin and eosin or May-Grünwald Giemsa (Yasutake and Wales 1983; Thoesen 1994). Upon microscopic evaluation, the presence of spores of the correct size and morphology in the cartilage confirms a *M. cerebralis* infection. The intensity of infection can

also be evaluated based upon severity and number of cartilaginous lesions, myxospore abundance, and host immune response (Hedrick et al. 1999b; MacConnell and Vincent 2002).

Polymerase Chain Reaction

Recent advances in molecular techniques have created valuable tools for the detection of *M. cerebralis* infections. For example, the polymerase chain reaction (PCR) specifically targets and amplifies a portion of *M. cerebralis* genomic DNA, allowing detection of all life stages of the parasite (Schisler et al. 2001; Andree et al. 1998, 2002). In addition, the assay is highly specific, preventing cross-reaction with other related *Myxobolus* spp. (Andree et al. 1998). This technique can be used detect early or light *M. cerebralis* infections in either the salmonid or oligochaete host. The assay has also been used to examine water filtrates for the presence of the parasite (Andree et al. 2002).

The first PCR assay for the detection of *M. cerebralis* involved amplification of ribosomal DNA. Andree et al. (1998) developed a nested PCR assay that utilizes two sets of primers for two separate amplification reactions. In the primary reaction, primers Tr 3-16 and Tr 5-16 amplify a 1300 base pair (bp) segment of the 18s rDNA. In the secondary reaction, primers Tr 3-17 and Tr 5-17 amplify the target 415 bp fragment. Subsequent PCR assays have been developed, most notably a single-round reaction utilizing a modified version of the Tr 5-16 primer (Tr 5-16m) and the Tr 3-17 primer (Epp and Wood 1998; Schisler et al.

2001). Several advantages are inherent in the single-round reaction and include reduced expenses (time, reagents, etc.), and most importantly, a decreased likelihood for sample contamination (Epp and Wood 1998). Additional PCR assays utilizing non-ribosomal DNA targets (e.g. heat-shock protein 70 gene) are also in development (Epp et al. 2002).

Both the nested and single-round PCR assays have been shown to have a 10-fold lower threshold than PTD for the detection of *M. cerebralis* (Andree et al. 2002). However, controversy exists over the ability of the PCR procedure to detect genomic DNA "which may or may not have been derived from a living pathogen" (Schisler et al. 2001). The sensitivity of the PCR assay also makes it susceptible to contamination (Schisler et al. 2001; Andree et al. 2002). Still, the assay is a very valuable tool for *M. cerebralis* research, and it was recently approved as an alternative to histological examination for the confirmation of *M. cerebralis* infection (Standard Procedures for Aquatic Animal Health Inspections, in press).

SUSCEPTIBILITY OF THE SALMONID HOST

While whirling disease research has focused primarily on the rainbow trout host, *M. cerebralis* is infectious to most salmonid species (MacConnell and Vincent 2002). However, the susceptibility to infection and disease appears to differ among species, and is dependent upon fish age, fish size, and parasite exposure dose (Halliday 1976; O'Grodnick 1979; Markiw 1991, 1992a; Hedrick et al. 1999b; MacConnell and Vincent 2002). Environmental factors, including water

temperature and flow, also play an important role in governing infection severity in the salmonid host (Halliday 1973; MacConnell and Vincent 2002). A recent review by MacConnell and Vincent (2002) details the effects of the *M. cerebralis* on the salmonid host and addresses the issues affecting susceptibility.

Species susceptibility

Numerous studies have investigated the variation in species susceptibility to *M. cerebralis* (O' Grodnick 1979; Hedrick et al. 1999a, 1999b, 2001b; Thompson et al. 1999; Sollid et al. 2002). The results of early studies, conducted before the elucidation of the life cycle of *M. cerebralis*, are often inconclusive due to missing information on fish age or exposure dose. However, there is a generally accepted ranking of species susceptibility. Rainbow trout are considered highly susceptible to infection and disease; sockeye salmon *Oncorhynchus nerka*, chinook salmon *Oncorhynchus tshawytscha*, Atlantic salmon *Salmo salar*, cutthroat trout *Oncorhynchus clarki*, and brook trout *Salvelinus fontinalis* are intermediate in their susceptibility; and brown trout *Salmo trutta*, bull trout *Salvelinus confluentus*, and coho salmon *Oncorhynchus kisutch* are less susceptible to the disease. Lake trout *Salvelinus namaycush* are considered refractory to infection (O' Grodnick 1979).

The mechanisms of the host immunity to *M. cerebralis* are not well understood, and variations in species susceptibility may be related to the innate differences in host immune responses. It appears that coho salmon are more effective than rainbow trout at preventing the attachment and penetration of the

parasite at the epithelial surface (Adkison et al. 2001). Brown trout appear to have a more efficient immune response than similarly exposed rainbow trout as suggested by a pronounced difference in spore concentration and lesion severity (Hedrick et al. 1999a). The authors suggest that the abundance of eosinophilic granular leukocytes in exposed brown trout, lacking in rainbow trout, may be key to deciphering the difference in immune mechanisms between these species (Hedrick et al. 1999a). The partial resistance of bull trout to whirling disease may also be modulated by a heightened immune response. Histological examination of bull trout found that early cartilaginous lesions, detected at 35 d postexposure, had subsequently resolved and were minimal at 5 months postexposure (Hedrick et al. 1999b).

Recent studies suggest that susceptibility differences may also exist between strains of the same species. Vincent (2002) investigated the relative susceptibility of several strains of rainbow trout from Montana and New York following laboratory challenges to graded doses of triactinomyxons. Lower infection intensities were observed among the DeSmet rainbow trout strain (Willow Creek, Montana), suggesting a decreased susceptibility to *M. cerebralis* for this strain. Laboratory studies undertaken by El-Matbouli et al. (2002) and Hedrick et al. (2002) have examined the potential resistance of a rainbow trout strain (Höfer strain) from Germany. Results from these studies indicate that the Höfer strain of rainbow trout may have acquired partial resistance to *M. cerebralis*, and further

studies are in progress to examine possible mechanism of resistance (El-Matbouli et al. 2002; Hedrick et al. 2002).

Age at exposure

Myxobolus cerebralis consumes cartilage as it progresses in its development within the fish host. Young fish have abundant amounts of cartilage and are very susceptible to the effects of infection (MacConnell and Vincent 2002). As fish age, ossification of cartilage presumably decreases the susceptibility of fish to disease (Halliday 1976; El-Matbouli et al. 1995). Investigations into age-related susceptibility have been conducted primarily with rainbow trout.

Markiw (1991) investigated the earliest life stage of rainbow trout susceptible to infection by *M. cerebralis*, challenging eyed eggs and 1-day-old fry. At 30 minutes postexposure, initial forms of the parasite were detected within the epithelium of eyed eggs and fry. However, examination at 4 months postexposure found the resulting fingerlings free of *M. cerebralis* spores. Two-day-old rainbow trout fry were the youngest fish that maintained infection by *M. cerebralis* to 4 months postexposure. Presumably, the more advanced physiological development of the fish permitted formation of spores (Markiw 1991).

Ryce et al. (1999) examined the effect of exposure age of rainbow trout fry on the development of whirling disease. Fry were exposed to triactinomyxons beginning at 1 week posthatch and continuing until 17 weeks posthatch. As exposure age increased, the development of clinical signs (including onset,

prevalence, and severity) and mortality rate decreased. The severity of infection (spore abundance and lesion score) was also inversely correlated with the exposure age. Fish exposed after 9 weeks of age had a significantly decreased susceptibility to disease. Data from this study clearly demonstrate that the susceptibility of rainbow trout is dependent upon age at exposure to *M. cerebralis*.

Although younger fish are most susceptible to infection and disease, adult rainbow trout can still become infected with *M. cerebralis*. Markiw (1992a) exposed adult rainbow trout (age: 1 year to 3.5 years) to a continuous dose of triactinomyxons for 3.5 months. While clinical signs of whirling disease did not develop in the adult fish, myxospores were recovered from fish at 7 months postexposure. Adult fish retain some amount of cartilaginous tissue after ossification converts most cartilage to bone, presumably enabling *M. cerebralis* infection to progress within adult fish (Markiw 1992a).

Size at exposure

While the age at exposure governs intensity of infection and susceptibility to disease, the size at exposure likely contributes to the outcome of *M. cerebralis* infection. In a study by Thompson et al. (1999), the smallest sizes of same-age, naturally-exposed rainbow and cutthroat trout suffered the highest mortality. Furthermore, laboratory exposures of age-matched rainbow trout (based on degree days) reared to different lengths demonstrate that size at exposure affects the prevalence of clinical signs (Ryce et al. 2001). However, the onset and severity of

signs was not different among these same fish. It appears that size at exposure plays a role in influencing age-dependent susceptibility to *M. cerebralis.*

Parasite exposure dose

The intensity of *M. cerebralis* exposure (number of triactinomyxons) also governs the degree of infection in susceptible fish. Markiw (1992a) did not recover myxospores from 2-month-old rainbow trout fry exposed to low doses of the parasite (1-10 triactinomyxons/fish). At higher triactinomyxon densities, a linear relationship between exposure dose and recovered myxospores was detected. However, final myxospore concentrations appeared to plateau at exposure doses of greater than 10,000 triactinomyxons/fish in 2-month-old rainbow trout.

In a study of brown trout susceptibility, different responses were observed among brown trout exposed to several parasite levels. At lower exposure doses (10-100 triactinomyxons/fish), brown trout maintained resistance and had fewer myxospores than rainbow trout exposed in parallel (Hedrick et al. 1999a). However, exposure of brown trout fry to high doses (1,000-10,000 triactinomyxons/fish) resulted in serious infection and clinical disease. These results indicate that heavy parasite exposure can overcome species resistance (Hedrick et al. 1999a).

Myxobolus cerebralis AND ANADROMOUS SALMONIDS

Anadromous salmonid species emerge in freshwater, migrate to the ocean to reside for several years, and return to freshwater to spawn (Groot and Margolis 1991). Salmonids with this life history pattern include species that are susceptible to *M. cerebralis* infection (e.g. chinook salmon and steelhead). Understanding the host-parasite interaction is difficult for these species due to their migratory movement. The impact of *M. cerebralis* on resident salmonids is obtained by close examination of year-class numbers, and this means of assessment is not possible with migratory (or endangered) species. Infected anadromous fish may also serve as a source of disseminating *M. cerebralis* if adults spawn and die in non-enzootic waters (Engelking 2002).

A study by Modin (1998) addressed the potential role of anadromous salmonids in disseminating *M. cerebralis*, and concluded that migratory salmonids have had little effect on parasite spread in the state of California. However, this premise was based solely upon the lack of new epizootics during 1965-1997. Because many factors are involved in the establishment of the parasite, it is difficult to discount the role of anadromous fish in *M. cerebralis* dissemination.

Surveys of adults returning to the Deschutes River, Oregon, indicate that 70% of hatchery steelhead originate from other river systems (Engelking 2002). Furthermore, more than 20% of the stray hatchery steelhead entering the river have detectable *M. cerebralis* myxospores. The potential for widespread dissemination of the parasite by migratory species was demonstrated by the recent introduction of

M. cerebralis into a non-enzootic river in Oregon. Stray adult salmonids, infected in upriver Columbia River tributaries, are the hypothesized source for the recent detection of *M. cerebralis* in the Clackamas River (Craig Banner, Oregon Department of Fish and Wildlife, personal communication).

Myxobolus cerebralis exposure may also have a direct effect on juvenile salmonid smolts, interfering with the ability to successfully adapt to saltwater. A recent study by Arkush et al. (2001) found an increased cumulative mortality among chinook salmon infected with *M. cerebralis* upon artificial transfer to seawater. Mortality also occurred among uninfected chinook salmon, but to a lesser degree. It is possible that infection, which causes significant damage to epithelial cells (Hedrick and El-Matbouli 2002), disrupts the ability of smolts to osmoregulate upon transfer to saltwater. Understanding the potential impacts of *M. cerebralis* exposure on saltwater adaptation and survival is important for the effective management of anadromous salmonid populations.

MANAGEMENT STRATEGIES FOR *Myxobolus cerebralis*

Management of *M. cerebralis* is difficult due to the complex life cycle of the parasite. Within the confines of an aquaculture facility, the options for control of *M. cerebralis* are more feasible. The potential for the treatment of water supply and modification of rearing substrate create opportunities to decrease or eliminate the source of parasite infection. However, complete eradication in natural systems is nearly impossible due to the longevity and resistance of the myxospore, the

cosmopolitan nature of *T. tubifex*, and the value of natural populations of fish (El-Matbouli and Hoffman 1991; Brinkhurst 1996). Human activities (agriculture, aquaculture, logging, mining, recreation, etc), which cause sediment deposition and organic input, also create aquatic environments suitable for dense populations of *T. tubifex* to flourish (Wagner 2002).

Aquaculture facilities

Several studies have shown the effectiveness of certain control measures within aquaculture facilities that reduce or eliminate *M. cerebralis* exposure. For example, elimination of earthen ponds and removal of organic waste and sediments in rearing facilities can prevent the establishment of dense *T. tubifex* populations (Wagner 2002). Chemical disinfection and drying of ponds has also been useful in aquaculture facilities in Europe (Hoffman and Hoffman 1972; Hoffman and O'Grodnick 1977).

Treatment of incoming water by chemical or physical means may be effective in reducing exposure of fish to the actinosporean stages (Hoffman 1990; Wagner 2002). Studies have shown that *M. cerebralis* infection is reduced among fish reared in water treated with ozone, chlorine, or ultraviolet irradiation (Hoffman 1974, 1975, 1990; Horsch 1987). Inactivation of the actinosporean stage at extreme pH values, high salt concentrations, and by electrical pulses was documented by Wagner et al. (2002). However, practical application of these latter control strategies has not been demonstrated.

Changes in fish rearing strategies can also reduce infection and disease. In Denmark, rearing fish to 5 cm on pathogen-free water prior to transfer to earthen ponds resulted in decreased incidence of disease (Rasmussen 1965). As discussed above, age-dependent susceptibility studies have reported that rainbow trout show significantly decreased clinical signs, spore loads and mortality when exposure to the parasite is delayed until at least 9 weeks posthatch (Ryce et al. 1999). These rearing strategies can be used to control the level of clinical disease where parasite exposure cannot be eliminated.

Natural ecosystem

Management of *M. cerebralis* within naturally-spawning salmonid populations is a daunting challenge for fisheries managers. As outlined above, control measures that may be effective in an aquaculture setting are inadequate for application to natural ecosystems. At this time, the only tool available to fisheries managers is the utilization of different stocking strategies, including preventing the stocking of fish infected with *M. cerebralis* (Wagner 2002).

The Colorado Division of Wildlife (CDOW) has undertaken intensive studies to examine the feasibility of mitigating the effects of *M. cerebralis* on streams, rivers, ponds, and lakes throughout the state (Nehring et al. 2001). Extensive water filtration studies demonstrated the prevalence of the actinosporean stage in natural settings. Direct comparisons between actinosporean prevalence and stocking events indicate that high parasite numbers in waterways are generally

witnessed one year after the stocking of fish infected with *M. cerebralis* (Nehring et al. 2001). The CDOW recently adopted policy changes that call for a reduction in the number of *M. cerebralis* infected fish stocked in 2002 and elimination of stocking infected trout by 2003 (Nehring et al. 2001).

Similar policies that deal with *M. cerebralis* management exist in other western states. In California, current policy limits the distribution of infected fish, either commercial or state-produced, to known enzootic waters (Modin 1998). Upon adoption of this policy in 1984, fisheries biologists in the state have found little evidence of population declines or clinical disease in enzootic waters. Fish health policy in Oregon prohibits the transfer of *M. cerebralis* infected fish into areas where the parasite is not established (OARs 635-007-0585). Although policies vary from state to state, the importance of preventing introduction is reflected in many state policies. The implementation of these regulatory policies demonstrates that effective management can limit the spread of *M. cerebralis.*

Salmonid species with a decreased susceptibility to *M. cerebralis* or successful life history pattern for avoidance of parasite exposure may serve an important role in management options. In Montana, surveys of rainbow trout populations found those that spawned earlier in the year and in tributaries were less affected by *M. cerebralis* (McMahon et al. 1999). Utilization of fish populations with this type of life history pattern may be useful in mitigating the impacts of *M. cerebralis* on natural populations. Furthermore, stocking species or strains with heightened resistance to *M. cerebralis* may also be a useful tool for fisheries

managers (Wagner 2002). Cutthroat trout are less susceptible to disease and produce fewer myxospores than simultaneously exposed rainbow trout (Walker and Nehring 1995; Hedrick et al. 1999b). Stocking cutthroat trout (as an alternative to rainbow trout) in waters where *M. cerebralis* is endemic may reduce the myxospore load in the system, serving to lessen disease severity in these systems (Wagner 2002).

RESEARCH OBJECTIVES

The impacts of *M. cerebralis* on Oregon salmonid populations have not been as severe as those witnessed in Montana and Colorado (Sandell et al. 2001). Yet, anadromous fish populations in Oregon are below reproductive thresholds and supplementation programs exist to restore threatened and endangered salmonid species. The goals of this research were to assess the susceptibility of several important salmonid species to *M. cerebralis* and examine current management practices that may lead to significant parasite exposure.

Objective 1) Determine susceptibility of resident and anadromous salmonids from the Deschutes River, Oregon to *M. cerebralis* and address population risks should establishment of the parasite occur. *Approach:* Controlled laboratory exposure of selected salmonid species from the Deschutes River to *M. cerebralis.*

Objective 2) Evaluate effects of sustained, low-dose *M. cerebralis* exposure and age-dependent susceptibility of chinook salmon. *Approach:* Controlled laboratory exposures of chinook salmon and rainbow trout fry to continuous, low doses of triactinomyxons for 4 weeks and at several ages (1, 3, 5, 7, and 9 weeks posthatch).

Objective 3) Determine if chinook salmon and steelhead are exposed to *M. cerebralis* during holding at acclimation facilities located in northeastern Oregon. *Approach:* Use sentinel fish to detect *M. cerebralis* exposure at chinook salmon and steelhead acclimation facilities. In addition, examine the prevalence of *M. cerebralis* infection among steelhead juveniles from a facility with known *M. cerebralis* exposure.

Objective 4) Assess effect of *M. cerebralis* exposure on juvenile steelhead mortality rates upon transfer to saltwater. *Approach:* Transfer steelhead juveniles to saltwater following natural and laboratory exposure to *M. cerebralis.*

RELATIVE SUSCEPTIBILITY OF SELECTED DESCHUTES RIVER, OREGON, SALMONID SPECIES TO EXPERIMENTALLY INDUCED INFECTION BY *Myxobolus cerebralis*

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Whirling Disease: Reviews and Current Topics

CHAPTER 2: RELATIVE SUSCEPTIBILITY OF SELECTED DESCHUTES RIVER, OREGON, SALMONID SPECIES TO EXPERIMENTALLY INDUCED INFECTION BY *Myxobolus cerebralis*

ABSTRACT

Laboratory challenges to determine the susceptibility of indigenous Deschutes River, Oregon, salmonids to *Myxobolus cerebralis* were conducted as part of a study to assess the risk of reintroducing anadromous salmon above a migration barrier on that river. Replicate groups of progeny from wild rainbow trout *Oncorhynchus mykiss*, steelhead (anadromous rainbow trout), kokanee *O. nerka*, and chinook salmon *O. tshawytscha* were exposed to doses of 0, 200, or 2,000 triactinomyxons per fish. Fish were evaluated at 5 months postchallenge for spore concentration in the cranial cartilage, severity of microscopic lesions in the cartilage, and clinical signs of disease. The wild rainbow trout (0.7 g at exposure) were most susceptible to infection, with infection prevalence and spore concentrations similar to those of a susceptible (Mt. Lassen) control rainbow trout strain (0.7 g at exposure), although clinical disease signs were less common in the wild strain. Two year classes of steelhead, exposed at different sizes (0.3 g and 1.0 g), both showed fewer clinical disease signs, a lower prevalence of infection, a lower spore concentration, and a decreased mean lesion score, compared with the control rainbow trout (0.6 g and 1.2 g). Kokanee (1.5 g at exposure) became infected but less severely than the control rainbow trout (1.8 g at exposure).

Clinical signs were not evident in the kokanee or the susceptible rainbow trout, possibly because of the large size at exposure. No signs of infection were detected in the chinook salmon (1.0 g at exposure) at either dose despite high infection prevalence in the control rainbow trout (0.6 g at exposure). These results demonstrate that the indigenous salmonids present in the Deschutes River, both above and below the barrier, are susceptible to infection, but the rainbow trout would be most at risk should introduction of the parasite occur in this system.

INTRODUCTION

The myxozoan parasite *Myxobolus cerebralis* is the causative agent of whirling disease, a condition first recognized for its effects in fish culture but more recently known for its impacts on wild trout populations (Höfer 1903; Nehring and Walker 1996; Vincent 1996; Hedrick et al. 1998). Spores of the parasite can develop in many species of salmonids, but the expressed severity of the disease may vary between species (Hoffman and Putz 1969; O'Grodnick 1979; Markiw 1992). Clinical signs of the disease manifest as cranial or spinal deformities, a darkened tail region (black tail), and the erratic swimming behavior that gave the disease its name. After penetration of the epidermis and migration through the nervous system, *M. cerebralis* preferentially attacks the cartilage, digesting it as the parasite progresses in its development (El-Matbouli et al. 1999a). Young fish are more susceptible to infection by *M. cerebralis* and the subsequent effects of the disease as they have proportionally more abundant cartilaginous tissue than adult fish (Halliday 1976; Markiw 1992).

A number of studies have examined the susceptibility of various salmonid species (O' Grodnick 1979; Hedrick et al. 1998, 1999b; Thompson et al. 1999). These reports are conflicting as some conclusions were derived from outcomes of natural epizootics and exposures in live cages rather than under the controlled conditions of laboratory studies. However, a generally accepted ranking of relative susceptibilities places rainbow trout *Oncorhynchus mykiss* as highly susceptible; sockeye salmon *O. nerka*, chinook salmon *O. tshawytscha*, Atlantic salmon *Salmo*

salmon *Salmo salar*, cutthroat trout *O. clarki*, and brook trout *Salvelinus fontinalis* as intermediate in their susceptibility; and brown trout *S. trutta* and coho salmon *O. kisutch* as having low susceptibility to the disease. Most resistant are lake trout *S. namaycush*, which are considered refractory to infection (O' Grodnick 1979). The ability to conduct controlled parasite challenges is beginning to result in a more clear understanding of species susceptibility. However, differences in age and size at challenge, fish strain, challenge dose, and exposure conditions continue to complicate interpretations. Nevertheless, these data are critical to understanding how this parasite might spread within a system and allow for the prediction of potential impacts on resident salmonid populations.

Since the late 1980s, *M. cerebralis* has been established in the upriver tributaries of the Columbia River (Lorz et al. 1989). Anadromous fish from wild or naturally-reproducing parents may become infected in rivers, where they hatch and complete their early rearing, or, as juveniles when they migrate through enzootic waters on their way to the ocean. However, hatchery-reared salmon often first encounter the parasite as juveniles, when released into their native rivers before commencing their seaward migration. Evidence that salmonids can become infected as juveniles is demonstrated by detection of *M. cerebralis* spores in adult hatchery fish returning to these rivers (Engelking 2002). Adult salmon are known to stray during their return migration (Groot and Margolis 1991), and stray adult steelhead (anadromous rainbow trout) and chinook salmon infected with *M. cerebralis* have been documented in the lower Deschutes River, a major tributary in

the mid-Columbia River basin. Some of these fish have differential fin clips and coded wire tags that identify their origin from upper Columbia and Snake River basin hatcheries (H. Mark Engelking, Oregon Department of Fish and Wildlife, personal communication).

On the Deschutes River, Oregon, the Pelton Round Butte (PRB) hydroelectric project serves as a barrier to natural migration, isolating upriver resident fish populations. Sampling of resident fish (H. Mark Engelking, Oregon Department of Fish and Wildlife, personal communication) and examination of *Tubifex tubifex* populations (authors' unpublished data) indicate that *M. cerebralis* is not established above the PRB project. However, transporting returning adult salmonids above the PRB project has been proposed in an effort to reestablish migration runs (Ratliff et al. 1998). This reintroduction brings with it the danger of stray adult salmon inadvertently introducing *M. cerebralis* (and other pathogens) into this isolated portion of the river.

Salmonid species considered for passage above the PRB project are spring-run chinook salmon, summer-run steelhead, sockeye, and bull trout *S. confluentus*. Prior to construction of the project, wild runs of these species spawned in the Metolius, Crooked, and upper Deschutes River tributaries, which are now inaccessible to returning adults. Wild sockeye runs were eliminated early in this century, but naturally-reproducing populations of kokanee *O. nerka* exist in the lakes and reservoirs above the project. Bull trout populations, that were once common throughout the Deschutes River, became extinct above Steelhead Falls by

the 1960s (D. Ratliff, Biologist, Portland General Electric Company, personal communication). However, passage is considered important, as it would provide the opportunity for genetic exchange that will permit the long-term conservation of this species.

As plans for fish passage were developed, critical uncertainties were identified (Ratliff et al. 1998). Along with likelihood for success of the effort, these uncertainties also included the risk of upstream pathogen transfer and the susceptibility to pathogens that are considered at risk for introduction. The information needed to assess these risks include habitat evaluation to determine if conditions exist for establishing the parasite life cycle, monitoring stray anadromous salmon to estimate the frequency and potential degree of introduction, and determining species susceptibility. This study focused on the latter objective and examined the susceptibility of indigenous rainbow trout, steelhead, chinook salmon, and kokanee, by controlled laboratory exposure, to the infectious stage of *M. cerebralis.*

METHODS

Fish Source

Gametes from Metolius River kokanee (DR-K), Crooked River rainbow trout (DR-R), Deschutes River summer steelhead (DR-S) and Deschutes River spring chinook salmon (DR-C) were collected from wild, spawning adults. Rainbow trout (RB-C) obtained as eyed eggs from Mt. Lassen Trout Farm (Red

Bluff, California) served as a susceptible exposure control for each test. Concurrent exposure of age-matched Mt. Lassen rainbow trout allowed for verification of parasite viability and for comparison of susceptibility between the test species. Eggs from each species were incubated and fry reared at the Salmon Disease Laboratory (SDL; Oregon State University, Corvallis, Oregon) in 12.8°C specific pathogen-free (SPF) well water. After absorption of the egg yolk, fry were fed a commercial trout food ad libitum. The source, age, and size at exposure for each species are presented in Table 2.1.

Table 2.1. Origin, age, and weight of species experimentally infected with *Myxobolus cerebralis*.

Species	Age[a] (weight) at exposure	Broodstock Origin
Experimental species		
Rainbow trout	63 d (0.7g)	Crooked River
Kokanee	129 d (1.5g)	Metolius River
Steelhead-1999	60 d (1.0g)	Deschutes River
Steelhead-2000	15 d (0.3g)	
Chinook salmon	104 d (1.0g)	Deschutes River
Control species		
Mt. Lassen rainbow trout	[b]	Mt. Lassen Trout Farm (Red Bluff, California)

[a] Fish age expressed as time based on a constant 12.8°C water temperature
[b] Control fish matched as closely as possible with the test species compared

Parasite Source

Susceptible *Tubifex tubifex* cultures were seeded with *M. cerebralis* spores harvested from the head cartilage of infected fish (Andree et al. 1998). Cultures were maintained in SPF water in a 15°C incubator, fed every 3 days and monitored for triactinomyxon production. At approximately 12 weeks, triactinomyxons were harvested as described by Hedrick et al. (1999b). For fish challenges, triactinomyxons were stained for viability, enumerated, and held briefly at 4°C prior to exposure.

Challenge

For each species, challenges were conducted as described by Hedrick et al. (1999b). Duplicate groups of 25 fish were used for each of three treatments: a high dose of 2000 triactinomyxons/fish, a low dose of 200 triactinomyxons/fish, and no dose (unexposed control group). The weight and age in degree-days of the RB-C were matched as closely as possible to that of each test species (Table 2.1). In 1999, the kokanee (1.5 g), wild rainbow trout (0.7 g) and steelhead (1.0 g) had separate RB-C groups. Because of space limitations in the isolation facility the following year, the steelhead (0.3 g) and chinook salmon (1.0 g) shared a RB-C (0.6 g). Fish were challenged in 5-L tanks and triactinomyxons were introduced with 500 ml of static aerated water. After a 2-h exposure, the fish and parasites were transferred to 25-L tanks and maintained for 5 months in 12.8°C SPF water to

allow for myxospore development. During this period, the fish were fed a semi-moist commercial trout food at a rate of approximately 2% body weight per day.

Evaluation

Fish were observed for clinical signs of the disease (black tail, skeletal deformities) and behavioral changes ("whirling"). Surviving fish were euthanized 5 months post-exposure by an overdose (500 mg/L) of tricaine methanesulfonate (Finquel, Argent Laboratories, Redmond, Washington) and examined for clinical signs. Heads were removed behind the operculum and divided into equal halves along a midsaggital plane. One half of each head was stored at 4°C for determination of spore concentration, and the remaining half was secured in a histological cassette and stored in 10% buffered formalin.

Spore concentration

For exposures conducted in 1999, cranial cartilage was disrupted for 30 seconds in 2 mL of filter-sterilized water using a Stomacher™ tissue homogenizer (VWR Scientific Products, Seattle, Washington). Spores were then enumerated following the homogenization of the sample. This procedure was used to enable subsequent use of the live spores to infect *T. tubifex* populations. Due to the necessity for protocol standardization, samples collected from exposures conducted in 2000 were processed by pepsin-trypsin digestion (Thoesen 1994). Following both extraction processes, samples were examined and duplicate spore counts were

made using a hemacytometer. Calculations were made for each half head as follows: # spores/half head = (# spores counted x dilution factor x mL sample)/# grids counted.

Histological assessment

A random selection of 10 half heads from each exposure dose was processed using standard histological methods. Two sections from each fish were stained with either hematoxylin and eosin or May-Grünwald Giemsa (Yasutake and Wales 1983) and examined microscopically. Cartilaginous lesions were evaluated as described previously by Hedrick et al. (1999b) based on parasite abundance, severity of lesions, and presence of host immune response.

Statistical analysis

The data collected following spore enumeration was $\log_{10}$ transformed in an attempt to bring the distribution closer to normal. Mean spore concentrations for each tank were then calculated using only those fish in which spores were observed (as described by Hedrick et al. 1999a). Using the S-PLUS 2000 statistical software package (Statsci 2000), a two-way analysis of variance (ANOVA) was performed to determine if fish species and/or parasite dose affected mean spore concentrations. Because the digest protocol was modified in the second year of this study, direct comparisons between spore concentrations were not made. Statistical comparison of the presence of clinical signs, prevalence of infection and mean

lesion score between fish species was not performed. For all tests, tanks were used as the experimental unit and significance was defined as $P < 0.05$.

RESULTS

Rainbow Trout

At both the high and low dose, all of the Deschutes River rainbow trout (DR-R) examined at 5 months post-challenge were infected with *M. cerebralis* (Table 2.2). Infection prevalence in the RB-C was similar, with spores detected in 93% of those challenged at the low dose and 100% at the high dose. There were few clinical signs of disease in either replicate group of DR-R exposed to a low dose (0%, 17%), and none of the RB-C showed clinical signs at that dose. In the high dose groups, however, clinical disease signs (whirling and black tail) were more evident in the RB-C strain (88% and 95%) compared with the DR-R strain (21% and 12%). Differences in lesion severity between the strains were evident only at the low dose, where the mean lesion score in the DR-R was 2.0 compared to mean lesion scores of 4.7 and 3.7 in the replicate RB-C groups. At the high dose, the mean lesion severity in the DR-R was not different from the RB-C groups with scores between 4.5 and 5.0. Mean spore concentrations were similar at the low dose for both strains of rainbow trout. However, at the high parasite dose, the RB-C groups had slightly higher mean spore concentrations. None of the unexposed control groups showed infection by *M. cerebralis*.

Table 2.2. Infection intensity as determined by percent of surviving fish with spores and clinical signs, mean lesion score and mean number of spores per half head of infected fish at 5 months post-exposure to 200 or 2000 triactinomyxons (TAMs) per fish. Unexposed fish were not infected with *Myxobolus cerebralis*.

	% with spores (% with signs)		Mean lesion score		Mean Spore Count (Method)	
Species (weight)	200 TAMs/fish	2000 TAMs/fish	200 TAMs/fish	2000 TAMs/fish	200 TAMs/fish	2000 TAMs/fish
Rainbow trout (0.7g)	100 (0) 100 (17)	100 (21) 100 (12)	2.0 2.0	4.5 5.0	3.7×10^5 (H) 1.8×10^5 (H)	8.9×10^5 (H) 8.2×10^5 (H)
Control rainbow trout (0.7g)	100 (0) 93 (0)	100 (88) 100 (95)	4.7 3.7	4.9 5.0	2.5×10^5 (H) 2.7×10^5 (H)	1.2×10^6 (H) 1.6×10^6 (H)
Kokanee (1.5g)	29 (0) 9 (0)	13 (0) 33 (0)	0.8 0.5	0.1 0.8	1.5×10^4 (H) 1.5×10^4 (H)	2.0×10^4 (H) 2.0×10^4 (H)
Control rainbow trout (1.8g)	75 (0) 75 (0)	96 (0) 100 (0)	2.6 2.4	4.2 3.0	3.0×10^4 (H) 3.7×10^4 (H)	1.2×10^5 (H) 5.7×10^4 (H)
Steelhead 1999 (1.0 g)	33 (0) 53 (0)	72 (0) [a]	2.4 0.6	1.5 [a]	4.5×10^4 (H) 5.5×10^4 (H)	4.5×10^4 (H) [a]
Control rainbow trout (1.2 g)	100 (5) 100 (10)	100(44) [a]	4.8 3.9	4.8 [a]	1.4×10^5 (H) 1.1×10^5 (H)	4.7×10^5 (H) [a]
Steelhead 2000 (0.3g)	4 (0) 16 (0)	70(13) 33 (0)	0.9 0.6	2.4 2.3	3.3×10^4 (P) 1.0×10^4 (P)	1.0×10^4 (P) 9.8×10^3 (P)
Control rainbow trout (0.6g)	92 (13) 100 (10)	92 (42) 30 (9)	3.9 4.1	4.2 0.8	1.1×10^5 (P) 1.0×10^5 (P)	3.9×10^5 (P) 1.9×10^4 (P)
Chinook salmon (1.0g)	0 (0) 0 (0)	0 (0) 0 (0)	0.0 0.0	0.0 0.0	ND (P) ND (P)	ND (P) ND (P)
Control rainbow trout (0.6g)	92 (13) 100 (10)	92 (42) 30 (9)	3.9 4.1	4.2 0.8	1.1×10^5 (P) 1.0×10^5 (P)	3.9×10^5 (P) 1.9×10^4 (P)

[a] Only one group of this species was exposed to 2000 TAMs/fish
(H): Processed by homogenization
(P): Processed by pepsin-trypsin digest
ND: None detected

The two-way ANOVA showed that the spore concentrations per half head were not affected by the difference in rainbow strains ($P = 0.2990$). For both strains, there was a significant effect due to differences in parasite dose upon the spore concentration ($P < 0.0010$). The interaction between strain and parasite dose had a nonsignificant effect on spore concentrations ($P = 0.4029$).

Steelhead

Two year-classes of Deschutes River steelhead (DR-S) were challenged in this study. In the 1999 exposure, all RB-C fish at both exposure doses were infected with *M. cerebralis,* as determined by the presence of spores (Table 2.2). The DR-S, which were 1.0g at exposure, had a lower prevalence of infection at the low dose (33%, 53%) than at the high dose (72%). The RB-C developed black tail and/or cranial deformities at both the low dose (5%, 10%) and the high dose (44%). None of the DR-S at either exposure dose displayed clinical disease signs. Mean lesion scores for the DR-S were lower (2.4, 0.6) in comparison to the RB-C (4.8, 3.9) at the low dose exposure. The mean lesion score in high dose exposure groups also showed lower levels of pathology in the cranial cartilage of the DR-S (1.5) than in the RB-C (4.8). The mean spore concentrations in the DR-S groups exposed to a low parasite dose were lower than the RB-C groups at the same exposure dose. At the high dose, the difference in cranial spore concentration between the species was 10-fold higher in the RB-C groups. None of the unexposed control groups showed infection by *M. cerebralis.*

The ANOVA showed a significant effect on the concentration of spores due to the difference in fish species ($P = 0.0240$). The parasite dose ($P = 0.0879$) and interaction between species and dose ($P = 0.0764$) both showed nonsignificant effects on spore concentrations.

In the 2000 exposure, the steelhead trout were smaller (0.3g), yet the data were similar to the steelhead exposures from the previous year. At the low exposure dose, relatively few of DR-S became infected (4%, 16%) compared with the high exposure dose (33%, 70%)(Table 2.2). Infection prevalence in the RB-C was high except for one exposure group at the high dose where only 30% of the fish became infected. This exposure group also had a low mean histology score. Signs of clinical disease were similar to those in the previous exposure, with none of the DR-S showing disease signs at the low dose exposure compared with 10-13% of the exposed RB-C. At the high dose, 42% of the RB-C showed clinical signs. Clinical signs were apparent only in 13% of one group of DR-S exposed at the high dose. Infection severity in the RB-C groups was again higher at the low dose (mean lesion scores of 3.9 and 4.1) compared with the DR-S (mean lesion scores of 0.9 and 0.6). Mean lesion scores among DR-S exposed at the high dose were 2.4 and 2.3, which was lower than the one RB-C group (4.2). Spore concentrations were lower for each exposure level in the DR-S than in the corresponding RB-C groups, even for the high dose group in which the infection prevalence was decreased. None of the unexposed control groups showed infection by *M. cerebralis.*

The two-way ANOVA showed nonsignificant effects on spore concentrations due to the difference in fish species ($P = 0.0928$) and parasite dose ($P = 0.5834$). The interaction between species and dose was also nonsignificant ($P = 0.6366$).

Kokanee

Kokanee (DR-K) from the Metolius River tributary showed a lower prevalence of infection compared to the RB-C at both parasite exposure doses (Table 2.2). At the low dose, infection prevalence in the RB-C was 75%, and in the DR-K was 29% and 9% for the replicate groups. At the high parasite dose, prevalence in the RB-C increased (96%, 100%), but remained low for the DR-K (13%, 33%). Clinical signs were not detected in either species at 5 months post-exposure. Mean lesion scores for the RB-C (2.6, 2.4) were higher than the DR-K (0.8, 0.5) at the low exposure dose. With an increase in exposure dose, the mean lesion scores of the RB-C (4.2, 3.0) were again higher than the mean lesion scores for the DR-K (0.1, 0.8). The mean spore concentrations for the DR-K were lower than those for the RB-C for both exposure doses. For both strains, the mean spore concentration was greater at the higher dose than at the lower dose. The unexposed control groups for this species did not show any evidence of infection by *M. cerebralis*.

For cranial spore concentrations, the ANOVA showed a significant effect due to the difference in fish species ($P = 0.0175$). There were nonsignificant effects

on the concentration of spores due to the parasite dose ($P = 0.0893$) and interaction between fish species and dose ($P = 0.3116$).

Chinook salmon

No signs of infection, as measured by presence of spores, clinical disease signs or histopathology, were detected in Deschutes River chinook salmon (DR-C) at either the high or low dose challenge despite the high infection prevalence and disease severity observed in the simultaneously exposed RB-C (Table 2 2).

DISCUSSION

Our experimental challenges showed a range of susceptibility to *M. cerebralis* among the salmonid species indigenous to the Deschutes River, Oregon. As demonstrated in other studies (O'Grodnick 1979; Hedrick et al. 1999a, b, 2001a, b), rainbow trout were the most susceptible species. The susceptibility of the wild Deschutes River rainbow trout tested in this study was not significantly different from that of the control rainbow trout strain although decreased clinical signs and mean spore count (at the high dose) and lesion severity (at the low dose) suggested a decreased sensitivity.

Results of challenges with steelhead in this study contrasted with other reports where this species was found highly susceptible (Hedrick et al. 2001a, b). Although differences in mean spore concentration between the steelhead and rainbow trout was only supported statistically for the 1999 brood, we believe that

these differences were also present in the 2000 brood but that significance was masked by a tank effect in the rainbow trout. One principle difference between studies that might account for this difference is the size at exposure. In this study the fish were considerably larger (1.0 g in 1999; 0.3 g in 2000) than those used in the studies by Hedrick et al. (2001 a, b), where fish ranged from 0.06 g – 0.14 g. The effects of size on infection severity are well-documented (Hoffman and Byrne 1974; Halliday 1976; Markiw 1992), and it would be expected that the larger steelhead in this study would show fewer signs of clinical disease than smaller steelhead. However, when compared with simultaneously exposed rainbow trout of approximately the same size, the Deschutes River steelhead appear to be more resistant to *M. cerebralis*.

The only published report on susceptibility of sockeye salmon, or its land-locked form the kokanee, is a study by O'Grodnick (1979) comparing the susceptibility of various salmonids to whirling disease. The age and size of fish at exposure was not specified and exposure consisted of a natural challenge to unknown numbers of infectious units. In that exposure, the number of fish infected and the presence of clinical signs was similar between the sockeye salmon and control rainbow trout, however, mean spore concentrations were lower for the sockeye. In this study, the size at exposure for both the rainbow trout control and the kokanee salmon was at least 1.5 g, and this may explain the absence of clinical disease signs. However, for all measures of infection severity (excluding clinical signs), kokanee salmon were more resistant than the rainbow trout control.

Results of exposures of chinook salmon also differ from those of Hedrick et al. (2001b) that demonstrated infection levels comparable to those seen in rainbow trout. Again, the difference between these studies is the fish size at challenge. In the study by Hedrick et al. (2001b), fish were exposed at 0.25 g; fish in the present study were 1.0 g. The challenge dose in that study was also slightly higher (2500 triactinomyxons/fish). In the only other published study of susceptibility of chinook salmon to *M. cerebralis*, O'Grodnick (1979) demonstrated an intermediate resistance, although the size at exposure was not stated.

One explanation for the disparity in the reported susceptibilities of chinook salmon and steelhead is that different species develop resistance at different rates, perhaps depending on cartilage ossification. Another possibility is that resistance developed against the myxozoan *Ceratomyxa shasta* might have resulted in a partial resistance against *M. cerebralis* infection, a hypothesis that was investigated by Hedrick et al. (2001a). *Ceratomyxa shasta* is enzootic throughout the Crooked and Deschutes Rivers and is found in portions of the Metolius River (Ratliff 1981). The parasite acts as a strong selection factor, and most of the salmonids in the Deschutes system have developed a high level of resistance. The rainbow trout tested in the study by Hedrick et al. (2001a) were of a hatchery strain originating from wild fish collected in the lower portions of the Deschutes River. Results in that study were similar to those seen here, with the lower Deschutes River rainbow trout strain showing fewer clinical signs, lower lesion scores and a decreased mean spore concentration compared to the control strain. However, in our study,

differences in susceptibility were less apparent in the native rainbow trout than among the other species. That this was not demonstrated for all species from this watershed makes this *C. shasta* resistance hypothesis unlikely; however, it seems plausible that resistance develops by different mechanisms between species. It also seems unusual that chinook salmon from this river system would have developed so complete a resistance and we feel that this challenge should be repeated before drawing any conclusions.

These data will play a critical role in assessing the potential risks involved in the proposed fish passage plans (Ratliff et al.1998) and contribute to our current understanding of susceptibility to *M. cerebralis* as it relates to fish age and species. Results of these challenges demonstrate that Deschutes River salmonids show variable susceptibility to *M. cerebralis* and suggest that if the parasite became established in this system there would be some risk of infection and disease for these populations. The most susceptible were the native rainbow trout, which are resident both above and below the PRB dam. We have established the presence of two key components necessary for the parasite to establish in this river, the alternate host *T. tubifex* and the susceptible salmonid host. However, other ecological factors are likely to contribute to the ability of the parasite to establish and persist in this system and further investigation of these other components is essential to determine the risk.

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CHAPTER 3: AGE-DEPENDENT SUSCEPTBILITY OF CHINOOK SALMON *Oncorhynchus tshawytscha* TO *Myxobolus cerebralis* AND EFFECTS OF SUSTAINED PARASITE CHALLENGES

INTRODUCTION

Whirling disease, caused by the myxozoan parasite *Myxobolus cerebralis* (Höfer 1903), has been implicated in recent declines of rainbow trout *Oncorhynchus mykiss* populations in Montana and Colorado (Nehring and Walker 1996; Vincent 1996; Hedrick et al. 1998). The parasite has a complex life cycle that requires two hosts, the oligochaete worm *Tubifex tubifex*, and one of the various salmonid species (Wolf and Markiw 1984). Infection of the fish host occurs when the actinosporean stage (triactinomyxon), released into the water column from infected *T. tubifex*, penetrates the epidermis of a susceptible salmonid (El-Matbouli et al. 1995). Heavy infection of rainbow trout can result in disease characterized by blacktail, erratic swimming behavior (whirling), and skeletal deformities (Uspenskaya 1957; Markiw and Wolf 1974; MacConnell and Vincent 2002). While much of what we know about *M. cerebralis* infection and disease is from controlled laboratory studies using the rainbow trout as a model (Markiw 1991, 1992; Ryce et al. 1999), *M. cerebralis* can infect most salmonid species (O' Grodnick 1979; Hedrick et al. 1999a, 1999b, 2001; Thompson et al. 1999; MacConnell and Vincent 2002; Sollid et al. 2002). However, the intensity of disease development varies

among species, and for some species, information on susceptibility is limited and/or contradictory.

The earliest reports of chinook salmon *Oncorhynchus tshawytscha* susceptibility were studies by Hoffman and Putz (1969) and O' Grodnick (1979). The authors concluded that chinook salmon are intermediate in susceptibility to whirling disease when compared to highly susceptible rainbow trout and resistant coho salmon *Oncorhynchus kisutch*. However, results from these studies are inconclusive because fish ages were not reported and challenges occurred under natural conditions in which exposure intensity could not be quantified.

These early susceptibility studies were conducted prior to the elucidation of the life cycle and the identification of *T. tubifex* as the alternate host (Markiw and Wolf 1983; Wolf and Markiw 1984), a discovery that has allowed the triactinomyxon stage to be harvested from laboratory cultures of infected oligochaetes. Controlled challenges with a known parasite dose have eliminated variables that can interfere with interpretation of results, allowing accurate assessment of species susceptibility. By using rainbow trout in a parallel exposure, a susceptibility baseline is generated for comparisons between the experimental and the control species. Indications of unequal susceptibility include differences in clinical signs, infection prevalence, myxospore abundance, and parasite-induced skeletal lesions.

Recent laboratory challenges investigating the susceptibility of chinook salmon to *M. cerebralis* have produced conflicting results. In a study by Hedrick et

al. (2001), chinook salmon fry were highly susceptible to infection and clinical disease. However, in a study on the susceptibility of salmonid species from the Deschutes River, Oregon, myxospores and lesions were not detected in chinook salmon following laboratory exposures (Sollid et al. 2002). In the latter study, age-matched exposed rainbow trout had high infection prevalence and disease severity. Similar indications of decreased chinook salmon susceptibility were observed by Vincent (2002) and Sandell et al. (2001). Cranial lesion severity in chinook salmon was low compared to rainbow trout exposed under similar conditions.

These discrepancies in chinook salmon susceptibility are likely due to variation in parasite dose and exposure age. To investigate the influence of these variables on susceptibility, we assessed infection and disease following challenges 1) at different ages and 2) to different levels and exposure conditions.

METHODS

Fish source

Chinook salmon were obtained as eyed eggs, fry, and juveniles from state hatcheries in Oregon (Table 3.1). Rainbow trout, used as either an exposure or susceptible species control, were obtained as eggs from Mt. Lassen Trout Farm (Red Bluff, California) or from Troutlodge, Inc. (Sumner, Washington). All fish were subsequently reared on specific pathogen free (SPF) water at 12°C at the Salmon Disease Lab (SDL, Oregon State University, Corvallis, Oregon). For challenges utilizing a susceptible species control, the age (in temperature units) of

the rainbow trout was matched as closely as possible with the age of the chinook salmon. The fish source and age at exposure for challenges are presented in Table 3.1.

Table 3.1. Source and age of chinook salmon *Oncorhynchus tshawytscha* and rainbow trout *Oncorhynchus mykiss* experimentally infected with *Myxobolus cerebralis*. TU=temperature unit(s)

Experimental Design	Species	Age at exposure (TU) [a]	Stock (Source)
Age-dependent Challenge	Chinook salmon		Upriver Bright (Bonneville Hatchery) [b]
1 wk		132	
3 wk		360	
5 wk		480	
7 wk		636	
9 wk		804	
	Rainbow trout		Troutlodge
1 wk		96	
3 wk		324	
5 wk		444	
7 wk		600	
9 wk		768	
Sustained Challenge	Chinook salmon	564	Marion Forks (Marion Forks Hatchery) [b]
	Rainbow trout	564	Mt. Lassen
Juvenile Challenge	Chinook salmon	~ 2520	Catherine Creek (Lookingglass Hatchery) [b]
	Rainbow trout	708 [c]	Troutlodge

[a] Fish age expressed in cumulative temperature units (°C).
[b] All hatcheries are located in Oregon
[c] Rainbow trout used only as a control for viability of triactinomyxons

Triactinomyxon source

Triactinomyxon stages of *M. cerebralis* were obtained from laboratory cultures of *T. tubifex* (origin, Mt. Whitney hatchery) infected by seeding with myxospores harvested from the cartilage of infected fish (Andree et al. 1998). For the collection of triactinomyxons, water from the cultures was filtered through 20-μm-mesh screen, and the concentrate was resuspended in a known volume of water. A sub-sample was stained with methylene blue to assess triactinomyxon viability and facilitate enumeration. The triactinomyxon suspension was held at 4°C until exposure of fish.

Challenge conditions

For challenges, each exposure group was placed in a 25-L tank. Fish were exposed for 2 hours to the appropriate number of triactinomyxons in 500 mL of static aerated water; 1000mL of static aerated water was used for challenge of juvenile fish. After exposure, the water flow was reestablished. Fish were maintained in SPF water at 12°C on a commercial trout diet.

Experimental Design

Age-dependent challenge

Chinook salmon and rainbow trout fry were challenged at five different ages (approximately 1, 3, 5, 7, and 9 weeks posthatch; Table 3.1). For each age point, triplicate groups of 20 chinook salmon fry and 20 rainbow trout fry were

exposed to a dose of 1,000 triactinomyxons/fish. Three duplicate groups of each species were maintained as unexposed controls. Following challenges, fish were monitored for onset of clinical signs and held for 5 months postchallenge to allow for myxospore development.

Sustained challenge

Replicate groups of 23 chinook salmon fry and 25 rainbow trout fry (Table 3.1) were used for each of three treatments: a high dose of 200 triactinomyxons/fish/day, a moderate dose of 50 triactinomyxons/fish/day, and a low dose of 5 triactinomyxons/fish/day. Fish were exposed for 2 hours, and challenges were repeated daily for 4 weeks (5 days a week). Replicate groups of each species were also maintained as unexposed controls. Fish were monitored for development of clinical signs. A sample (n=5) was taken from each tank after 5 months and the experiment was terminated at 11 months postchallenge.

Juvenile challenge

Triplicate groups of 10 chinook salmon juveniles (Table 3.1) were exposed to a dose of 10,000 triactinomyxons/fish. Triplicate groups were also maintained as unexposed controls. Rainbow trout fry were exposed simultaneously only to ensure the viability of triactinomyxons and not as a susceptibility control. After challenge, fish were monitored for development of clinical signs and held for 5 months to allow for myxospore development.

Sampling

At sample and termination points, fish were euthanized with an overdose (500 mg/L) of tricaine methanesulfonate (MS-222; Argent Laboratories, Redmond, Washington), and clinical signs (ie. caudal melanosis, skeletal deformities) were recorded. Heads were severed from the body using disposable razor blades (one blade per fish) to prevent cross-contamination. One half of each head was fixed in 10% neutral buffered formalin for histological examination, and the remaining half head placed into a 50-mL conical centrifuge tube. To determine if myxospores localized differently between the species in the age-dependent challenge, vertebrae were collected posterior to the dorsal fin and placed into separate 50-mL conical centrifuge tubes.

Evaluation

Myxospore extraction and enumeration

Half heads and caudal vertebrae were immersed in tap water and placed in a heated shaker bath at 45°C. Frequent agitation was applied during the heat-soaking process until soft tissues were separated from the skeletal elements (Thoesen 1994). Samples were decanted through a gauze filter and rinsed with tap water. Bony fragments were recovered and returned to the 50-mL tube; the rinse water was also added to the sample tube. Pepsin-trypsin digestion was performed on each sample (Markiw and Wolf 1974) with the following modifications. Following trypsin digestion, samples were passed through a gauze filter to remove undigested

fragments. The filtrate was centrifuged at 1200 x g for 10 min, the supernatant decanted, and the pellet resuspended in phosphate buffered saline (PBS). Samples were preserved in 10% buffered formalin upon completion of digestion.

Individual digest samples were diluted with water (1:2-1:5), and aliquots were placed on both sides of a hemocytometer. Myxospores of the correct morphology and size were enumerated under 400x magnification. Total myxospores were calculated for each half head or vertebral sample as (myxospores counted x dilution factor x mL sample x 10^4) / grids counted.

Histological examination

Preserved half heads were processed using standard histological methods. For each fish, two 5-μm sections were stained with hematoxylin and eosin, and two 5-μm sections were stained with May-Grünwald Giemsa (Yasutake and Wales 1983). Lesion severity, myxospore abundance, and host immune response were evaluated, and scores are reported using a scale from 0 (none) to 5 (severe) as described by Hedrick et al. (1999b).

Five fish from each triplicate tank of both species were evaluated for each exposure in the age-dependent challenge. For the sustained challenge, only fish exposed to 200 triactinomyxons/fish/day were evaluated. For the juvenile exposure, only fish in which myxospores were detected were evaluated.

Statistical analysis

Prior to statistical analyses, myxospore counts were $\log_{10}$ transformed in an attempt to bring the distribution closer to normal. For each tank, the mean myxospore concentration was calculated using only those fish in which myxospores were observed (as described by Hedrick et al. 1999a). Using the S-PLUS 2000 statistical software package (Statsci 2000), a two-way analysis of variance (ANOVA) was performed to determine if fish species and/or fish age affected mean myxospore concentrations. For all tests, tanks were the experimental unit and significance was defined as $P < 0.05$. Statistical analysis of prevalence of clinical signs and lesion severity was not performed.

RESULTS

Age-dependent challenge

Clinical signs

Appearance of clinical signs was dependent upon fish species and exposure age. Among chinook salmon exposed at 1 week posthatch, whirling behavior was first observed at 14 weeks postexposure; blacktail developed earlier at 10 weeks postexposure, and at 5 months, 89% of the fish displayed clinical signs of disease (Figure 3.1). Blacktail and whirling were detected at 6 weeks postexposure among rainbow trout exposed at 1 week posthatch, and at 5 months, 100% of the fish showed clinical signs of disease (Figure 3.1). Of the chinook salmon exposed at 3 weeks posthatch, whirling behavior was not observed and 8% had blacktail at

termination. Among rainbow trout exposed at 3 weeks posthatch, whirling behavior was observed at 8 weeks postexposure and 100% developed blacktail. Chinook salmon exposed at 5, 7, and 9 weeks posthatch did not develop any clinical disease signs. Rainbow trout exposed at 5 and 7 weeks posthatch displayed whirling behavior at 11 weeks postchallenge and blacktail was observed at termination (27-31%). Clinical signs were not apparent in rainbow trout exposed at 9 weeks posthatch.

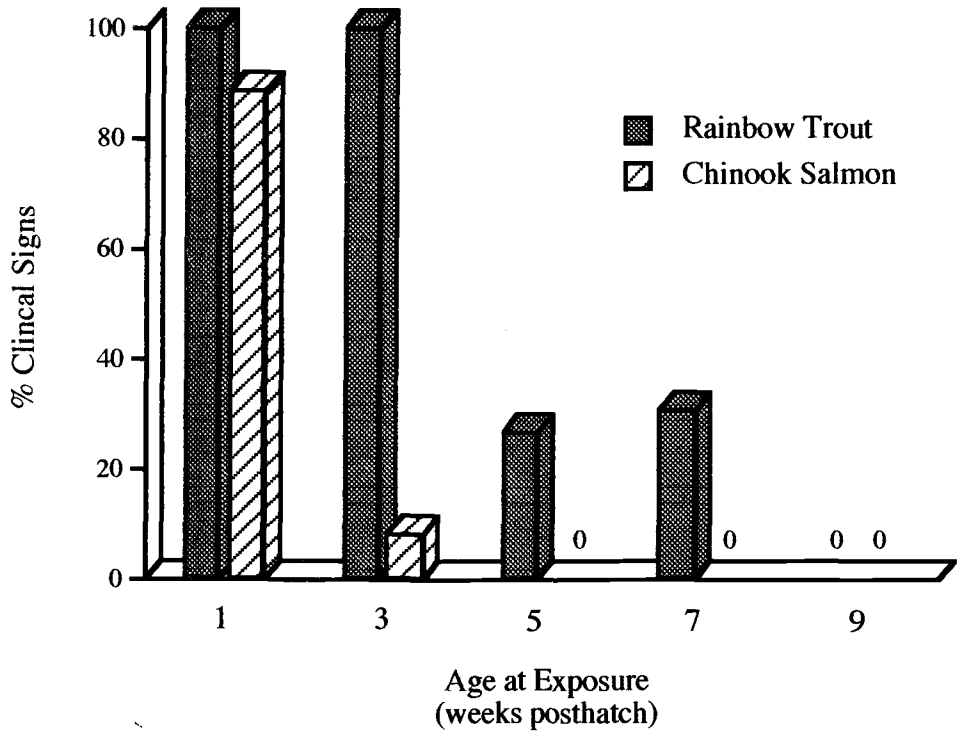
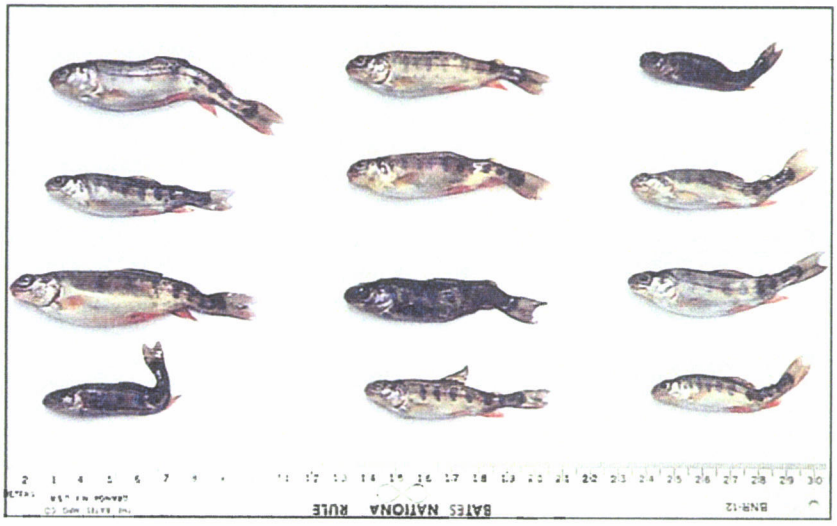


Figure 3.1. Prevalence of clinical signs at 5 months postexposure of chinook salmon *Oncorhynchus tshawytscha* and rainbow trout *Oncorhynchus mykiss* exposed to 1,000 *Myxobolus cerebralis* triactinomyxons per fish at 1, 3, 5, 7, and 9 weeks posthatch.

The severity of clinical signs was different between rainbow trout and chinook salmon following exposure at 1 week posthatch. At termination, rainbow trout had severe spinal deformities and blacktails (Figure 3.2a); spinal deformities were less severe and blacktail was less apparent in chinook salmon (Figure 3.2b).

(a)



(b)

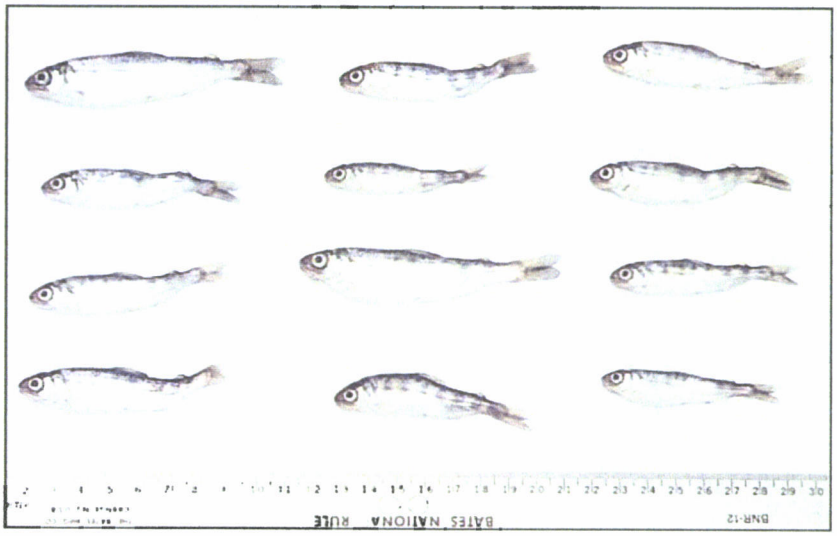


Figure 3.2. Clinical signs at 5 months postexposure for (a) rainbow trout *Oncorhynchus mykiss* and (b) chinook salmon *Oncorhynchus tshawytscha* exposed to 1,000 *Myxobolus cerebralis* triactinomyxons per fish at 1 week posthatch.

Infection Prevalence

At 5 months postexposure, 100% of chinook salmon and rainbow trout exposed at 1 week posthatch were infected (as determined by presence of myxospores) (Figure 3.3). The prevalence of *M. cerebralis* infection among chinook salmon decreased during subsequent challenges from 90% (3 weeks) to 1.7% (9 weeks). The prevalence of infection among rainbow trout was 100% for fish exposed between 1-7 weeks of age and 96% for fish exposed at 9 weeks.

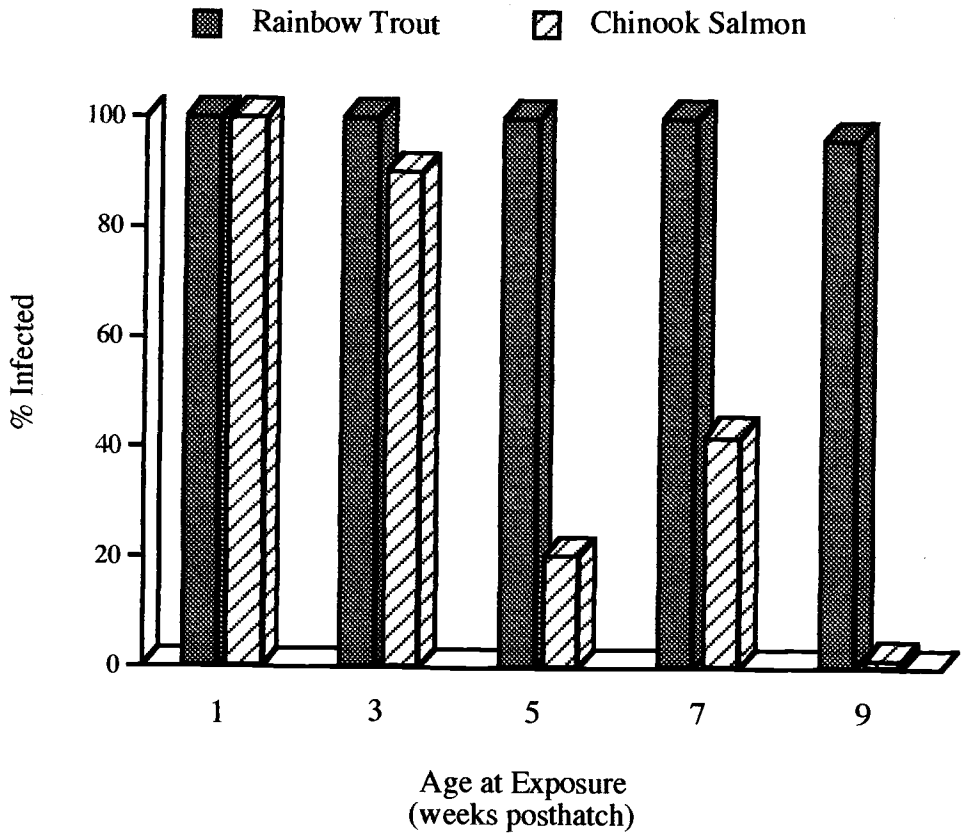


Figure 3.3. Prevalence of infection at 5 months postexposure of chinook salmon *Oncorhynchus tshawytscha* and rainbow trout *Oncorhynchus mykiss* exposed to 1,000 *Myxobolus cerebralis* triactinomyxons per fish at 1, 3, 5, 7, and 9 weeks posthatch.

Myxospore abundance

Cranial samples

At each exposure age, the mean cranial myxospore burden was higher among rainbow trout than among chinook salmon. The greatest mean myxospore abundance (7.09×10^5 myxospores/half head) was observed in rainbow trout exposed to *M. cerebralis* at 3 weeks posthatch (Figure 3.4a). The greatest mean myxospore burden among chinook salmon was 6.35×10^4 myxospores per half head for fish exposed at 1 week posthatch. With the exception of a small increase among fish exposed at 7 weeks, mean myxospore burden among chinook salmon decreased as exposure age increased.

Statistical analysis (two-way ANOVA) demonstrated that the difference in species had a significant effect upon the mean myxospore concentrations per half head ($P < 0.00001$). For both species, there was also a significant effect due to differences in exposure age upon myxospore concentration ($P < 0.00001$). The interaction between species and exposure age also had a significant effect on myxospore abundance ($P = 0.007$).

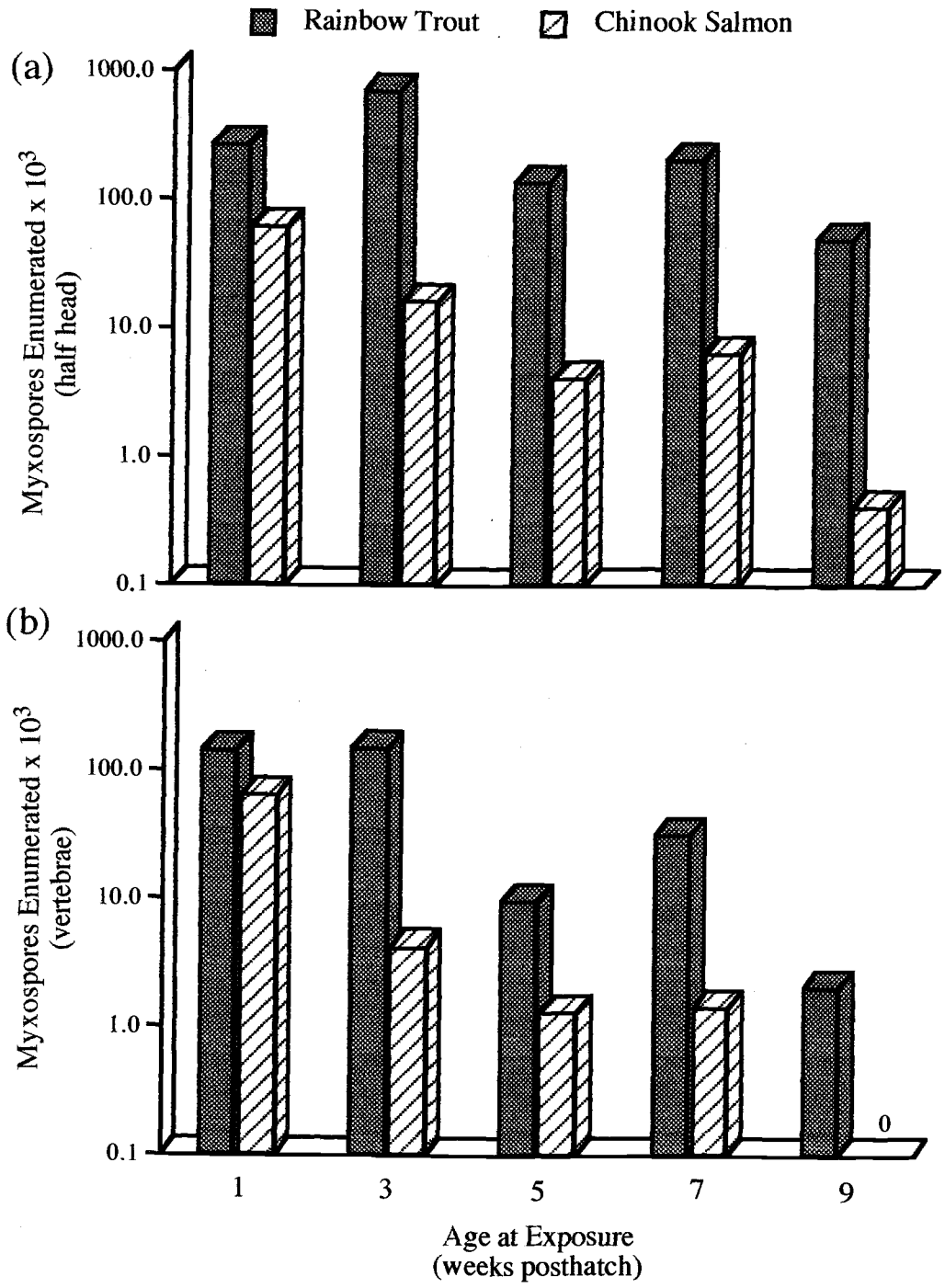


Figure 3.4. Mean myxospore abundance in (a) half head and (b) vertebral samples at 5 months postexposure of chinook salmon *Oncorhynchus tshawytscha* and rainbow trout *Oncorhynchus mykiss* exposed to 1,000 *Myxobolus cerebralis* triactinomyxons per fish at 1, 3, 5, 7, and 9 weeks posthatch.

Vertebral samples

The mean myxospore burden in the caudal vertebrae was higher among rainbow trout than among chinook salmon for all exposure ages. The greatest mean myxospore abundance (1.50×10^5 myxospores/vertebrae) was observed in rainbow trout exposed to *M. cerebralis* at approximately 3 weeks posthatch (Figure 3.4b). The greatest mean myxospore burden among chinook salmon was 6.58×10^4 myxospores for fish exposed at 1 week posthatch. As exposure age increased, mean myxospore abundance decreased for chinook salmon. Myxospores were not observed in the vertebrae of chinook salmon exposed at 9 weeks posthatch.

Statistical analysis (two-way ANOVA) demonstrated that the difference in species had a significant effect upon the mean myxospore concentrations in the vertebrae ($P < 0.00001$). For both species, there was also a significant effect due to differences in exposure age upon myxospore concentration ($P < 0.00001$). The interaction between species and exposure age had a nonsignificant effect on myxospore abundance ($P = 0.111$).

Microscopic pathology

Lesions associated with *M. cerebralis* were more severe among rainbow trout than among chinook salmon for all exposure ages. Mean lesion severity among rainbow trout was high (>4.0) for three exposure ages (1, 3, 7 weeks posthatch) (Figure 3.5). For chinook salmon, the mean lesion score was 2.6 (range 0-5) among fish exposed at 1 week posthatch and 1.3 (range 0-4) among fish

exposed at 3 weeks posthatch. Lesion severity was low (<1.0) for chinook salmon exposed after 3 weeks of age.

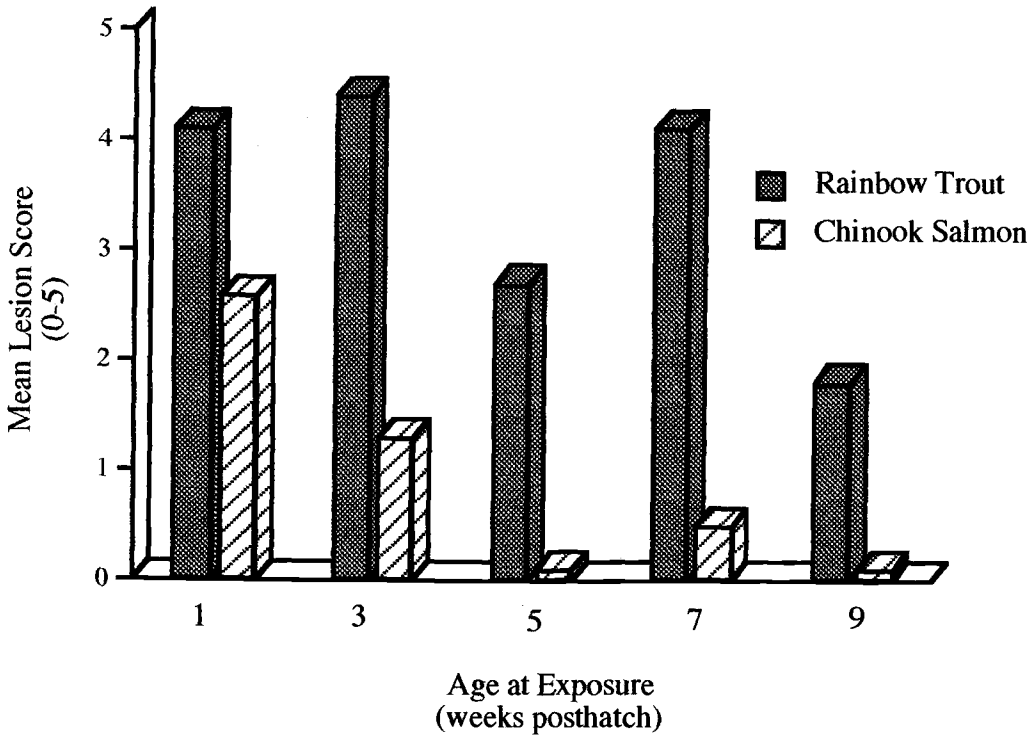


Figure 3.5. Severity of microscopic lesions at 5 months postexposure of chinook salmon *Oncorhynchus tshawytscha* and rainbow trout *Oncorhynchus mykiss* exposed to 1,000 *Myxobolus cerebralis* triactinomyxons per fish at 1, 3, 5, 7, and 9 weeks posthatch.

Parasite-induced lesions were concentrated in the cranial cartilage of rainbow trout and chinook salmon at most exposure doses. However, among chinook salmon exposed at 1 week posthatch, lesions were also observed in the cartilage of gill arches (Figure 3.6).

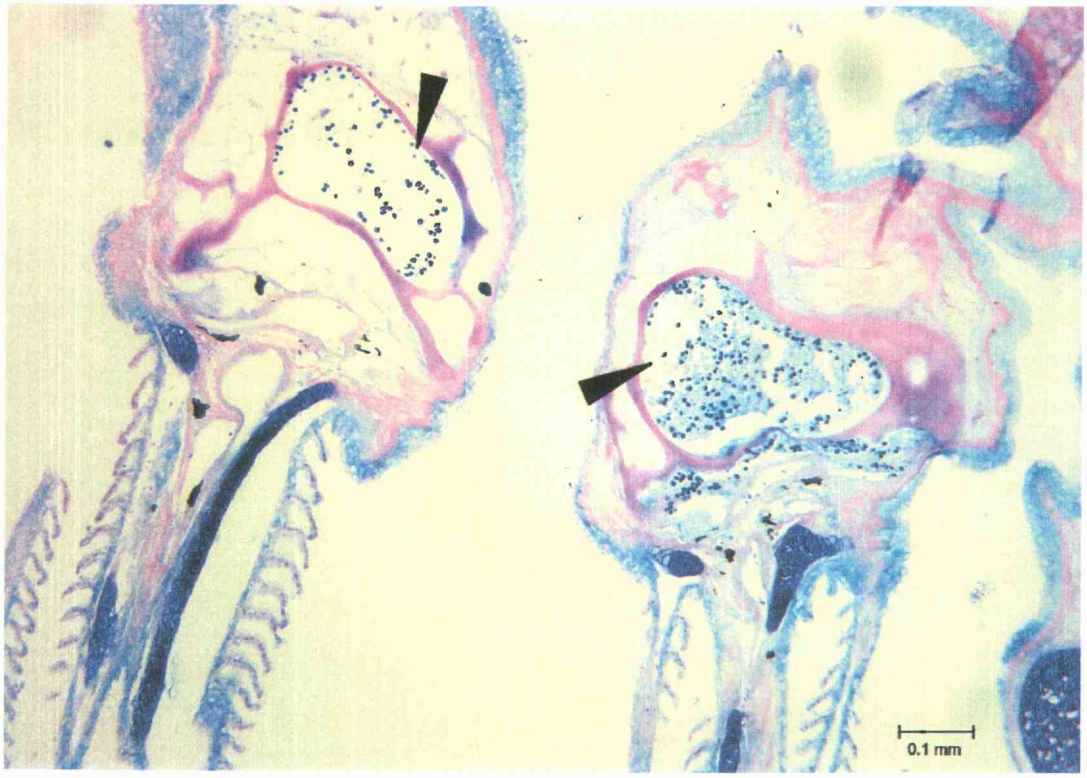


Figure 3.6. *Myxobolus cerebralis* (arrowheads) in gill arches of chinook salmon *Oncorhynchus tshawytscha* exposed at 1 week posthatch to 1,000 *M. cerebralis* triactinomyxons per fish.

Sustained Challenge

At 5 months postchallenge, infection (defined as the presence of myxospores) and parasite-induced lesions were not detected in chinook salmon (7 weeks posthatch; 564 TU) at any parasite dose following exposure to sustained, graded doses of *M. cerebralis*. The prevalence of infection for age-matched rainbow trout exposed to the low dose (5 triactinomyxons/fish/day) was 10%, and 100% of the fish were infected following exposure to moderate (50

triactinomyxons/fish/day) and high (200 triactinomyxons/fish/day) parasite doses. Mean myxospore abundance in rainbow trout ranged from 4.00 x 10^4 (low dose) to 3.96 x 10^5 (high dose). Mean lesion scores were 0.2, 2.0, and 3.0 for rainbow trout exposed to low, moderate, and high parasite doses, respectively. Clinical signs (cranial deformities) were detected only among rainbow trout exposed to a moderate parasite dose. Infection was not detected in the unexposed control fish of either species.

Results at 11 months postchallenge were similar to those obtained at 5 months postchallenge. However, a single chinook salmon exposed to a sustained high dose (200 triactinomyxons/fish/day) was infected with 1.20 x 10^4 myxospores per half head. Microscopic lesions were not observed in this fish. Neither myxospores nor lesions were detected in any of the remaining chinook salmon. At 11 months postchallenge, the prevalence of infection for the rainbow trout exposed to sustained doses (low, moderate, high) was 37%, 90%, and 100%, respectively. Mean myxospore abundance among rainbow trout ranged from 2.31 x 10^4 (low dose) to 1.63 x 10^5 (high dose). Mean lesion severity was 0.6, 1.6, and 2.2 for rainbow trout exposed to low, moderate, and high parasite doses, respectively. Clinical signs (cranial deformities) were only observed in rainbow trout exposed to moderate and high parasite doses. Infection was not detected in the unexposed control fish of either species.

Juvenile challenge

Myxospores were detected 5 months postchallenge in chinook salmon juveniles exposed to a high dose of 10,000 triactinomyxons/fish. The prevalence of infection was 10% and the mean myxospore burden among infected fish was 3.3 x 10^3 myxospores per half head. Cranial lesions were not detected in infected juveniles. Clinical signs were not observed in the juvenile chinook salmon throughout the experiment. Myxospores and cranial lesions were also detected in rainbow trout fry used as a triactinomyxon viability control. Infection was not detected in the unexposed groups of either species.

DISCUSSION

Laboratory challenges at different ages, parasite levels, and exposure durations demonstrate chinook salmon are less susceptible to *M. cerebralis* than control strains of rainbow trout (Mt. Lassen and Troutlodge). As indicated by lack of clinical signs, resistance to disease developed earlier in chinook salmon (after 3 weeks posthatch) than in rainbow trout (after 7 weeks posthatch). For fish exposed at 1 week posthatch, the prevalence of infection was 100% for both species. However, all measures of infection severity, including myxospore burden and lesion score, were lower in chinook salmon. This increased resistance was also reflected in the high dose challenge of juveniles and in the sustained exposures, indicating an overall decreased susceptibility to *M. cerebralis* for this species.

While the report of high susceptibility by Hedrick et al. (2001) seems to conflict with the outcome of the current study, both studies confirm that very young chinook salmon fry (<14 d posthatch) are susceptible to whirling disease. The exposure dose in the previous study (2,500 triactinomyxons/fish) was higher than the exposure dose utilized in the present study (1,000 triactinomyxons/fish), and this likely contributed to the high intensity of infection and disease. The lack of infection in chinook salmon exposed at 15 weeks posthatch observed in a previous study by our laboratory (Sollid et al. 2002) may also be explained by the rate at which resistance to infection developed. In the current study, the prevalence of infection among chinook salmon exposed at 9 weeks posthatch was only 1.6%. It is likely that resistance to *M. cerebralis* continues to increase as chinook salmon exposure age increases.

A number of studies have investigated the influence of exposure age on *M. cerebralis* infection and disease in rainbow trout, providing a basis for comparison with the current study (Markiw 1991; 1992; Ryce et al. 1999). Markiw (1991) reported that the earliest susceptible age for rainbow trout was 2 d posthatch. An intensive study of age-dependent susceptibility (Ryce et al. 1999) found that the development of clinical disease in rainbow trout was dependent upon exposure age. When parasite exposure was delayed until 9 weeks posthatch, the severity of disease was significantly reduced. The present study corroborates this information for rainbow trout and defines the range of age-dependent susceptibility for chinook salmon.

In addition to the reduced severity of *M. cerebralis*-associated lesions in chinook salmon, histological examination also revealed differences in lesion location. Whereas cranial cartilage was the predominant localization site for lesions in rainbow trout, lesions were also abundant in the cartilage of gill arches in chinook salmon exposed at 1 week posthatch. Previous studies have reported localization of lesions in the gill arches of brown trout, indicating that triactinomyxons preferentially attach to gill epithelium in these species (Baldwin et al. 1998; Hedrick et al. 1999a; Baldwin et al. 2000). Baldwin et al. (2000) suggested that the localization of *M. cerebralis* in the gill arches may inhibit the onset and severity of clinical disease (Baldwin et al. 2000). However, blacktail and whirling were observed in chinook salmon exposed at 1 week posthatch, demonstrating that there were sufficient lesions in other areas to cause clinical disease.

The challenge strategies in this study were designed to provide information necessary to assess the effects of *M. cerebralis* on chinook salmon populations. The age-dependent challenges were undertaken to determine the age at which chinook salmon most susceptible and the rate at which resistance develops. However, the high, single-dose exposure used to determine relative susceptibility in laboratory challenges does not reflect conditions that fish would encounter in a natural exposure. To simulate a more-realistic *M. cerebralis* exposure, a laboratory model with daily challenge to low triactinomyxon densities was developed. The apparent

resistance of chinook salmon was not overcome by sustained exposure, suggesting that heavy infection may not result under natural exposure conditions.

In a study by Sandell et al. (2002), chinook salmon (age: 6 weeks) were exposed under natural conditions in the Lostine River, Oregon for 14 d. While the prevalence of infection was 37.5% (as determined by presence of *M. cerebralis* DNA), clinical signs of disease, cranial myxospores and lesions were not detected in the chinook salmon at 5 months postexposure. Further, chinook salmon in the Lostine River emerge during a time of decreased exposure incidence (Sandell et al. 2002). However, juveniles of the species can reside for up to 1 year in the river and would repeatedly encounter the parasite during this time. The current study demonstrates that chinook salmon can withstand a prolonged exposure to *M. cerebralis* with only evidence of low infection levels.

In some regions, chinook salmon do not encounter *M. cerebralis* until they are yearlings migrating through enzootic areas or until released from rearing facilities into streams and rivers. High-level exposure of juveniles did not result in clinical disease or a high prevalence of infection, indicating that *M. cerebralis* infection may not cause direct effects on juveniles. However, the impact of exposure and infection on smoltification and saltwater residence is unknown. Schisler et al. (2000) demonstrated that *M. cerebralis* infection increases mortality upon exposure to multiple stressors. Since smoltification is a stressful physiologic change (Maule et al. 1987), infection may interfere with the successful adaptation of chinook salmon to saltwater. Furthermore, detection of myxospores in juvenile

chinook salmon raises concerns about dissemination of the parasite. If infected adults migrate into non-enzootic waters to spawn, introduction of *M. cerebralis* could place highly susceptible rainbow trout populations at risk.

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CHAPTER 4: RISK OF *Myxobolus cerebralis* INFECTION FOR SALMONID JUVENILES ACCLIMATED IN NORTHEASTERN OREGON

INTRODUCTION

Whirling disease was historically considered a concern only for fish in culture facilities; yet, the disease was implicated in devastating declines in rainbow trout *Oncorhynchus mykiss* populations in Montana and Colorado rivers during the 1990s (Nehring and Walker 1996; Vincent 1996; Hedrick et al. 1998). *Myxobolus cerebralis* (Höfer 1903), the etiologic agent of whirling disease, has a complex, two-host life cycle (Wolf and Markiw 1984). Following replication and maturation within its oligochaete host, *Tubifex tubifex*, the triactinomyxon stage of *M. cerebralis* is released into the water column (Wolf and Markiw 1984). Upon encountering a susceptible salmonid, the triactinomyxon penetrates the epidermis of the fish to initiate a second developmental cycle that culminates in the myxospore stage of the parasite. The myxospore is ingested by the oligochaete host, completing the life cycle of the parasite (El-Matbouli et al. 1995). Heavy infection of rainbow trout can result in blacktail, erratic swimming (whirling), and skeletal deformities (Uspenskaya 1957; Markiw and Wolf 1974; MacConnell and Vincent 2002).

Myxobolus cerebralis was first detected in the Pacific Northwest in 1986 during an inspection of a private trout hatchery located in northeastern Oregon (Lorz et al. 1989). The Oregon Department of Fish and Wildlife (ODFW)

subsequently sampled wild and feral fish from rivers and ponds located in the Grande Ronde and Imnaha River basins. Since the initial discovery in 1986, *M. cerebralis* has been detected in fish collected from several tributaries of the Grande Ronde River (Catherine Creek, Lostine River, and Wallowa River) and in one tributary of the Imnaha River (Little Sheep Creek)(ODFW, unpublished data). *Myxobolus cerebralis* is presently considered widespread throughout both river basins in this region (Sandell et al. 2001); however, because fish may migrate from the site of infection, the extent of parasite establishment in the region is not known.

Management policies for chinook salmon *Oncorhynchus tshawytscha* and steelhead *Oncorhynchus mykiss* (anadromous rainbow trout) in northeastern Oregon have been altered to limit the effects of whirling disease on these species. Susceptibility to *M. cerebralis* is influenced by fish age (Markiw 1991; MacConnell and Vincent 2002, and delaying exposure until 9 weeks posthatch decreases the intensity of infection and disease (Ryce et al. 1999). To prevent exposure of young fish, gametes collected from captive broodstock and hatchery populations are fertilized and reared on well water at hatcheries located outside of the enzootic region. At approximately 3 months posthatch, chinook salmon fry are returned to the enzootic region and reared at a hatchery supplied with spring water (William Noll, Captive Broodstock Assistant Project Leader, Oregon Department of Fish and Wildlife, personal communication); steelhead fry are reared outside of the region on well water. As yearlings, chinook salmon and steelhead are transferred from these hatcheries to acclimation facilities located on their natal

streams. The goal of the acclimation program is to increase survival after transport and to improve homing accuracy of returning adults by providing an extended period for imprinting (Schroeder et al. 2001). After holding for 4-6 weeks, the juveniles are released from the acclimation facilities to begin their seaward migration.

This study was undertaken with a two-fold purpose: 1) to provide information relevant to the management of salmonid populations by identifying those acclimation sites that represent a high risk for *M. cerebralis* exposure and 2) to further define the geographic distribution of the parasite in northeastern Oregon. Sentinel rainbow trout fry were exposed at each of the acclimation sites in the region to detect the presence of the parasite. Steelhead juveniles held at a facility with known *M. cerebralis* exposure were examined after acclimation to determine if parasite exposure results in infection of juveniles.

METHODS

Acclimation sites

Within northeastern Oregon, seven holding facilities currently operate for the acclimation of juvenile salmonids (Figure 4.1). These facilities are supplied with water from adjacent rivers and streams. Acclimation sites and holding periods for steelhead and chinook salmon stocks are presented in Table 4.1.

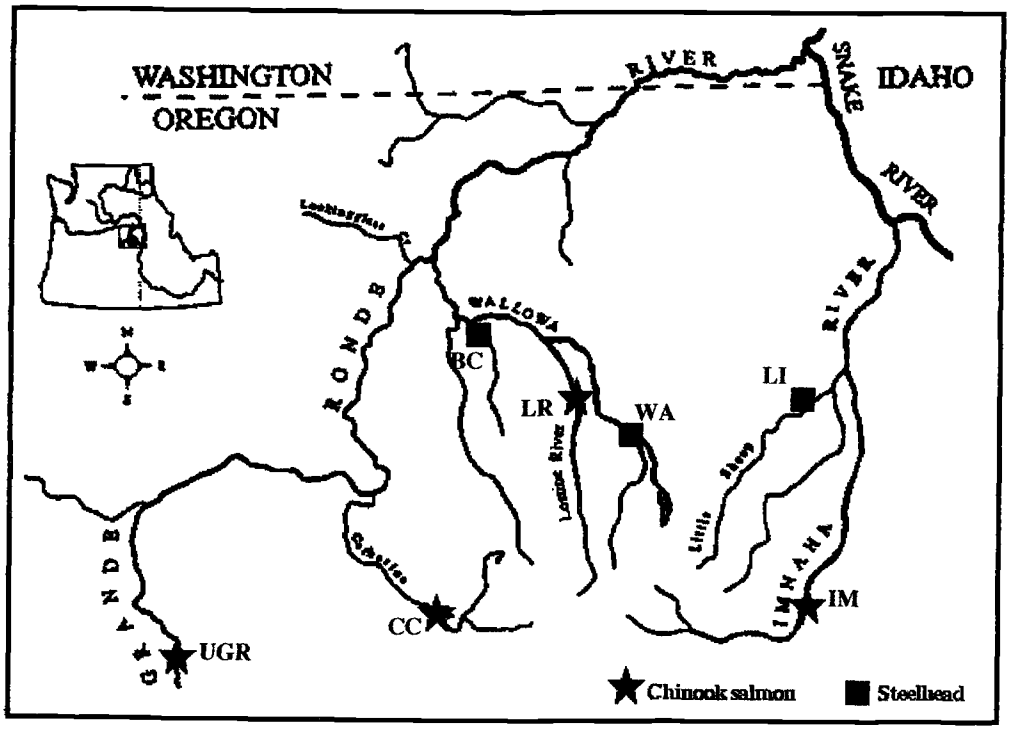

Figure 4.1. Salmonid acclimation facilities in northeastern Oregon.

Table 4.1. Acclimation periods in 2001 for chinook salmon *Oncorhynchus tshawytscha* and steelhead *Oncorhynchus mykiss* at facilities located in northeastern Oregon.

Acclimation Facility	Species (Stock)	Acclimation Period (month/day)[a]
Wallowa[b] (WA)	Steelhead (Wallowa)	Feb 20-April 16 April 9-May 23
Big Canyon[c] (BC)	Steelhead (Wallowa)	Feb 26-April 13 April 16-May 24
Little Sheep (LI)	Steelhead (Imnaha)	Mar 8-April 12 April18-May 10
Imnaha (IM)	Chinook salmon[d]	Mar 1-April 15
Catherine Creek (CC)	Chinook salmon[d]	Feb 26-April 15
Upper Grande Ronde (UGR)	Chinook salmon[d]	Feb 26-Mar 24
Lostine (LR)	Chinook salmon[d]	Feb 16-April 15

[a]Two groups of yearlings were acclimated at steelhead facilities
[b]water source: Spring Creek
[c]water source: Deer Creek
[d]Chinook salmon stocks were acclimated at facilities located on their natal streams

Sentinel fish exposures

Rainbow trout fry (Troutlodge, Inc., Sumner, Washington) were transported at approximately 4 weeks posthatch to northeastern Oregon. A control sample of fry (n=40) was collected prior to initiation of exposures in the spring and fall.

Replicate fry cages (Sandell et al. 2001) with 100 sentinel rainbow trout fry each were placed at the inflows of the seven acclimation sites in March 2001, a time that coincided with the transfer of steelhead and chinook salmon juveniles to the facilities. Water temperature was measured at each site when sentinel cages were deployed. Sentinel rainbow trout fry were held for the duration of the acclimation period with samples taken from each site after approximately 14, 28, and 50 d of exposure. For each exposure period, 20 fry were collected from each replicate cage and euthanized with an overdose (500 mg/L) of tricaine methanesulfonate (MS-222; Argent Laboratories, Redmond, Washington). At the steelhead acclimation facilities where two groups of juveniles are held, sentinel fry were retained and an additional sample was collected after approximately 60 d of exposure. At sampling, each fish was placed into a microcentrifuge tube with tissue lysis buffer (400uL).

Sentinel rainbow trout fry exposures were repeated in September 2001. After a 14 d exposure, 20 fry were collected from each replicate cage and euthanized with an overdose (500 mg/L) of MS-222. Each fish was placed into a microcentrifuge tube with tissue lysis buffer (400uL).

Steelhead juvenile exposure

Ninety-five steelhead juveniles were collected after a 6-week exposure during the second acclimation period at the Wallowa facility. The fish were transported to the Hatfield Marine Science Center in Newport, Oregon, and maintained for 8 weeks in freshwater on a commercial trout diet. Mortalities were

collected daily and survivors were euthanized with an overdose (500 mg/L) of MS-222. Using precautions to prevent cross-contamination, heads were severed behind the operculum, placed into individual bags, and frozen.

Polymerase chain reaction (PCR) analysis

Juvenile steelhead samples (n=50) were prepared for PCR analysis as described by Schisler et al. (2001) with the following modifications. After removal of soft tissues, bone and cartilage fragments were placed in a clean 15-mL screw capped tube. Tissue lysis buffer (2 mL) and proteinase-K (1.8 mg/mL) were added and samples were incubated at 55°C for 60 min with occasional agitation. Digestion and extraction of DNA from juvenile and sentinel fry samples was conducted as described by Sandell et al. (2001). Individual fish were assayed for the presence of *M. cerebralis* DNA using the nested PCR assay as described by Andree et al. (1998).

Analysis of Data

Infection was determined by presence of *M. cerebralis* DNA detected by PCR analysis. The prevalence of infection was calculated as number of samples with detectable *M. cerebralis* DNA/ total number of samples assayed. Mean prevalence of infection is reported for replicate exposure groups.

RESULTS

Sentinel exposures

Steelhead acclimation facilities

During the spring of 2001, *M. cerebralis* DNA was detected in sentinel rainbow trout fry held at two of three steelhead acclimation facilities (Table 4.2). At the Wallowa facility, the prevalence of infection was high (48% after 14 d; 65% after 28 d). High mortality due to problems with water flow at this site prevented collection of fish at 60 d. Samples collected from the Little Sheep facility at 13 d were contaminated during PCR preparation and excluded from analysis. For 27 d and 62 d exposures, sentinel fry held at the Little Sheep facility had a low prevalence of infection, 7.5% and 2.5%, respectively. *Myxobolus cerebralis* DNA was not detected in sentinel fry held at the Big Canyon acclimation site. Control rainbow trout fry, taken prior to initiation of exposures, were negative for parasite DNA. Upon deployment of cages, water temperature was 5.5°C at the Wallowa, 4°C at the Little Sheep, and 0°C at the Big Canyon facilities (Table 4.2).

Table 4.2. *Myxobolus cerebralis* infection in sentinel rainbow trout fry exposed during 2001 at northeastern Oregon steelhead *Oncorhynchus mykiss* juvenile acclimation facilities. NR=not recorded

	Spring			Fall [a]	
Facility	Temp [b]	Exposure Period	% Positive [c]	Temp [b]	% Positive [c]
Wallowa	5.5°C	14 d	48	9°C	2.5
		28 d	65 [d]		
		60d	- [e]		
Little Sheep	4°C	13 d	- [f]	NR	10
		27 d	7.5		
		62 d	2.5		
Big Canyon	0°C	14 d	0	15°C	10
		28 d	0		
		60 d	0		

[a] Fall exposure period was 14 d
[b] Temperature measured when sentinel cages were deployed
[c] Prevalence of infection for n=40 samples
[d] High mortality prevented an adequate number of samples to be collected from second cage; only samples from one cage (n=20) were assayed by PCR
[e] High mortality due to problems with water flow prevented collection of fish
[f] Prevalence of infection was not determined due to contamination during PCR preparation

Myxobolus cerebralis DNA was detected in sentinel rainbow trout fry held at each of the steelhead acclimation facilities in the fall. The prevalence of infection following the 14 d exposure was 2.5% at the Wallowa facility, and 10% at the Big Canyon and Little Sheep facilities (Table 4.2). Water temperature was 9°C at the Wallowa and 15°C at the Big Canyon facilities when exposures were initiated; water temperature was not recorded at the Little Sheep facility (Table 4.2).

Chinook salmon acclimation facilities

Myxobolus cerebralis DNA was detected in sentinel rainbow trout fry held during the spring of 2001 at the chinook salmon acclimation facilities located on the Lostine, Imnaha, and Upper Grande Ronde Rivers (Table 4.3). The highest prevalence of infection was 7.7% for the Lostine (28 d), 17.5% for the Imnaha (40 d), and 17.5% for the Upper Grande Ronde (24 d) facilities. Parasite DNA was not detected in fry held at the Catherine Creek acclimation site. Samples collected from this facility at 14 d were contaminated during PCR preparation and excluded from analysis. Control rainbow trout fry, taken prior to initiation of exposures, were negative for *M. cerebralis* DNA. Water temperature at time of cage deployment was 5°C or lower at all sites (Table 4.3).

Table 4.3. *Myxobolus cerebralis* infection in sentinel rainbow trout fry exposed during 2001 at northeastern Oregon chinook salmon *Oncorhynchus tshawytscha* juvenile acclimation facilities.

	Spring			Fall [a]	
Facility	Temp [b]	Exposure Period	% Positive [c]	Temp [b]	% Positive [c]
Lostine	1°C	14 d	0	14°C	10
		28 d	7.7		
		46 d	0		
Imnaha	5°C	12 d	12.5	13°C	2.5
		27 d	2.5		
		40 d	17.5		
Catherine Creek	2°C	14 d	- [d]	16°C	17.5
		28 d	0		
		35 d	0		
Upper Grande Ronde	1°C	13 d	5	19°C	10
		24 d	17.5		

[a] Fall exposure period was 14 d
[b] Temperature measured when sentinel cages were deployed
[c] Prevalence of infection for n=40 samples
[d] Prevalence of infection was not determined due to contamination during PCR preparation

Following the 14 d exposure conducted in the fall, *M. cerebralis* DNA was detected in sentinel fry held at each of the chinook salmon acclimation sites. The prevalence of infection was 17.5% at the Catherine Creek, 2.5% at the Imnaha, and 10% at the Lostine and Upper Grande Ronde facilities. Water temperatures ranged from 13-19°C when exposures were initiated (Table 4.3).

Steelhead juvenile exposure

Myxobolus cerebralis DNA was detected in steelhead juveniles (Wallowa stock) acclimated during the spring of 2001 for 6 weeks on Spring Creek water (Wallowa facility). The prevalence of infection, as determined by PCR analysis, was 52% (n=50). Signs of clinical disease (blacktail, skeletal deformities) were not observed.

DISCUSSION

This study demonstrates that *M. cerebralis* is established in tributaries of the Grande Ronde and Imnaha Rivers, supporting a suspected presence throughout both river basins. Surveys of free-ranging salmonids conducted by ODFW detected *M. cerebralis* infection among juvenile fish collected from Little Sheep and Catherine Creeks, and the Lostine and Wallowa Rivers. The current sentinel studies confirm the establishment of *M. cerebralis* in these waters, as well as in the main-stem of the Grande Ronde and Imnaha Rivers, and Deer Creek, a tributary of the Grande Ronde River.

Results from this study also demonstrate the potential for parasite exposure at all chinook salmon and steelhead acclimation sites. During the acclimation period for both species, infection prevalence in sentinel fry was relatively low (<17.5%) indicating that low numbers of triactinomyxon stages were present in the water. However, an increased prevalence of infection in sentinel fry held at the

Wallowa facility (65%) identified this site as presenting a high risk for exposure of juvenile steelhead to *M. cerebralis* than other sites.

At most acclimation sites, the presence of the parasite was verified by its detection in sentinel rainbow trout fry exposed in the spring when temperatures were below 6°C. Detection of *M. cerebralis* in Deer and Catherine Creeks was limited to the fall exposures when temperatures were 15°C and 16°C, respectively. Laboratory studies suggest that the development and release of triactinomyxons from *T. tubifex* is prolonged at water temperatures between 5°C and10°C and optimal at about 15°C (El-Matbouli et al. 1999b). Thus, detection of the parasite in fish exposed at Catherine and Deer Creeks during the fall was likely enhanced by favorable water temperatures for triactinomyxon release. In a study to address the relationship between temperature and infection, a positive correlation was demonstrated for fish exposed under natural conditions in Willow Creek, Montana (Baldwin et al. 2000). Unlike the study in Montana, infection prevalence in most exposure groups was low, possibly reflecting low overall parasite abundance. This is supported by a low prevalence of infection among sentinel fish exposed in the Lostine River from July to November when temperatures ranged from 12.2°C to 3.4°C (Sandell et al. 2001). However, the limited temperature data available in the current study prevents the identification of a relationship between infection prevalence and temperature.

During spring exposures, the prevalence of infection among sentinel fry did not increase as exposure duration was lengthened. The small sample size (n=40)

may have masked an increase in parasite prevalence. However, triactinomyxon density fluctuates with water conditions (temperature, flow) and changing dynamics of *T. tubifex* populations (Kerans and Zale 2002), and sentinel fish held for a longer period would not receive a constant, continual exposure to *M. cerebralis*.

An unexpected decrease in infection prevalence was observed among sentinel rainbow trout fry held at the Wallowa facility during the fall exposure period. One explanation for this may be flow alterations influenced by an irrigation system of interconnected canals in the agricultural area surrounding the Lostine and Wallowa Rivers. Anecdotal evidence suggests that during juvenile acclimation at the Wallowa facility, high water flows resulted in a diversion of Wallowa River water (a source with suspected high parasite levels) to the facility's water source (William Knox, Assistant District Fish Biologist, Oregon Department of Fish and Wildlife, personal communication). Thus, the low infection prevalence observed among sentinel rainbow trout following the fall exposure was likely based solely upon exposure to Spring Creek water.

Detection of *M. cerebralis* DNA in the cartilage of steelhead juveniles held at the Wallowa facility from April to May confirms that juveniles are at risk of infection. The prevalence of infection among the juvenile steelhead was similar to that among rainbow trout sentinels held at the facility. This indicates that the prevalence of *M. cerebralis* infection among sentinel fish can be used as an estimate of the actual parasite exposure incurred by juveniles. Utilizing young,

susceptible fish for sentinel exposures allows for a clear interpretation of results with minimal interference from confounding variables (e.g. fish age, species). For this study, sentinel exposures also eliminated the need to lethally sample valuable fish populations.

Most information on susceptibility of steelhead and chinook salmon is derived from laboratory studies using very young fish, and indicate that steelhead are more susceptible to *M. cerebralis* infection and disease (MacConnell and Vincent 2002; Sollid et al. 2002). Laboratory challenges of two strains of steelhead fry by Hedrick et al. (2001) demonstrated a degree of susceptibility to infection and disease comparable to rainbow trout. However, previous studies in our laboratory suggest that differences in strain susceptibility might exist (unpublished data). Although cranial myxospore abundance was similar in the two strains tested, a lower prevalence of infection was detected among Wallowa steelhead (67%) than among Imnaha steelhead (100%) following a 6-week exposure under natural conditions in the Lostine River. This apparent decrease in susceptibility may offer an advantage for Wallowa steelhead acclimated at the Wallowa facility where risk of parasite exposure is high.

While the susceptibility of chinook salmon is not as clearly defined, chinook salmon are intermediate in their susceptibility to *M. cerebralis* (MacConnell and Vincent 2002; Sollid et al. 2002; Vincent 2002). Laboratory challenges indicate that chinook salmon fry are susceptible infection and disease when exposed at an early age (Hedrick et al. 2001). However, a study of age-

dependent susceptibility demonstrated that chinook salmon exposed at 1, 3, 5, 7, and 9 weeks of age incur less severe infections than age-matched rainbow trout (Sollid 2002). Further, resistance to clinical disease developed in chinook salmon when exposure was delayed until after 3 weeks of age.

For both steelhead and chinook salmon, little information exists that addresses the effects of infection on juveniles. Managed populations of chinook salmon and steelhead in northeastern Oregon do not encounter *M. cerebralis* until transferred to acclimation sites in the enzootic region. Steelhead juveniles held at the Wallowa facility did not develop clinical disease signs, indicating that delayed exposure reduces the direct effects of *M. cerebralis* infection. Although not likely to succumb to clinical disease and mortality, these fish did become infected.

It has been demonstrated that infection by *M. cerebralis* affects the ability of rainbow trout to survive multiple stressors (Schisler et al. 2000). This raises the possibility that infection may interfere with the ability of salmonid smolts to successfully adapt to saltwater, a process associated with physiologic stress (Maule et al. 1987). In a recent study (Kristen D. Arkush, University of California Bodega Marine Laboratory, personal communication), mortality among chinook salmon infected with *M. cerebralis* increased upon transfer to saltwater. Mortality also occurred among uninfected chinook salmon, but to a lesser degree. These findings are supported by similar studies conducted with steelhead smolts in our laboratory (unpublished results). It is possible that the process of infection affects the ability of smolts to osmoregulate upon transfer to saltwater; however, more research is

needed to determine the cause of increased mortality witnessed in these preliminary studies.

Pathologic examinations of hatchery stocks have demonstrated a greater prevalence of *M. cerebralis* infection among steelhead than chinook salmon adults returning to northeastern Oregon (ODFW unpublished data). While laboratory studies show that steelhead have a heightened susceptibility to *M. cerebralis* infection, other factors may also play a role in the increased incidence of infection observed among returning steelhead adults. It is certain that juveniles of both species will encounter *M. cerebralis* during their migration out of the enzootic region. Differences in timing, duration, and intensity of exposure during migration may contribute to the increased prevalence of infection observed among returning steelhead adults. While fish infected as juveniles do not display overt signs of clinical disease, they may become carriers of the parasite with the potential to disseminate *M. cerebralis* during migration. Data suggest that steelhead adults are more likely to stray from their natal streams than chinook salmon adults (Oregon Department of Fish and Wildlife 1996). In a study by Engelking (2002), more than 20% of stray hatchery steelhead adults and 10% of stray hatchery chinook salmon adults returning to the Deschutes River, Oregon, carry spores of *M. cerebralis.* Thus, the increased susceptibility of steelhead combined with increased stray frequencies by the species could have a detrimental effect on management efforts to contain *M. cerebralis* within northeastern Oregon.

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SUMMARY

The susceptibility of select Oregon salmonid species to *Myxobolus cerebralis* was investigated to provide information for assessing the effects of parasite exposure on salmonid populations. In the Deschutes River, Oregon, where *M. cerebralis* is not established, susceptibility studies were used to determine the risks for populations if the parasite became established. Laboratory challenges indicated a range of susceptibility to *M. cerebralis* among the salmonid species indigenous to that river. However, the degree of susceptibility of chinook salmon *Oncorhynchus tshawytscha* and steelhead *Oncorhynchus mykiss* observed in this study indicates that establishment of the parasite in the Deschutes River may not result in clinical disease in these species. This is substantiated by the low incidence of clinical signs observed in chinook salmon and steelhead in the Grande Ronde and Imnaha River basins where *M. cerebralis* is enzootic.

In northeastern Oregon where *M. cerebralis* is considered widespread, assessment of chinook salmon susceptibility supports observational data and indicates the species is at low risk for development of clinical disease. Age-dependent studies demonstrate that chinook salmon are less susceptible than rainbow trout to severe infections and become resistant to disease after 3 weeks posthatch. Sandell et al. (2001) suggested that chinook salmon fry emerge in areas where parasite exposure is low, further reducing the potential impacts of infection. The prevalence of infection among chinook juveniles exposed to a high parasite

level was low, and simulation of natural exposure conditions demonstrates that chinook salmon can withstand a prolonged exposure to *M. cerebralis* without developing clinical disease.

While *M. cerebralis* infection may not result in clinical disease in chinook salmon and steelhead in areas where the parasite is enzootic, the impacts of exposure on smoltification and saltwater adaptation have not been thoroughly investigated. We have demonstrated that juvenile steelhead and chinook salmon held for acclimation in northeastern Oregon are exposed to the parasite. Schisler et al. (2000) reported that *M. cerebralis* infection increases mortality when exposure to multiple stressors occurs, and the physiological stress associated with smoltification is well-documented (Maule et al. 1987). Upon transfer to saltwater, increased mortality was observed among steelhead and chinook salmon exposed to *M. cerebralis* (Arkush et al. 2001). This suggests that exposure of juveniles during acclimation in enzootic areas may have negative impacts on the survival of these fish during saltwater adaptation.

The impact of infection in chinook salmon and steelhead extends beyond the potential to interfere with saltwater adaptation. The exposure of anadromous salmonids to *M. cerebralis* may contribute to the dissemination of the parasite if infected adults stray and spawn in waters where the parasite is non-enzootic. Further, survey data suggests that up to 70% of adults returning to the Deschutes River are strays from upriver tributaries of the Columbia River (Engelking 2002). Exposure and infection of anadromous salmonids may have a detrimental effect on

efforts to reestablish wild populations of these species, and may interfere with management efforts to prevent the spread of *M. cerebralis* into non-enzootic areas.

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APPENDIX

APPENDIX: EFFECT OF *Myxobolus cerebralis* EXPOSURE ON JUVENILE STEELHEAD *Oncorhynchus mykiss* UPON TRANSFER TO SALTWATER

INTRODUCTION

Myxobolus cerebralis, a parasite of salmonids (Höfer 1903), is considered widespread throughout the basins of the Grande Ronde and Imnaha rivers in northeastern Oregon. Within this region, managed populations of chinook salmon and steelhead first encounter the parasite upon transfer as juveniles to acclimation facilities for holding on their natal rivers. While the effects of *M. cerebralis* exposure on juveniles have not been thoroughly investigated, studies have demonstrated that infection can increase mortality upon exposure to multiple stressors (Schisler et al. 2000) This information suggests that the parasite may compromise the survival of juvenile salmonid smolts during the stressful period of saltwater adaptation (Maule et al. 1987).

A recent study (Arkush et al. 2001) found an increased cumulative mortality among chinook salmon *Oncorhynchus tshawytscha* smolts infected with *M. cerebralis* upon artificial transfer to seawater. Mortality also occurred among uninfected chinook salmon smolts, but to a lesser degree. To investigate the effect of exposure on the ability of steelhead *Oncorhynchus mykiss* juveniles (of smolt age) to adapt to saltwater, we assessed two types of *M. cerebralis* exposure: 1) a controlled laboratory challenge and 2) a natural exposure at an acclimation facility.

METHODS

Fish source

Steelhead juveniles (Wallowa strain) were from the same cohort. All fish were hatched and reared on specific-pathogen free water at Irrigon Hatchery, Oregon.

Natural exposure

In April 2001, juvenile steelhead were transferred to the Wallowa acclimation facility in northeastern Oregon where *M. cerebralis* is enzootic. After a six week holding period, fish (n=95) were transported to an isolation facility at the Hatfield Marine Science Center (HMSC) in Newport, Oregon. The fish were maintained in aerated freshwater on a commercial trout diet.

Laboratory exposure

One-hundred juveniles were retained at Irrigon Hatchery, and did not receive exposure. These fish were transported to HMSC and divided into two groups: laboratory challenge (n=50) and control (n=50). The laboratory challenge group was exposed to a high-dose of *M. cerebralis* (11,000 triactinomyxons/fish) in static water for 2 hours. The challenge was repeated under the same conditions at 24 d post-transfer to HMSC to ensure exposure that may have been compromised by high chlorine levels.

Saltwater transfer

Steelhead juveniles were maintained in freshwater until 32 d postexposure. At this time, seawater was gradually introduced over 24 hours. The experiment was terminated 15 d post-transfer to saltwater.

Evaluation

Mortalities were recorded daily. Serum and gill filament samples were collected from the naturally-exposed (n=2), laboratory challenged (n=2), and control fish (n=4) two days following transfer to full-strength saltwater. These samples were tested for plasma sodium concentrations and gill ATPase activity.

RESULTS

Prior to transfer of steelhead juveniles to saltwater, high percent cumulative mortality was observed among juveniles naturally exposed to *M. cerebralis* (Figure A.1). At time of saltwater transfer, survival was 28% among the steelhead juveniles obtained from the acclimation facility. Few mortalities were recorded among the laboratory challenged and unexposed control groups prior to saltwater transfer.

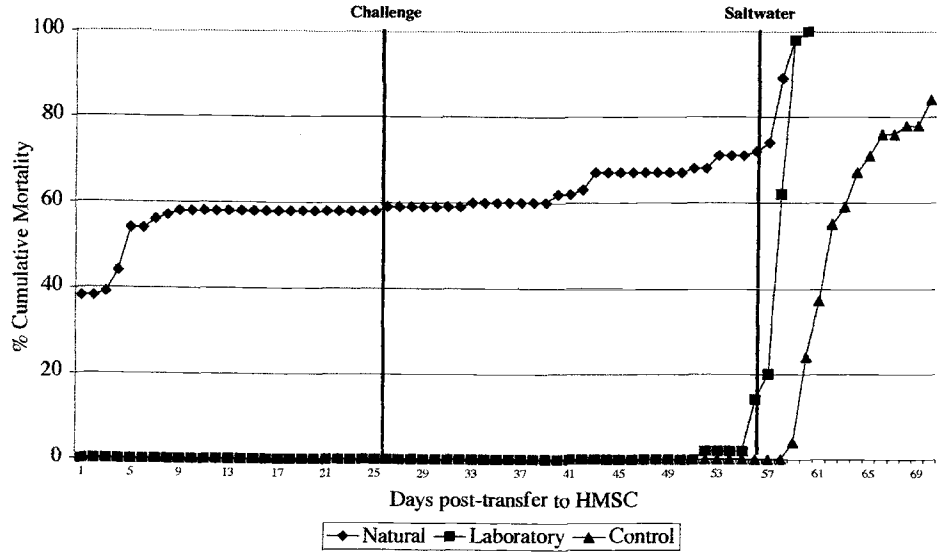

Figure A.1. Percent cumulative mortality among steelhead *Oncorhynchus mykiss* juveniles, exposed to *Myxobolus cerebralis* (natural exposure or laboratory challenge) or unexposed, following transfer to Hatfield Marine Science Center (HMSC). Laboratory challenge and transfer to saltwater indicated by vertical lines.

Steelhead juveniles had high mortality rates upon transfer to saltwater (Figure A.2). At 4 d post-saltwater-transfer, percent cumulative mortality for juveniles exposed to the parasite under laboratory conditions reached 100%. Steelhead juveniles naturally exposed to *M. cerebralis* had 100% cumulative mortality at 5 d post-saltwater-transfer. Percent cumulative mortality of controls at 5 d post-saltwater-transfer was 37%. When the experiment was terminated at 15 d post-saltwater-transfer, percent cumulative mortality among unexposed controls was 84%.

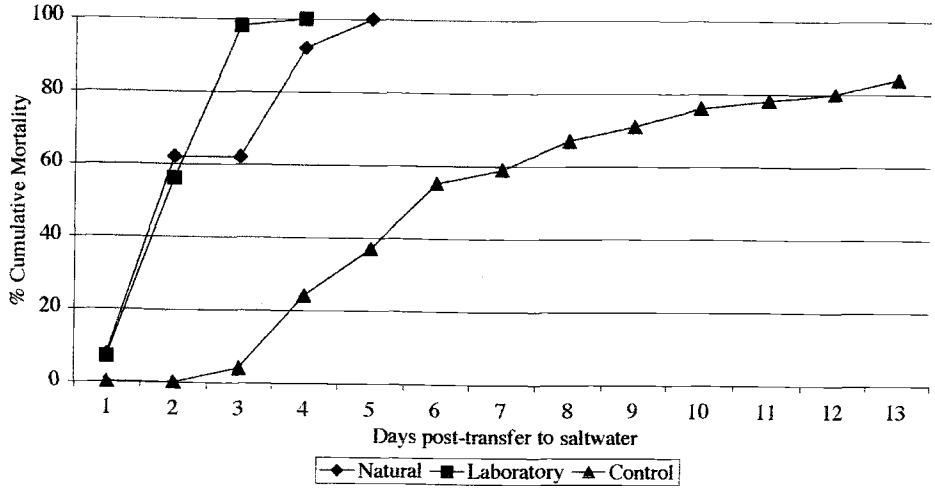

Figure A.2. Percent cumulative mortality among steelhead *Oncorhynchus mykiss* smolts, exposed to *Myxobolus cerebralis* (natural exposure or laboratory challenge) or unexposed, following transfer to saltwater.

DISCUSSION

The results from this study suggest that exposure to *M. cerebralis* likely contributed to an increased mortality rate in steelhead juveniles upon transfer to saltwater. However, high mortality also occurred in the unexposed controls upon transfer to saltwater. Clearly, adaptation to saltwater was not successful among the steelhead, and this was confirmed by high sodium concentrations (data not presented) in serum samples collected from all three groups of fish. Further, low gill ATPase activity indicated that smoltification was not occurring in the steelhead juveniles. This may have resulted from the delayed transfer to saltwater.

The focus of the study by Arkush et al. (2001) was assessment of saltwater introduction on fish with established *M. cerebralis* infections. In the present study, we examined the effect of exposure that occurs just prior to migration and saltwater

entry. While both studies were complicated by unsuccessful saltwater adaptation, they suggest that *M. cerebralis* can compromise survival of juvenile salmonids upon transfer to saltwater.

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