LITERATURE REVIEW
MYXOBOLUS CEREBRALIS,
THE CAUSATIVE AGENT OF WHIRLING DISEASE

PELTON ROUND BUTTE HYDROELECTRIC PROJECT

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INTRODUCTION

Whirling disease has recently become the most widely known disease of salmonids. Salmonid whirling disease was discovered in Europe in 1893 and has since been spread around the world with shipments of cultured and wild fish. It causes lesions of salmonid skeletal tissues, particularly in the head. It can cause skeletal deformities as it destroys the cartilage in young fish. The head skeletal structures are involved, and darkening of the tail , a loss of equilibrium, and tail chasing behavior are other signs of disease. Mortality is often the result of such infections in young fish. Survivors are often deformed with blunt noses or curvatures of the spine. *Myxobolus cerebralis* (Myxozoa: Myxosporea), the causative agent of whirling disease, has a complex two-host life cycle, which begins when waterborne triactinomyxon spores released from the infected oligochaete *Tubifex tubifex* contact a susceptible salmonid. Diagnosis of the disease is straightforward, but can be complicated by the presence of other *Myxobolus* species and by other parasites or pathogens that cause fish to whirl. It is very difficult to detect fish with no overt signs of disease. These carrier fish have been suspected as responsible for most introductions of the parasite into new areas. The pathogen is difficult to eradicate but can be managed for in cultured fish if they are cultured in specific pathogen free (spore-free) water, in concrete raceways with strong water flows, or in ponds that are regularly disinfected, and if they are constantly monitored for the presence of spores. Fish can carry *M. cerebralis* spores and still be healthy. Such fish have been considered suitable for stocking into waters already containing *M. cerebralis*, and even for human consumption (Hoffman 1990). The situation for wild or feral fish is quite different. Improvements in fish husbandry practices reduced the impacts to aquaculture, but the parasite is not so manageable in the environment. Control of the water supply, elimination of the intermediate host worms, and transferring the young to areas with few or no spores is not possible in the field.

Serious declines have now been noted in both Montana and Colorado among several populations of non-hatchery rainbow trout (*Oncorhynchus mykiss*). Studies of a three year decline (1991-1994) in self sustaining rainbow trout populations on the Madison River in Montana, suggested that in 1995 the losses were associated with young fish being infected with *M. cerebralis.* As of 1999, the populations of rainbow trout in the Madison River have not recovered but remain at levels of 20-25 percent that of the populations prior to infection. It is believed that some young rainbow trout survive because certain spawning beds lie in regions where lower levels of the triactinomyxons exist. They thus escape early infection and survive, as they become less susceptible with age. The more resistant brown trout (*Salmo trutta*) populations have remained relatively stable with some slight increase. This indicates they have assumed all

available habitat for their life history and cannot utilize that which the rainbow trout formerly inhabited (Vincent and Byroth 1999).

Upon contact, the triactinomyxon spores of *M. cerebralis* attach to the fish and release their sporoplasm cells into the epidermis. At approximately six weeks postinfection, sporogenesis begins, resulting in a large number of *M. cerebralis* spores in the cartilage of infected fish. The spores of *M. cerebralis* can be released from infected fish only after the fish die or are eaten by predators. In both cases, spores released into the aquatic environment can be ingested by *T. tubifex* and then develop into the actinosporean triactinomyxon stage in the intestine within about three months. The triactinomyxon is the only stage infectious for salmonid fish.

GEOGRAPHIC DISTRIBUTION

World Distribution

Myxobolus cerebralis has been found in Germany, Italy, Russia, United States of America, Bulgaria, Sakhalin Island of Russia, Yugoslavia, Sweden, South Africa, Scotland, New Zealand, Ecuador, Columbia, Lebanon, Ireland, Spain, and England (Hoffman 1990). It is generally believed to be of central European origin, being first reported in 1903 in Germany (Post 1987).

A typical survey of fish in an endemic region or country often reveals that the area affected is larger and involves more species of fish. The following report is such an example. Whirling disease was diagnosed in rainbow trout at Silverstream Fish Hatchery near Christchurch New Zealand in 1980. As a consequence, a nationwide survey for *M. cerebralis* in salmonids was conducted by examination of wild and hatchery fish. In addition, sentinel rainbow trout were used at six locations to test for the presence of the parasite This survey and other studies revealed *M. cerebralis* at locations in two watersheds and provided the first New Zealand documentation of *M. cerebralis* in brook trout (*Salvelinus fontinalis*), chinook salmon (*O. tshawytscha*), and sockeye salmon (*O. nerka*); (Boustead 1993).

USA Distribution

Whirling disease was first detected in the United States in Pennsylvania in 1958 and in Nevada about the same time (Hoffman 1990). Since then the disease has been detected in twenty one other states: Alabama, California, Colorado, Connecticut, Idaho, Maryland, Massachusetts, Michigan, Montana, Nevada, New Hampshire, New Jersey, New York, Ohio, Oregon, Utah, Virginia, Washington, West Virginia, Virginia and Wyoming (Nickum 1999, Wolf 1986).

A summary of the detection and distribution of whirling disease in California illustrates the transfer of infected fish as the most significant manner in which the parasite is transferred among watersheds. In fact, the transfer of fish is the only manner that has been documented for the introduction of *M. cerebralis*. *Myxobolus cerebralis* has become widely established in self sustaining California salmonid populations since its initial discovery in Monterey County in 1965. Most significant is the occurrence of the parasite in the "blue ribbon" trout waters of the Owens Valley basin of the eastern Sierra. From the Lahontan basin on the north to the Owens Valley basin to the south, the parasite has become well established. In spite of the presence of the parasite, streams of the eastern Sierra are considered by many to support high quality trout populations which attract thousands of anglers to the region. Observations and surveys suggest that fish populations are healthy in the Owens Valley drainage and in the *M. cerebralis*-positive waters of the Lahontan and Pacific drainages. These observations are supported by population data comparing populations of rainbow trout (*O. mykiss*) and brown trout (*Salmo trutta*) on Sagehen Creek and the lower Truckee River in the Lahontan basin, and rainbow trout populations in *M. cerebralis* positive and negative sections of the Carmel River on the central California coast near Monterey. The chronological appearance and distribution of *M. cerebralis* strongly implicates dispersal of live or processed, publically and commercially produced fish as a major factor in the spread of the parasite in California. Infected anadromous stocks have not appeared to spread *M. cerebralis* into numerous coastal waters and waters entering directly into San Francisco Bay. A severe epizootic of *M. cerebralis* at the Mt. Whitney State Fish Hatchery in the spring of 1995 confirmed the virulent potential of *M. cerebralis* in California. Spores of *M. cerebralis* can no longer be detected in wild populations at three locations since elimination of the source of infection in those waters (Modin 1998).

Oregon Distribution

Whirling disease was first detected in December 1986 in a private hatchery located in the Grande Ronde watershed in northeast Oregon. Rainbow and brook trout were found infected. An extensive survey was started in 1987 to determine the distribution of *M. cerebralis* in Oregon. Lorz, Amandi, Banner, and Rohovec (1989) found whirling disease in two watersheds; Grande Ronde (including Wallowa and Lostine rivers) and Imnaha. Steelhead trout (*O. mykiss*) and chinook salmon (*O. tshawytscha*) were found infected in these areas along with rainbow and brook trout. According to routine sampling by Oregon Department of Fish and Wildlife the disease appears to remain in the original watersheds. The only new occurrence of *M. cerebralis* since 1988 has been in wild steelhead at Catherine Creek in the Grande Ronde (Holt 1996).

Deschutes Distribution

In 1987, *M. cerebralis* was detected in hatchery stray summer steelhead trout at Warm Springs Hatchery. Spores of *M. cerebralis* were found again in stray hatchery steelhead trapped at Pelton trap in 1997 and 1998 (Engelking et al. 1999). The parasite has not been detected in any Deschutes hatchery stock or resident Deschutes fish including brown trout bull trout (*Salvelinus confluentus*) mountain whitefish (*Prosopium sp.*), kokanee salmon (*O. nerka*), or redband rainbow trout. However, one unmarked steelhead (1998) and an unmarked chinook salmon (1998) were confirmed to be infected with *M. cerebralis* by histological examination. Fish pathologists for Oregon Department of Wildlife suspect the fish were either stray wild fish or mismarked hatchery fish from an upper Snake River facility. *Myxobolus cerebrali*s is established in the Grande Ronde and Imnaha rivers of the upper Columbia River basin and Idaho streams of the Snake River basin where these straying fish may have originated. Few signs of disease and no population effects on resident fish during limited surveys have been noted in northeastern Oregon. Three adult hatchery stray spring chinook salmon were detected with presumptive whirling disease spores in 1998 (Table 1). The three infected stray chinook salmon were coded wire tagged and came from Dworshak, McCall and possibly (tag lost after preliminary reading) Rapid River hatcheries. To date the Deschutes watershed is considered not to have *M. cerebralis* established in it.

Table 1. Groups of fish found to be infected by M. cerebralis from surveys that included resident, anadromous and stray fish in the Deschutes River watershed below Pelton Round Butte Project.

Fish Species Stock[a]	Year	Number Tested	Number Presumptive *M. cerebralis* Infected	Number Confirmed *M. cerebralis*
Summer Steelhead Trout				
Unmarked	1998	13	1	1
Hatchery Stray	1997–1998	150	32	8
Hatchery Stray	1998–1999	25	3	No Results
Spring Chinook Salmon				
Unmarked	1997	57	1	1
Unmarked	1998	14	4	No Results
Hatchery Stray	1998	23	3	No Results
Rainbow Trout	1998	59	0	
	1999	60	0	
Mountain Whitefish	1998	60	0	
	1999	60	0	

a. Unmarked fish are of unknown origin; samples were taken for genetic analysis. The fish could be wild, wild stray fish, or unmarked hatchery or hatchery stray fish.

SUSCEPTIBILITY

Currently, there are twelve known salmonid species and two non-salmonid fish that are susceptible to whirling disease: rainbow trout (*O. mykiss)*, lake trout (*Salvelinus namaycush*), sockeye salmon (*O. nerka*), brook trout , chinook salmon (*O. tshawytscha)*, brown trout, coho salmon (*O. kisutch*), golden trout (*O.mykiss aguabonita*) (Anonymous 1988), cutthroat trout (*O. clarki)* (Yasutake and Wolf 1970), Atlantic salmon (*S. salar*) (Hoffman 1990), steelhead trout (*O. mykiss*) (Horsch 1987), bull trout (*Salvelinus confluentus*), Arctic grayling (*Thymallus thymallus*) and whitefish (*Prosopium williamsoni*) (Nichum 1999).

There is a large degree of variation in the susceptibility of whirling disease between species and among strains. Whirling disease susceptibility studies have demonstrated fish to range

in the degree to which they are susceptible to the parasite. This range has been denoted as: highly susceptible, intermediate in susceptibility, carriers, and resistant or refractory to the disease. O'Grodnick, in 1979, performed a field study on seven salmonid species. Susceptible species that did not show signs of disease were found to have spores of the parasite present in the cartilage without significant tissue damage. Resistant, or refractory species did not show the presence of any spores in the cartilage after exposure. He found rainbow trout to be the most susceptible to whirling disease. Lake trout were completely refractory. Brown trout and coho salmon did not break with clinical disease signs but were susceptible. Sockeye salmon rated second in susceptibility with brook trout and chinook salmon rating intermediate. Contrary to O'Grodnick's study, Hoffman and Putz (1969) found *M. cerebralis* spores in lake trout. Three separate field studies presented during the 1997 Whirling Disease Symposium revealed cutthroat trout to be highly susceptible (Elle; Vincent; MacConnell et al.). One of the field studies showed Arctic grayling to be susceptible to infection, but resistant to disease (MacConnell et al. 1997). Studies of a variety of hybrids of resistant and susceptible species revealed that the hybrids were capable of becoming infected (Wilson et al. 1997). This indicates that resistance is not a dominant genetic trait.

TRANSMISSION AND LIFE CYCLE

Tubificid worms are required as an alternate host to complete the life cycle of the parasite; as was demonstrated by Markiw and Wolf (1983) to achieve experimental infections with *M. cerebralis* tubificid worms were necessary. These authors were later able to show that the infectious form was a triactinomyxon spore (Wolf and Markiw, 1982). Antigenic evidence suggested that the two spore forms were in fact from a single organism (Markiw and Wolf, 1989). Molecular studies by El-Matbouli and Hoffman in 1998 and Hedrick in 1990, confirmed the genetic identity of the triactinomyxon and myxosporean forms. The alternating myxosporean and actinosporean stages of *Myxobolus cerebralis* (Hofer 1903) have been compared for sequence homology of the small subunit (18S) ribosomal RNA genes. A 99.8% similarity between the sequences of these two stages was significantly greater than that of *M. cerebralis* compared to two other *Myxobolus* species from salmonid fish. The two different taxonomic classes (Myxosporea and Actinosporea) were thus forms of the same organism as shown by this molecular evidence confirming the alternating stages for the life cycle of *M. cerebralis* (Andree et al. 1997a). The alternate host appears to be restricted to *T. tubifex* as six other genera of oligochaete worms have proven thus far to be unable to support infection (Hedrick et al. 1997, Wolf and Markiw 1982). Field studies support the idea that tubificid worms are the alternate host. These studies of worms in natural areas where *M. cerebralis* is endemic have shown that the *T. tubifex* is infected with the parasite. Aquatic oligochaetes from a whirling disease enzootic

area in southwest Montana were examined for infection with *M. cerebralis*. *Tubifex tubifex* were infected with *M. cerebralis* as determined by the nested PCR assay (Rognlie and Knapp 1998). Finally, other parasitic organisms of fish have been detected that also have alternating life cycles indicating similar evolutionary strategies among the members of these groups (Upenskaya 1995)

ETIOLOGICAL AGENT

Whirling disease is caused by a myxosporean parasite, *Myxobolus cerebralis* (Hofer 1903). The myxospores are almost circular in front and lenticular from the side. The two vaulted shell valves are joined along a suture line. Often the spores are somewhat irregular and asymmetrical. A furrow parallel to the suture is visible in both valves. The spores are quite small, 8 to 10 µm in length and width, and about 6-7 µm in thickness. In contrast other *Myxobolus* species are somewhat larger, 10.5 to 13 um in length and width and 9.5 um in thickness. Examined under scanning electron microscopy, the other species were morphologically similar although distinctive in size. A mucous plug has been reported to cover the posterior end of the spore. This plug is not always present (Hedrick et al. 1991). Two oval polar capsules of equivalent size are contained in the spore. These capsules contain polar filaments and are no greater in length than one half the length of the spore. The two nuclei reside in the sporoplasm contained in the remainder of the spore (Lom and Hoffman 1971).

The spores of *M. cerebralis* are extremely stable. The myxospores have been found to remain viable from three to even thirty years under environmental conditions (Hoffman et al. 1962, Halliday 1976). The spores are also resistant to physical extremes and survive freezing to -20°C (El-Matbouli and Hoffman 1991b), various chemical treatments (Hoffman and Hoffman 1972) and passage through the digestive tracts of predators (Taylor and Lott 1978). High temperatures and extreme desiccation appear to be more detrimental to the viability of the spores (Wolf and Markiw 1982). In transmission experiments with tubificids *Tubifex tubifex* as primary hosts and fry of rainbow trout (*O. mykiss*) as secondary hosts, it was shown that *Myxobolus cerebralis* spores can tolerate freezing at -20°C for at least 3 months, aging in mud at 13°C for at least five months, and passage through the guts of northern pike (*Esox lucius*) or mallards (*Anas platyrhynchos*) without loss of infectivity (El Matbouli and Hoffmann 1991a).

Other molecular studies have defined the stage of replication of the parasite DNA. From DNA concentration studies of different life stages, it was demonstrated that meiosis occurs only once in the developmental cycle of *M. cerebralis*. This takes place within the pansporocyst found in *T. tubifex*. The genome is diploid in the sporoplasm cells of the triactinomyxon spores in *T. tubifex* and *M. cerebralis* in trout (El Matbouli et al. 1998b). This suggests replication of the genome (DNA) occurs only during the stage found within the alternate worm host.

Taxonomy

The phylum Myxozoa has previously been considered to comprise two classes, Myxosporea Butschli, 1881 (primarily of fishes) and Actinosporea Noble. It has recently been demonstrated that the life cycle of *Myxobolus cerebralis* Hofer, 1903 (Myxobolidae: Platysporina) of salmonid fishes requires transformation of the myxosporean into an actinosporean stage in the oligochaete worm *T. tubifex* (Tubificidae) (Wolf and Markiw 1984). The stage infective to fish is the actinosporean spore (triactinomyxon). This type of two-host life cycle has now been confirmed, or suggested, for fourteen myxosporean species. The taxonomy of the Myxozoa has been revised, and the following taxonomic changes have been made: suppression of the newer class Actinosporea and the order Actinomyxidia Stolc, 1899; and suppression of all families in the Actinosporea except Tetractinomyxidae (Kent et al. 1994).

EPIZOOTIOLOGY

Infection of fish occurs following from exposure to the triactinomyxon stage (TAMS). The tubificid worms begin releasing TAMS when held at 12.5°C fourteen to sixteen weeks after infection from the myxospore. For the next two to nine weeks, the worms release the greatest numbers of TAMS, the number then declines but continues for up to nine months. However fish placed in exposure tanks with the infected worms become infected as long as one year after the initial exposure of the worms to spores (Markiw 1986). Unlike the myxospore, TAMS are relatively fragile and remain viable only three or four days at 12.5°C. Higher temperatures lead to faster inactivation of these spores (Markiw 1992b).

Ecological variables may influence the course of infection in fish populations. The effects of environmental stresses such as flow, temperature extremes, pollution, biological alteration, and changes in species diversity are important variables in influencing infection and disease. Highly productive streams appear to favor establishment of whirling disease, while relatively infertile mountain streams with low fish numbers do not (O'Grodnick, 1978). Waters with high sediment and organic matter favor tubifex worms, this environment may lead to an increase of the infective dose to which fish are subjected (Modin 1998).

Temperature also plays a major role in the production of TAMS (Gilbert and Granath 1998). Optimal temperature for triactinomyxon production appears to be 15°C; lower temperature delaying production and higher temperatures (< 20°C) destroying the developmental stages (El-Matbouli et al 1998b). Field studies also indicate that temperatures have an important role in infection. In live box studies, young of the year rainbow trout had more severe infections at temperatures of 9 to 14°C, than at 17°C (Vincent 1998). Whirling disease can spread from a point infection throughout a watershed. In three years whirling disease spread six miles

downstream and 1,500 feet upstream from the initial infection site in a Michigan River (Yoder 1972). Examination of other data from the field suggests that fish farther from sources of infection have fewer clinical signs of disease (Schisler et al. 1997). Means of spread other than infected fish are possible, but have not been documented (Hoffman 1990). In the United States whirling disease has spread primarily through the transfer of live fish and the movement of infected fish within watersheds.

Field studies have presented a variety of outcomes for whirling disease exposures. In both New York and Oregon, very little in the way of clinical signs of disease and population effects have been noted on wild trout (Schachte and Hulbert 1998; Holt 1996). Significant impacts have been observed in the Rocky mountain states, including Montana, Colorado and some locations in Idaho (Elle 1997; Nehring et al. 1997; Vincent 1996). The severity of the infection has decimated these wild trout populations with little or no indication of recovery.

The intensity and prevalence of whirling disease was tested by exposure of two month-old fry and one, two and three and one half year-old adults of rainbow trout to a known number of laboratory-produced *M. cerebralis* at the actinosporean triactinomyxon stage. Fry exposed to graded concentrations of infectivity (triactinomyxons) for three hours were individually examined for spores of *M. cerebralis* five and six months later. Exposure of fish to the lowest doses, one and ten triactinomyxons per fish, did not result in detectable myxosporean spores. Fish that became lightly infected by a dose of 100 triactinomyxons per fish experienced a decrease in the incidence of infection between five and six months after exposure. Adult fish continuously exposed to the highest dose of triactinomyxons for three and one half months were infected and asymptomatic; however, the severity of myxosporean infection decreased with increased age of fish. A linear relationship was revealed from these studies between the numbers of recovered myxosporean spores and doses of 100-10,000 triactinomyxons per fish. The spore burden appeared to plateau at doses of 10,000-100,000 triactinomyxons per fish (Markiw 1992a).

The minimum age at which eggs or fish could be infected with the parasite was determined by early exposure experiments. Various developmental stages of eyed eggs and newly hatched sac fry of rainbow trout (*O. mykiss*) were exposed to several concentrations of laboratory produced spores of the triactinomyxon stage of *M. cerebralis*. Exposed eggs and sac fry and unexposed controls were examined microscopically immediately after challenge for the presence of initial forms of the disease and four months later for the presence of spores of the myxosporean stage of *M. cerebralis* in resulting fingerlings. Although initial forms of whirling disease shown as intracellular aggregates of small sporozoites (sporoplasms) 1.5-2.0 µm in diameter were found in the epithelium of eyed eggs a few hours before hatching and in one day-old sac fry, the resulting fingerlings examined four months later were free of *M. cerebralis* spores. The youngest trout that became infected with whirling disease and yielded spores of *M. cerebralis* was the two day-old sac fry. These experiments suggest that transfer of eyed eggs from locations where *M. cerebralis*

is endemic should not pose a risk of spreading the parasite if the eggs are properly disinfected (Markiw 1991).

In areas with barely detectable levels of tubificid worms, significant fish losses to whirling disease have occurred. Detection of the intermediate oligochaete worm host is often problematic. Millions of worms per river mile may be distributed at densities of only of fifty worms per square yard. This low density prevents easy detection of the worm. In many streams the worms, spores and young fish are concentrated in localized areas, which leads to a high magnitude of pathogen transfer.

Low risk areas of parasite transfer and infection include streams with diverse insect communities that are very poor *T. tubifex* habitat. Increasing water temperatures may decrease tubificid worm populations and limit sources of TAMS. Lake outflows from large natural lakes appear to greatly reduce the number of *T. tubifex*. Many of these outflow streams have temperatures or other factors that impact the worm populations. Lakes also seem to block the downstream movement of free parasites. Abundant *Rhyacophila* species at these sitesoften reduce *T. tubifex* populations.

High risk areas for the amplification of *M. cerebralis* include spring creeks in which *T. tubifex* is normally present; tailwater streams which have reduced biodiversity, especially in the insect community, and waters impacted by urban areas, agriculture or forestry. Grazing and other uses often lead to nutrient loads that promote *T. tubifex* growth. Lakes that are warm and shallow probably have few *T. tubifex,* but deeper colder lakes and those with springs may support *T. tubifex* populations (Gustafson 1997).

DETECTION

Diagnostic Procedures

Fish with obvious signs of whirling disease can be infected with various pathogens other than *M. cerebralis*. Definitive diagnosis is a combination of observing signs of disease and demonstration of spores in the cartilage or bone of susceptible fishes. There are several diagnostic procedures for finding *M. cerebralis* spores. In spite of the validated diagnostic methods, occasionally misdiagnoses are made when based only upon signs exhibited by diseased fish. These observations must be critically examined and the diagnosis based upon confirmation of spores in cartilage tissues by histology. Whirling disease was suspected in losses of salmonids in South America, but these suspision was proved to be incorrect. Reports described whirling disease of salmonids in Venezuela, Colombia, Ecuador, and Chile. The disease(s) were shown not to be associated with infections with *M. cerebralis*In Ecuador and Chile observatons of

salmonid diseases with clinical signs resembling those of whirling disease further demonstrated that other diseases can mimic *M. cerebralis* infections. In Chile, the diseases in coho salmon and rainbow trout were attributed to bacterial infections, whereas no infectious agent was associated with afflicted fish in Ecuador. Myxosporean whirling disease of salmonids has not been demonstrated in South America. Another example of a parasitic infection that caused disease signs similar to those of whirling disease was described in Atlantic salmon smolts in Ireland. Based on light microscopy, the detected parasite differed from *M. cerebralis* (Frasca et al. 1998). Mixed infections of multiple parasites can complicate diagnoses. Three myxosporeans were encountered in the cranial tissues of a California population of rainbow trout examined for the presence of *M. cerebralis*. Typical spores of *M. cerebralis* and a previously undescribed species of *Myxobolus* were found in the cranial tissues by the enzyme digestion method. *Henneguya zschokkei* was also detected in digest preparations of cranial tissues. This parasite has a double tail structure. Microscopic examinations of tissues of individual rainbow trout showed occasional infections with both myxobolid species (Hedrick et al. 1991). These types of findings illustrate the need for careful diagnostic procedures. The causes of several other diseases of salmonids with clinical signs similar to those of myxosporean whirling disease (e.g. whirling behavior, skeletal deformities, blackened tails) include bacterial or microsporidial infections of the meninges or brain, myxobacterial infections of the vertebrae, and dietary deficiencies (Margolis et al. 1996). A diagnosis of myxosporean whirling disease, therefore, cannot be made on clinical signs alone. Laboratory confirmation of the diagnosis through demonstration of the presence of *M. cerebralis* is necessary. There are molecular and microscopic methods that have been developed to diagnose whirling disease. Spores can be identified from fish with heavy infections by simply scraping the inside of the cranium and reading wet mounts. Polymerase Chain Reaction (PCR) techniques can detect light infections by detection of DNA that corresponds to that of the parasite. The plankton centrifuge and proteolytic enzymatic digestion methods degrade the cartilage and release spores of the parasite from sections of the skull, gill arches and vertebrae. Histological examination of preserved tissues and skeletal fragments allows direct microscopic detection of *M. cerebralis* spores in infected tissues and bone. Pathological changes in bone and cartilage are also evident from histological sections. The Giemsa stained spores in these tissue sections will reveal polar capsules and lightly stained sporoplasm in the oval shaped spores (Post 1987). Histology remains the confirmatory methodology.

Plankton Centrifuge

The plankton centrifuge method consists of grinding the bone and cartilage samples in a laboratory blender. The resultant solution of tissues and bone are separated by centrifugation in a plankton centrifuge (O'Grodnick 1975). The spores imbedded in these tissues are released into the supernatant fluid. This method is used frequently to prepare infectious viable spores and when

fish are known to have moderately heavy infections. Markiw and Wolf (1980) developed a modified plankton centrifuge method which includes a partial trypsin digestion step. This method can be completed in two to three hours and increases the harvest of viable *M. cerebralis* spores.

Proteolytic Digestion with The Enzymes Pepsin and Trypsin

Pepsin and trypsin enzyme digestion is considered a sensitive and validated diagnostic procedure for *M. cerebralis.* This method was developed in 1974 by Markiw and Wolf. Heads from juvenile or small adult fish are cut in half. Large adult fish are sampled using the core method and the core is divided into two equal parts (Lorz et al. 1989). One half of the head or core is preserved in 10% buffered formalin for histological conformation of spores. The remaining half is frozen or processed immediately with the enzymes. The tissues and bone are treated for periods of time separately with pepsin and trypsin. The resulting material is microscopically examined to detect the presence of myxosporean spores. A presumptive infection is defined as the microscopic observation of spores that appear morphologically (size; 8 to 10 µm in diameter, shape; round or oval with polar capsules not greater than one half the spore diameter, and other specific characteristics, see below) similar to those of *M. cerebralis*. Two separate microscopic examinations should be conducted by two fish health specialists on each enzyme digest sample to verify the presence or absence of spores (Post 1987).

Histology

Histology is considered the most accurate method of detection. The center of the fish head or a core sample of this area is sampled and preserved in a histological fixative solution such as 10% buffered formalin. This fixed tissue and bone sample is cut in very thin sections and mounted on to microscope slides. The slides are processed and then stained with Geimsa or other stain. Spores of the dimensions and morphology that correspond to those of *M. cerebralis* and are associated with lesions in the cartilage confirm the presence of *M. cerebralis* infection in the fish (Lorz and Amandi 1994).

Spore Identification

Most diagnostic procedures for whirling disease require morphologic identification of *M. cerebralis* spores. Mature spores are mostly round, biconvex or lenticular and are 8 to 10 µm in diameter. The polar capsules are equal in size and are less than or equal to half the length of the spore. The capsules measure in length by width about 5µm x 3 µm. A mucous envelope can sometimes be seen around the posterior end of the spore, and a sporoplasm fills the rest of the

spore. Spores in fixed tissue are smaller, 6µm to 8µm in diameter, than fresh spores. Spores of myxosporeans are often confused because of similarities in morphology.

Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction is a relatively new *M. cerebralis* detection method developed by Andree, MacConnell, McDowell, Gresoviac and Hedrick (1997b). This molecular procedure can be used for detecting the presence of *M. cerebralis* DNA in fish with extremely light to heavy infections. Deoxyribonucleic acid (DNA) is extracted from enzymatically digested cartilage samples. This DNA is enzymatically amplified by the procedure to concentrations that are detectable. The amplified DNA is applied to an electrophoretic gel that allows separation based upon the molecular weight of the DNA molecules. If the DNA isolated from the sample and amplified is from *M. cerebralis*, a characteristic band will be evident on the gel. This band will represent DNA of a specific molecular weight that corresponds to DNA from *M. cerebralis*. The absence of a band of the characteristic molecular weight indicates no DNA from the parasite was detected. The PCR procedure carried out on these samples allows the detection of extremely minute quantities of the parasites' DNA. The extreme sensitivity of this method allows for the detection of very light infections, very early infection periods, and very few spores. The main drawback of the methodology is the possibility of false positive results from cross contamination of PCR samples. This lack of specificity may identify fish as being infected when, in fact, they are not infected. Validation of the procedure is underway.

In situ Hybridization

in situ hybridization is a molecular diagnostic extension of the histological procedure. Rather than staining the sample and microscopically examining the tissue, the tissue is reacted with a *M. cerebralis* specific DNA probe, which will bind to *M. cerebralis* specific DNA sequences. If the DNA probe binds, it can be visualized by radioactive tags or fluorescent methods. As with the PCR methodology, the method is very sensitive and specific, but awaits validation (Antonio et al. 1997). It also requires very accurate and fastidious technique to obtain acceptable results.

PATHOLOGY

In the last five years many aspects have been studied regarding the host-pathogen relationship of this parasite. The parasite's histozoic development causes significant damage to cartilage and induces central nervous system (CNS) symptoms by pressure on the brain and spinal cord. Two different stages of sporogony occur, one in each host. Early developmental stages in

the fish can be found multiplying in the epidermis and peripheral and central nervous systems. The presporogenic stages then migrate to vertebral and cranial cartilage where the first sporogonic phase occurs. The spores in the cartilage of infected fish become embedded in the fish's skeleton as ossification (bone deposition) occurs. Spores have also been reported in other tissues and organs, including brain, muscles, liver, and the gall bladder (Uspenskaya 1957). Mature *M. cerebralis* spores found in fish cartilage are infectious for *T. tubifex* when ingested by the oligochaete after release from the infected fish. The spores are released into the environment by decomposition of dead fish or after passing through the digestive tract of predators (Markiw, 1992c). External cysts containing *M. cerebralis* spores on the operculum of infected cutthroat trout may rupture and release spores from live fish (Taylor and Haber 1974). In the gut lumen of the worm, the spores extrude their polar capsules and attach to the gut epithelium by polar filaments. The shell valves then open along the suture line, and the sporoplasm penetrates between the gut epithelial cells. The binucleate sporoplasm multiplies by schizogony, producing many one-cell stages which begin gamogonic development. As a result of the multiplication process, the intercellular space of the epithelial cells in more than ten neighboring worm segments may become infected. The uninucleated cells begin to appear after ten to twenty five days following infection. This stage may last for a year or sporogenesis proceeds (El-Matbouli and Hoffman 1998). If sporogenesis proceeds (60-90 days post infection), pansporocysts with eight zygotes start the sporogonic phase. The final stage of this development is a pansporocyst containing eight folded triactinomyxon spores. The spores are liberated into the gut lumen. The spores reach the water either by ingestion by fish or following the death of the infected worm. Infected tubificid worms can release triactinomyxons for at least one year (El Matbouli and Hoffmann 1998).

The triactinomyxon is 15 to 20 times larger than the myxospore. It has a structure like that of a grappling hook which can attach to the fish host. The upper structure, the epispore, is about 36 µm long and contains 64 spherical sporoplasms, three polar capsules cap the structure. The style extends below the epispore for 90 µm and attaches to three tapering tail like appendages about 170 µm in length. The arms allow the TAMS to float the spore and attack the epithelium of the fish (Wolf and Markiw 1984).

Within five minutes of contact with TAMS whether by ingestion or waterborne contact, the triactinomyxon attaches to the epidermis and extrudes the polar filaments into the tissue. After being anchored to the host in this manner, the sporoplasms penetrate into the fish within ten minutes (El-Matbouli et al. 1995). The initial infection occurs in the skin, fins, gill tissues, and/or the digestive tract (Markiw 1989).

Penetration of triactinomyxon-sporoplasms of *Myxobolus cerebralis* through skin, fins, gills and buccal cavity have been demonstrated experimentally in rainbow trout. The TAMS reach the cartilage via peripheral nerves and the central nervous system (CNS). It had previously been

thought that the TAMS reached the cartilage by way of the blood, lymph, and/or coelomic fluid. During the first hour following penetration, the sporoplasm migrates between the epidermal cells. Then, it enters the epithelia and multiplies intracellularly. These stages migrate deeper into the subcutis, then through the peripheral nerves and CNS. It takes about three weeks for the parasites to reach the head cartilage. During this period of movement through the host, they also multiply (El-Matbouli et al. 1995.).

Disease Signs

The signs of whirling disease vary from inapparent to gross structural abnormalities and death. The whirling behavior is common in susceptible small fish when infected with the parasite. Rose and co-workers (1998) have suggested such behavior is the result of damage to cartilage and a granulomatous response around the organs of equilibrium. The spinal cord and axons may be damaged. Older fish show less or no such behaviors. Dark tails are common from infections and may result from pressure on nerves in the tail area which control the caudal pigment cells. Again, less darkening is seen in older infected fish. The disease has been called "black tail disease" in young fish (Post 1987). Permanent skeletal deformities of the head and the spine occur from infection caused damage to cartilage. Interference with normal development of bone structure in juvenile fish leads to these deformities. The cartilage is destroyed or damaged and normal ossification does not occur. Inflammatory response to the infection may also lead to the misshapen skulls, short opercula, misaligned jaws, and spinal curvatures. In highly susceptible young fish, death often results from infection with the parasite. It must be noted that subclinical infections and infections of older or less susceptible fish may show no signs of disease.

Histological examination of infected fish reveals the extensive damage caused by the parasite internally. Visible erosion of cartilage tissue is evident from action of the trophozoite forms of the parasite. The immune response of the fish also lead to granulomatous lesions (an inflammatory response) (Hedrick et al. 1991). The destruction of the cartilage is believed to occur from extracellular digestion of the cartilage matrix and phagocytosis of cartilage cells (Uspenskaya 1982).

Mortality from *M. cerebralis* infection is most often associated with fry or very young fish. High mortality has been reported in the literature (Hoffman 1962; Roberts and Elson 1970; Hastein 1971). Recent experimental work confirms these reports; immediate mortality was observed by some researchers (Markiw 1991), and severe deformities were found by others (Hedrick et al. 1991). Mortality among fish in culture facilities will commonly reach 100%. Mortality decreases with age, however deformed fish are incapable of feeding and have overall decreased performance. Death often occurs from secondary infections and predation. Age one

year class susceptible fish that are infected in general show no signs of disease, although the infection rate may reach 100% (Post 1987).

As with other diseases, there is a continuum of signs associated with disease. Reduced performance and abnormal behaviors are often found in infected fish. Lower growth rates among infected fish has been noted (Hoffman 1974). Increased susceptibility to other pathogens and reduced capacity to endure stresses have been observed in fish infected by whirling disease parasites (Hoffman et al. 1962; Goede 1986). Reduced fecundity and premature mortality are among other effects of infection that have been suggested to occur.

Many factors impact the outcome of infection. Environmental stresses are known to increase the susceptibility of fish to disease (Goede 1986). Specifically, higher water temperatures are known to enhance *M. cerebralis* infections. The parasite develops more rapidly and disease signs are more severe in infected fish. However as was noted above, higher temperatures also inactivate the TAMS more rapidly in the water. There appears to be a dose response to the number of TAMS to which a fish is exposed. It has been reported that as few as 10 TAMS per fish will produce 68% mortality in rainbow trout fry (Markiw 1991). The upper limit of infectious TAMS to which a fish can be infected with appears to be on the order of 10,000 to 100,000 per fish. There are significant differences in age susceptibility of fish. The older the fish, the greater the amount of ossification has occurred, thus there is less susceptible cartilage tissue for the parasite to attack (Halliday 1976). Eggs and sac fry appear to be resistant to infect for unknown reasons. Young fish remain the most vulnerable. Older fish may be less susceptible for physiological reasons, such as increased development of the immune system or changes in the epithelium which decreases the ability of the parasite to gain entrance to the host (Markiw 1992a, El-Matbouli et al. 1995).

Fish Immune Response

Fish mount an immune response to infection *M. cerebralis* as was noted first in rainbow trout (Griffin and Davis 1978). In early stages of infection, the parasite may be surrounded by round cells, and macrophages and then removed by these immune defense cells. Those parasite cells that reach the nervous tissue enter cellular areas not protected by immunocompetent cells and thus avoid destruction (El-Matbouli et al. 1995). There is also evidence of acquired immunity to the parasite. Fish once exposed to *M. cerebralis* at a level of 1000TAMS were resistant to reinfection four to eight weeks later. However fish exposed to low doses of TAMS (200 per fish) did not show such resistance (Hedrick et al. 1997).

There is some indication that long term nonspecific resistance may develop in fish populations. Juvenile fish from parents that had recruited prior to a populations' effects of whirling disease and those from parents recruited after whirling disease effects were used in field

exposure experiments. The spore loads in the juveniles from adults pre-whirling disease were greater than the other juveniles. Unfortunately the survival of the two groups was similar. This does suggest some level of genetic resistance may be present in the surviving fish (Thompson et al. 1997). This resistance may be a heritable characteristic. As is seen with sickle cell anemia, disease resistance can also impart phenotypes that have other undesirable consequences.

Therapy and Control

A variety of methods have been employed to control whirling disease especially in fish culture facilities. Chemical, physical and pharmaceutical drugs have been employed to inactivate or kill the parasite or treat exposed fish. There is no approved therapy for whirling disease (Post 1987). The disease can be reduced or eliminated under certain conditions by quarantine and restriction of movement, surveillance and destruction of infected stocks, sanitation and disinfection in artificial propogation facilities.

Physical methods and chemicals to inactivate spores that have been effective are heat, ultraviolet light, ozone, potassium hydroxide, calcium hydroxide, chlorine and Roccal. The spores are very resistant, and high levels or long exposure times with these agents are often required (Hoffman and Hoffman 1972).

Effective drug therapy for myxosporeans remains an unsolved problem. Fumagillin is the only effective drug which can be successfully applied against several myxosporeoses in the form of prolonged feeding. It has been used with good results against sphaerosporosis of common carp, hoferellosis of goldfish, myxidiosis of eel, and proliferative kidney disease, whirling disease, and ceratomyxosis of salmonids (Molnar 1993). Feeding of Fumagillin DCH salt did prevent clinical outbreak of whirling disease in experimentally infected rainbow trout fry. In 70 to 100% of nonmedicated fish severe infections occurred, while only 10 to 20% of medicated trout harbored *M. cerebralis* spores at a very low levels in experimental tests. In contrast to those from non-medicated fish, *M. cerebralis* spores from Fumagillin-treated trout had distinct morphological defects. Fumagillin seems to be an effective oral drug for the control of whirling disease in salmonid fish (El Matbouli and Hoffmann 1991b). The search for other effective chemical treatments requires rapid, sensitive, and accurate screening methods. *In vitro* staining of triactinomyxon spores with vital fluorescein diacetate correlated with the ability of the spores to infect fry of rainbow trout. This method has potential for quickly screening therapeutants intended to control myxosporean infection of fish (Markiw 1992b). However, drug therapy remains elusive.

Disease can be minimized in fish culture facilities by several management control methods such as the use of concrete raceways, early rearing of fish until they are six centimeters in length on specific pathogen free water, and the use of resistant species of fish. If the fish have been

exposed to *M. cerebralis*, they may not have clinical signs but still may be carriers in spite of these measures (Hoffman 1990).

In the natural environment, control of whirling disease is problematic. Exclusion of infected fish remains the best method to prevent the spread of whirling disease. However some potential management techniques to deal with the parasite in the environment are being considered. Rather than attacking the parasite directly, some consider attacking the alternate host. The *T. tubifex* worm may be a weak link in the life cycle of the parasite. Blocking the disease cycle at this point may be practical and have the advantage of reducing the threat to all salmonid stocks. A strain of the worm has been shown not to allow replication of the parasite. Other potential ways to stop the parasite at the worm stage are under consideration (Gustafson 1997).

There is potential for reducing the impacts in infected natural areas. If the parasite load can be reduced for very young susceptible fish, acceptable levels of fish populations may be maintained. In 1994, a good escapement of the Madison River young rainbow trout allowed a relatively good yearling class in 1995. It has been suggested that strong sediment flushing prior to emergence of young and steady or increasing flows may reduce the contact and infection with TAMS. These high waters may reduce worm populations through mortality. Lower water levels may concentrate the worms with the young fish in shallow waters. These conditions will increase the number and severity of infections. Water temperatures that favor rapid growth of the young may allow them to escape infection or show reduced pathology because the tissue damage may be less (Gustafson 1997). Of course the ability to control water flows and levels is not easily attained.

Once established in a watershed *M. cerebralis* is considered to be permanently present. Heroic efforts in Michigan and Utah failed to eradicate the parasite (Hnath 1988, Wilson et al. 1997). On the bright side, several formerly *M. cerebralis* positive locations in California have seen the parasite diminish to levels below detection. Eliminating the source of infection, which reduces the parasite numbers, may prevent the parasite from sustaining itself (Modin 1998).

SUMMARY

Whirling disease remains a serious problem for natural and artificially propagated salmonid fishes. Spread of this parasite among rearing facilities and natural watersheds is an extremely serious environmental and economic concern. Every effort should be made by fish health professionals, in conjunction with those responsible for the fisheries resources, to hold the geographic spread of this pathogen and aim to reduce its present distribution (Post 1987).

Salmonid fish are the primary or final host of *M. cerebralis* and *T. tubifex* serves as the intermediary or alternate host. In the primary host, the parasite reaches maturity. In the alternate host, the parasite passes through a larval stage, and multiplication occurs. Hosts and parasites that have long evolutionary histories in common tend to exhibit relationships that may be benign or even beneficial to each other. However, those relationships of host and parasite that are relatively recent often are detrimental to the host. This may be the case of *M. cerebralis* and rainbow trout in which the host can exhibit severe signs of disease from infection. Epizootic losses in natural fish populations from long term endemic parasite infections have been suggested to be a form of natural population control in populations that have exceeded the carrying capacity of the environment. Such losses also may be indicators that environmental conditions have degraded to favor the parasite and long term balances have been upset (Post, 1987). The losses from *M. cerebralis* in Montana may provide an example of the introduction of an exotic pathogen to a naive host population (Vincent and Byroth 1999).

The wealth of information that is presently being developed will hopefully be the groundwork of control methods and improvements in detection of the parasite. Identification of the parasitic infection by *M. cerebralis* relies on tedious methodology that may be relatively insensitive though highly accurate (histology). New methodologies, such as PCR and *in situ* hybridization, should allow for better, faster, and possibly non-lethal testing. Standardization of field and laboratory methodologies will allow better coordination and comparison of research being conducted. Increased knowledge of the intermediate host worm may allow for control methods to be devised that will be applicable to the natural environment. Greater understanding of the susceptibility of different species may lead to ways to circumvent infection in fish stocks (Anacker and Winton 1999).

Current knowledge already indicates some ways to reduce the effects of *M. cerebralis*. Worm research points to reduction or prevention of habitat degradation, because this favors *T. tubifex.* Although, it must be noted that in California and Colorado *M. cerebralis* occurred under relatively pristine conditions. Maintaining native invertebrate community diversity is important, because this will constrain the *T. tubifex* population. Reducing the organic enrichment in the watershed also inhibits the intermediate host worm. High levels of sediment also favor the worm. Often dams and hydroelectric projects simplify the insect community, encourage sedimentation and release water with high organic loads. These factors all favor the intermediate host worm of *M. cerebralis*. Similarly, irrigation ditches support large worm populations and can hold dead fish that may release spores (Executive Summary 1998 Whirling Disease Symposium).

It is believed, by further research, information can be generated upon which rational decisions for whirling disease prevention and/or control can be made. The data suggest variations in outbreaks of this disease because of a variety of biotic and abiotic factors. With time the manner *M. cerebralis* expresses itself within a watershed also changes as conditions change. There exist

three key problems fisheries managers are confronted with are the presence of the parasite, *M. cerebralis* in a watershed; the presence and signs of the disease, whirling disease; and, finally, negatively impacted fish populations. The potential options for whirling disease control are prevention (exclusion of the pathogen from the watershed), control (reducing the disease effects to acceptable levels), and eradication (elimination of the pathogen, or reducing its reproductive success to below levels of detection). Eradication remains a goal of many researchers, but the ability to detect the pathogen in all hosts and to interrupt transmission of *M. cerebralis* does not exist. Thus prevention and control methods must currently be relied on to manage the disease. Such programs require understanding the disease sufficiently to identify targets to be controlled. Effective methods must be put in place that will reduce or prevent the disease without unacceptable ecological or economic consequences. Detection of infected individuals, populations of fish, and watersheds is crucial to control. Infrastructure and methodology to support surveillance, control and prevention efforts must be present. Sufficient financial resources are necessary to begin and maintain control efforts over long periods of time and large areas (Stephens 1999).

Studies to identify ecological, management or population factors that influence the impacts of *M. cerebralis* are underway. Risk factors may be identified by these studies and used to manage the pathogen. Surveillance programs, by qualified investigators, to identify the patterns of *M. cerebralis* and whirling disease distribution and prevalence are required to characterize fish population status. Different populations will likely require different control programs. Ultimately, the control of this pathogen may only be achieved by resolution of the scientific information with the social and ecological obstacles that may arise to action. Prevention and/or control of whirling disease will require union of many approaches and jurisdictions in a common goal to maintain and protect a watershed and its salmonid fish inhabitants (Stephens 1999).

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