Environmental and molecular aspects of salmonid whirling disease
by Stacie Marie Clark

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Veterinary Molecular Biology
Montana State University

Abstract:
Whirling disease, caused by the metazoan parasite Myxobolus cerebralis, is a significant international problem for feral and hatchery-raised salmonids. Whirling disease continues to have devastating impacts on local economies and recreational activities in certain regions of Russia, British Columbia, Czechoslovakia, Ireland, Europe, New Zealand, Scotland, Denmark and in the United States. In the U. S., whirling disease has been found in 22 states. In the upper Madison River of Montana, whirling disease has been found responsible for a 90% decline in young rainbow trout. The life cycle of M. cerebralis is complex involving several developmental stages in 2 hosts, an oligochaete intermediate host and a salmonid definitive host. To date, there are still no effective means of preventing or managing the disease. One of the major tasks currently underway in Montana and other regions of the U. S. is to develop a rapid and sensitive test that can be used easily for detecting whirling disease in both oligochaete and fish hosts. In this study, a specific DNA-based polymerase chain reaction (PCR) assay was used to detect M. cerebralis DNA in both salmonid and oligochaete hosts. The assay involved isolating and purifying parasite DNA from fish and oligochaetes, using M. cerebralis specific primers, and amplifying a region of parasite rDNA sequence using a nested-PCR approach. Two rounds of PCR were used on each test sample to amplify a known region of parasite 18 S ribosomal DNA sequence. . The amplified parasite product was detected by gel electrophoresis and visualized at approximately 410 bp. Also during this study, an improved method for processing parasite DNA in a background of oligochaete or salmonid DNA was developed. The improved method involved digesting host and parasite tissue with a detergent lysis buffer and testing the resulting material,' without further purification of parasite DNA, directly in the PCR reaction,

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MONTANA STATE UNIVERSITY-BOZEMAN
Bozeman, Montana

March 1998

APPROVAL

of a thesis submitted by

Stacie Marie Clark

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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ACKNOWLEDGEMENTS

My deep gratitude is expressed to the numerous people who have assisted me throughout my studies, especially my major professor, Dr. C. A. Speer, for his support and expertise. The technical assistance of Andy Blixt, Kathy Prokop and Gayle Callis was immensely helpful. I would like to extend my appreciation to the members of my committee, Dr. Robert White and Dr. Mark Jutila, for their valuable input and time. I wish to thank Dr. Stuart Knapp for his role in the beginning stages of this project, especially for sponsoring the environmental studies and initial PCR work using the first-generation primers.

I wish to express heartfelt thanks to my family, especially my mother who taught me the incredible joys of flyfishing, Doug for all his encouragement, and to my son for inspiration and who will someday find his own path along a lazy river.

I would like to thank Drs. Ron Hedrick and Karl Andree at U.C. Davis for providing technical training and PCR primer sequences, Crystal Hudson and Linda Vanneset at the U. S. Fish and Wildlife Service, Bozeman Fish Health Laboratory, who allowed me to collaborate with them on this project. My sincere appreciation is expressed to Dick Vincent at Montana Fish Wildlife and Parks for providing the Willow Creek Study Site, and as an example of unending dedication to the battle against whirling disease. This research was sponsored in part by research grant 291560 from the Whirling Disease Foundation and the National Partnership on Management of Wild and Native and Cold Water Fisheries.

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ABSTRACT

Whirling disease, caused by the metazoan parasite *Myxobolus cerebralis*, is a significant international problem for feral and hatchery-raised salmonids. Whirling disease continues to have devastating impacts on local economies and recreational activities in certain regions of Russia, British Columbia, Czechoslovakia, Ireland, Europe, New Zealand, Scotland, Denmark and in the United States. In the U. S., whirling disease has been found in 22 states. In the upper Madison River of Montana, whirling disease has been found responsible for a 90% decline in young rainbow trout. The life cycle of *M. cerebralis* is complex involving several developmental stages in 2 hosts, an oligochaete intermediate host and a salmonid definitive host. To date, there are still no effective means of preventing or managing the disease. One of the major tasks currently underway in Montana and other regions of the U. S. is to develop a rapid and sensitive test that can be used easily for detecting whirling disease in both oligochaete and fish hosts. In this study, a specific DNA-based polymerase chain reaction (PCR) assay was used to detect *M. cerebralis* DNA in both salmonid and oligochaete hosts. The assay involved isolating and purifying parasite DNA from fish and oligochaetes, using *M. cerebralis* specific primers, and amplifying a region of parasite rDNA sequence using a nested-PCR approach. Two rounds of PCR were used on each test sample to amplify a known region of parasite 18 S ribosomal DNA sequence. The amplified parasite product was detected by gel electrophoresis and visualized at approximately 410 bp. Also during this study, an improved method for processing parasite DNA in a background of oligochaete or salmonid DNA was developed. The improved method involved digesting host and parasite tissue with a detergent lysis buffer and testing the resulting material, without further purification of parasite DNA, directly in the PCR reaction.

CHAPTER 1

INTRODUCTION

Historical Perspective

Whirling disease, caused by the metazoan parasite *Myxobolus cerebralis*, is considered a major disease of the fishes in the family Salmonidae (Markiw, 1992). Since whirling disease was first described in Germany by Hofer (1903), it has spread to over 28 countries on 5 continents (Halliday, 1976). Whirling disease was first diagnosed in the U. S. in 1958 at the Benner Spring Fish Research Station in Pennsylvania. It is speculated that *M. cerebralis* contaminated frozen trout were imported to the station from Europe (Hoffman, 1962; Margolis, 1981; Horsch, 1987; Yasutake, 1970). Subsequently, the disease spread to Nevada (1958), Connecticut (1961), Virginia (1965), California (1966), and Massachusetts (1966) (Hoffman, 1990). Since then, whirling disease has spread to 22 states, including Montana.

In December, 1994, *M. cerebralis* was first discovered in Montana's upper Madison River. There has been additional confirmation of 52 positive *M. cerebralis* Montana waters since that time (anonymous, 1997) (Table 1). Major Montana drainages contaminated with *M. cerebralis* include the Beaverhead, Madison, Jefferson and Yellowstone.

Table 1. Summary of 52 Montana waters which have tested positive for *M. cerebralis* from December 20, 1994 through July 29, 1997. (Montana Fish Wildlife and Parks, 1997).

LOCATION	DRAINAGE	SPECIES
Alder Gulch Creek	Beaverhead	BNT
Beaverhead River	Beaverhead	BNT
Big Hole River	Big Hole	RBT
Big Sheep Creek	Beaverhead	BNT
Birch Creek Reservoir	Beaverhead	RBT
Blacktail Deer Creek	Beaverhead	RBT, BKT
Blaine Spring Creek	Madison	RBT
Boulder River	Jefferson	RBT, BNT, BKT
Canyon Pond	Beaverhead	RBT, BNT
Cherry Creek	Madison	RBT
Clark Canyon res.	Beaverhead	RBT
Clark Fork River	Clark Fork	RBT
Cottonwood Creek	Blackfoot	RBT
Culver Pond	Beaverhead	BKT
E. Fork Rock Creek	Clark Fork	RBT, BKT, WCT
Flint Creek	Clark Fork	RBT, BNT, BKT
Flint Creek, N. Fork	Clark Fork	RBT, BKT
Georgetown Lake	Clark Fork	KOK
Grasshopper Cr. (upper)	Beaverhead	RBT, BNT
Hells Canyon Creed	Jefferson	RBT, BNT
Horse Creek	Madison	RBT, BNT
Horse Prairie Creek	Beaverhead	BKT
Hound Creek	Smith	BNT
Jack Creek	Madison	BNT

Table 1 Cont.

Jefferson River	Jefferson	RBT, BNT
Little Prickly Pear Cr.	Missouri	RBT
Lost Creek	Clark Fork	BNT
Madison River	Madison	RBT
Missouri River	Missouri	RBT
Moore Creek	Madison	BNT
Moose Creek	Madison	BNT
O'dell Creek	Madison	RBT, BNT
Papoose Creek	Madison	RBT
Poindexter Slough	Beaverhead	BNT
Racetrack Creek	Clark Fork	BNT, BKT
Red Rock R. (Springs)	Beaverhead	RBT, BNT
Red Rock R. Creek	Beaverhead	BKT, RBX
Rock Creek	Clark Fork	RBT, BNT, BKT, RBX
Ruby River	Beaverhead	BNT
Ruby Reservoir	Beaverhead	RBT
Soap Creek	Madison	RBT
South Boulder River	Jefferson	RBT
Squaw Creek	Madison	unknown
Stuart Mill Creek	Clark Fork	BKT
Swan River	Flathead	RBT
Warm Spring Creek	Clark Fork	BKT
West Fork Madison River	Madison	RBT, BNT
Whitehall Creek	Jefferson	BNT
Willow Creek (above Res.)	Jefferson	RBT
Willow Springs Creek	Jefferson	BNT
Wolf Creek	Madison	RBT
Yellowstone River	Yellowstone	RBT

Species Codes: **RBT**- Rainbow Trout
BNT- Brown Trout **KOK**- Kokanee
BKT- Brook Trout **RBX**- RBT x Cutthroat hybrid
WCT- Westslope Cutthroat

Economic Impacts

Whirling disease threatens some of Montana's best wild trout fisheries, and is responsible for tremendous economic and recreational losses. The economic value of sport fishing in Montana has been estimated to be $500,000,000 with sport fishing on the Madison River alone generating approximately $50,000,000 (Table 2) (R.Vincent, pers. commun., 1997). According to R. Vincent, whirling disease research coordinator for Montana Fish Wildlife and Parks, the number of angler days spent on southwest Montana streams in 1996 declined 10 to 15%. Although poor weather conditions may have been a contributing factor, Vincent (pers. commun., 1997) believes this decline can be partly attributed to whirling disease.

Table 2. Estimated economic value of selected Montana streams in 1993 (Brooks, pers. commun., 1996).

RIVER	*TOTAL ANGLER DAYS*	*TOTAL USE VALUE*
Madison	146,039	$ 47,756,238
Beaverhead	20,736	$ 4,619,394
Big Hole	63,247	$ 13,744,591
Gallatin	71,129	$ 19,944,504
Jefferson	16,865	NOT AVAILABLE
Upper Yellowstone	79,718	$ 27,728,476

Host Susceptibility

Whirling disease is a chronic disease of salmonids which may cause high mortality rates, especially in rainbow (*Oncorhynchus mykiss*) and brook (*Salvelinus fontinalis*) trout under one year of age (Hoffman, 1974; Markiw, 1991, 1992; Putz and Hoffman, 1966). Rainbow trout, cutthroat trout (*Oncorhynchus clarki*), brook trout, kokanee salmon, and chinook salmon have been shown to exhibit clinical signs of whirling disease. In contrast, brown trout (*Salmo truttta*) , lake trout (*Salvelinus namaycush*) and coho salmon appear to be relatively resistant (O' Grodnick, 1979). Other salmonids susceptible to whirling disease include golden trout (*Oncorhynchus aguabonita*) (Yasutake and Wolf, 1970) and Atlantic salmon (Hoffman, 1990).

Pathogenesis

Clinical signs of whirling disease appear at approximately 35 to 80 days after initial exposure to *M. cerebralis* spores depending on the intensity of infection and water temperature (Markiw, 1997). Earlier initial clinical signs may appear at higher water temperatures (up to 17° C) or in the presence of a higher concentration of spores. In a study by Halliday (1973), symptomatic signs of whirling disease (whirling and black tail) appeared approximately 70 days post exposure at 12° C. In contrast, fry exposed to approximately the same number of *M. cerebralis* spores at 17° C exhibited clinical signs of disease at 55 days post exposure. Additionally, a greater percentage of trout with whirling disease symptoms was observed at 17° C than at 12 °C.

The caudal peduncle and tail may become dark or even black ("blacktail") due to neural damage from lesions and deterioration of cartilaginous tissue (Fig. 2) (Elson, 1969; Halliday, 1973; Hastein, 1971; Hoffman, 1966; Roberts, 1970). When alarmed or feeding, some infected fish show an abnormal whirling behavior, hence the origin of the disease name. Since *M. cerebralis* has a selective attraction to cartilage it mainly affects young fish in which bone ossification has not progressed to maturity (Halliday, 1973; Lucky, 1970).

Although internal organs appear normal, histological sections of cartilage, particularly gill, skull, and vertebrae show areas of inflammation and deterioration (Fig. 3). Spores from *M. cerebralis* may be visible around cartilage lesions (Fig. 4). The presence of *M. cerebralis* spores in cartilage is considered pathognomonic, but subsequent confirmation by polymerase chain reaction testing is required by the U.S. Fish and Wildlife Service as part of the newly implemented National Wild Fish Health Survey (anonymous, 1997).

Figure 1. Skeletal deformities in a rainbow trout infected with *M. cerebralis*.

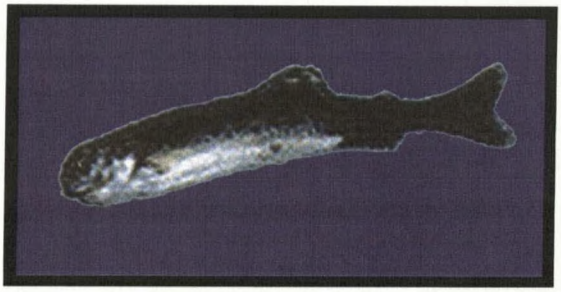


Figure 2. Darkened tail region, "blacktail", due to *M. cerebralis* infection in a rainbow trout.

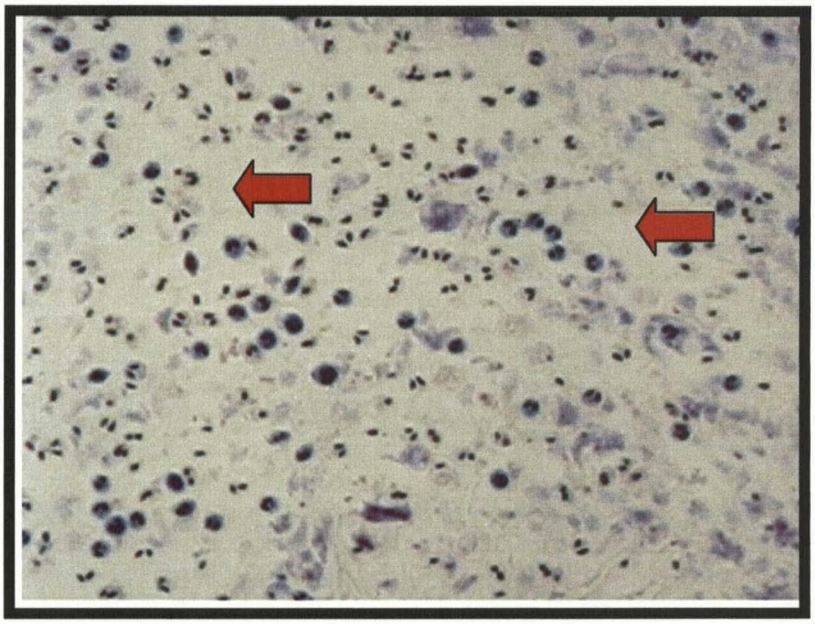


Figure 3. Photomicrograph of spores of *M. cerebralis* infected rainbow trout showing areas of cartilage deterioration (arrows). Hematoxylin and eosin stained. Courtesy of U.S. Fish and Wildlife Service. (40x).

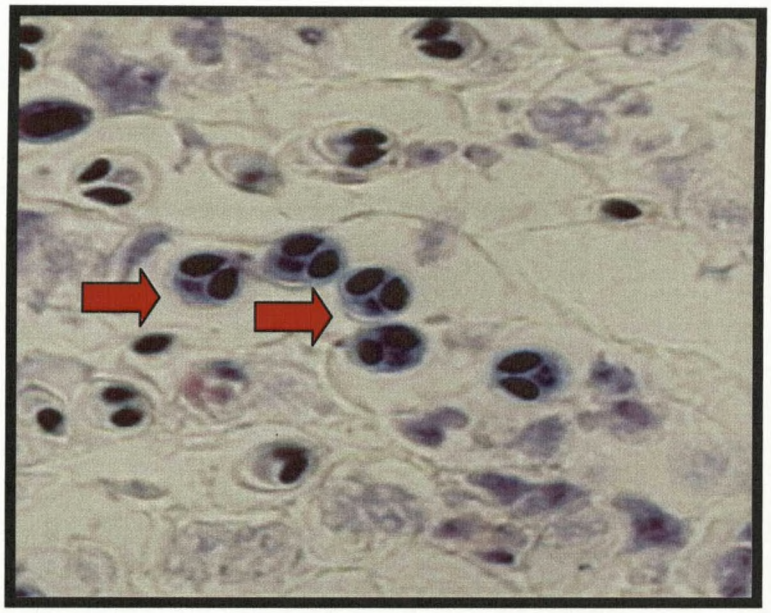


Figure 4 Photomicrograph of spores (arrows) of *M. cerebralis* in rainbow trout cartilage. Hematoxylin and eosin stained. Courtesty of U. S. Fish and Wildlife Service (100x).

Life Cycle

M. cerebralis has a complex life cycle consisting of an oligochaete intermediate host and a salmonid definitive host. The oligochaete, *Tubifex tubifex*, releases the actinosporean triactinomyxon (TAM) stage of *M. cerebralis* which in turn infects salmonid hosts and undergoes sporogenesis in cartilaginous tissues. Infection of the definitive host occurs when the waterborne TAMs come in contact with the host or *M. cerebralis* infected oligochaetes or TAMs are ingested (Wolf and Markiw, 1984) (Fig. 5).

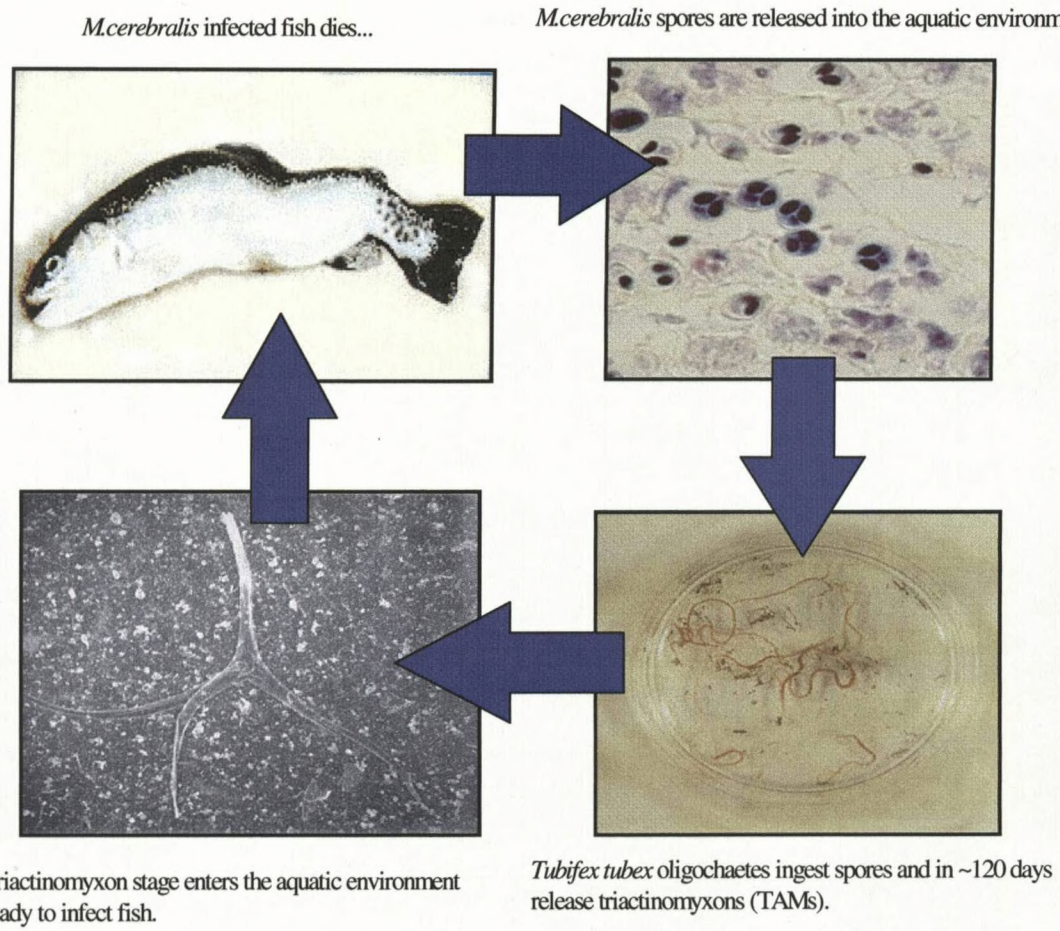


Figure 5. Life cycle diagram of whirling disease. *M. cerebralis* photomicrograph courtesy of U. S. Fish and Wildlife Service.

Initial penetration of TAMs takes place within 5 min. post-exposure. TAMs begin to attach in the epithelium of fins, skin, gills, buccal cavity, or lining of the digestive tract. After extruding their polar filaments into the epithelium, which is believed to help anchor the parasite in place, sporoplasms penetrate superficial tissues within 10 min. post exposure (El-Matbouli et al., 1995). Within the first 60 min. after penetration sporoplasm aggregates undergo mitotic development and migrate in the epidermis and gill epithelium Individual sporoplasms then penetrate an epidermal or gill epithelial cell

and undergo an endogenous cleavage to produce a secondary cell within a surrounding primary cell (El- Matbouli et al., 1995).

The large parasitic aggregate proliferates through synchronous mitosis to produce new cell-doublets that possess enveloping and inner cells (El-Matbouli et al., 1995). These cell-doublets rupture the membrane of the original primary cell and enter the host cell cytoplasm. (El-Matbouli et al., 1995; Daniels et al., 1976). The cell doublets then exit the host cell and become intercellular. At this point, the extracellular doublets either penetrate adjacent epithelial cells to start the cycle anew or migrate deeper into the dermis. After approximately 4 days post-exposure, the parasites migrate intercellularly into the central nervous system, an immunologically privileged site, while continuing the proliferative cell doublet cycle. At days 6-14 most parasites are found in the spinal cord; by day 20 cell-doublets begin to move from nervous tissue into cartilaginous tissue (El-Matbouli et al., 1995).

Once in cartilaginous tissue, the primary cell nucleus divides to form many vegetative nuclei, while the inner secondary cell divides to produce generative cells. This forms the plasmodium or trophozoite stage, which digests the surrounding cartilaginous matrix. Release and transformation of secondary cells, which produce new trophozoites up to approximately 80 days post-exposure, dissipate the plasmodium. At this time, one secondary cell may engulf another and form a pansporoblast, which will ultimately produce two mature spores (El-Matbouli et al., 1995). Completion of sporogenesis into mature *M. cerebralis* spores requires about 2.6 months at a water temperature of 12.5 ° C (Markiw, 1997).

When infected fish die, the mature spores (myxospore) of *M. cerebralis* are released into the aquatic environment either by gradual decomposition of infected fish or by being consumed by predators and scavengers, in which they may pass through the digestive tract intact (Markiw, 1992). Myxospores may settle in sediments and be ingested by *T. tubifex*. Within the oligochaete, the myxospore extrudes its polar filaments into the gut wall and the sporoplasms penetrate the gut epithelial cells and form pansporoblasts. Pansporoblasts develop into TAMs, (Fig 6) which are released from the oligochaete. The interval between ingestion of myxospores and the release of TAMs is approximately 3.5 months at 12.5 ^{o} C (El-Matbouli and Hoffman, 1989; Wolf and Markiw, 1984). Peak release of TAMS occurs within 15-60 days after TAM release has commenced, followed by a rapid decline. However, low levels of TAM release may continue for several months. For example, Markiw (1986) demonstrated that experimental fish became infected with whirling disease after existing in an aquarium with oligochaetes, which had been infected one year prior to the experiment.

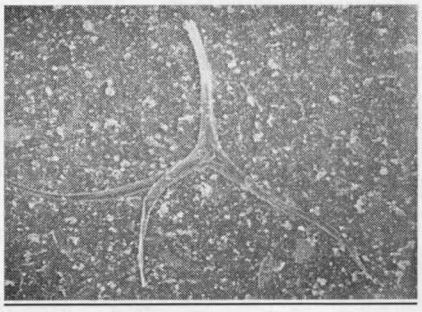


Figure 6. Scanning electron micrograph of a triactinomyxon that was released from a *M. cerebralis* infected oligochaete. (400x)

Distribution

The nearly world-wide distribution of whirling disease has been primarily attributed to the intercontinental shipping of infected fish and fish products to areas where *M. cerebralis* did not occur naturally (Halliday, 1976). In the U. S., whirling disease has been found in 22 states: Alabama, California, Colorado, Connecticut, Idaho, Maryland, Massachusetts, Michigan, Montana, Nevada, New Hampshire, New Jersey, New York, Ohio, Oregon, Pennsylvania, Utah, Virginia, Washington, West Virginia, and Wyoming (Fig 7). In Montana, 52 waterways have been identified as *M. cerebralis* positive transmission sites (anonymous, 1997).

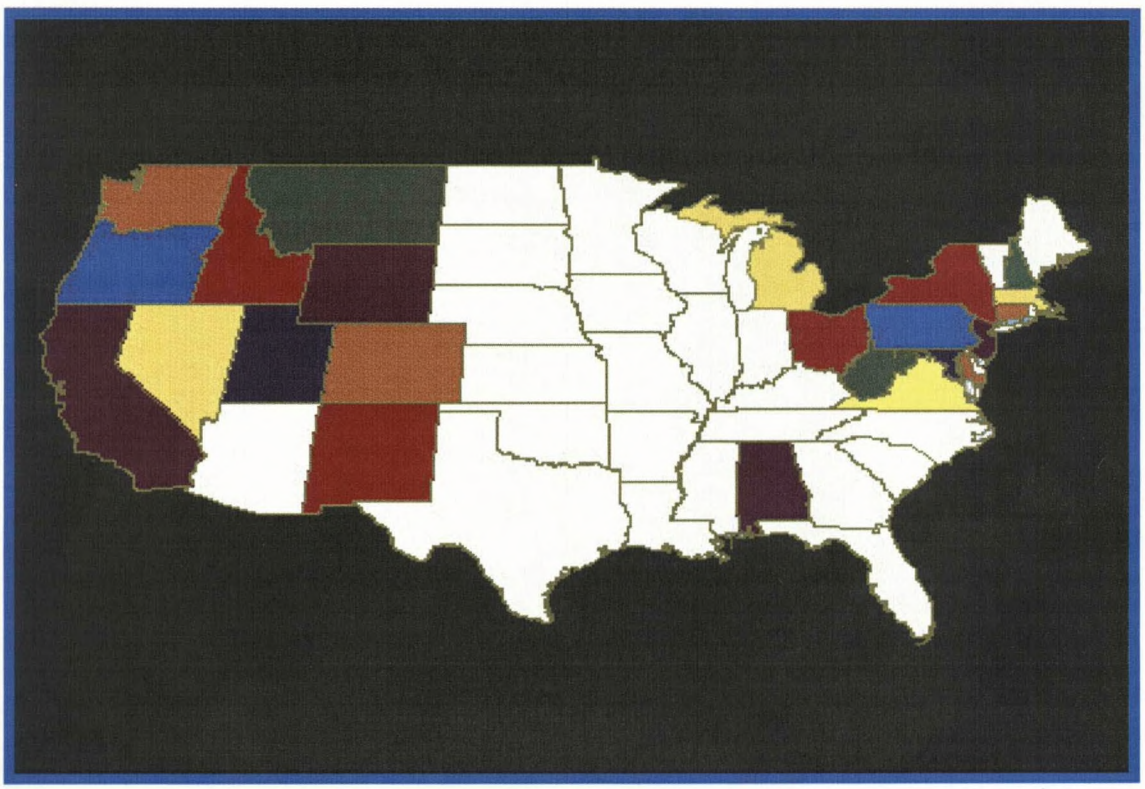


Figure 7. States in the U.S. where whirling disease has been reported.

Oligochaete Microhabitat Distribution

Since *T. tubifex* serves as an intermediate host for *M. cerebralis* (Markiw, 1984), distribution of this species is an important indicator of potential transmission sites of whirling disease. Knowledge concerning oligochaete habitat requirements is important to understanding the interactions between the intermediate and definitive hosts that contribute to disease transmission. For example, small fry were observed swimming in oligochaete collection locations in Willow Creek. Fry often seek refuge in shallow areas to avoid predation by larger fish (Allen, 1995). Oligochaetes also favor these areas of high sedimentation and low water velocity (Armitage, 1984; Brinkhurst, 1969; Della, 1955). Thus, these sites are believed to be the primary sites where salmonid fry become infected via the TAMs of *M. cerebralis*.

Oligochaetes are continual filter feeders that rapidly turnover river bottom sediment (Fisher et al., 1980; Della, 1955) through the siphon-like action of their gut mechanism. The increased rate of sediment recycling by oligochaetes exceeds the rate of sediment accumulation in the Great Lakes by an order of magnitude (Fisher et al., 1980). In this manner, sediments can be recycled through the gut of such organisms many times before complete burial in the substratum (Robbins et al., 1989). Thus, *T. tubifex* individuals may continually ingest spores of *M. cerebralis* thereby causing sustained oligochaete infections. Due to this reinfection, oligochaetes may also release TAMs over longer periods of time.

Oligochaetes have been used as pollution indicators in sediment bioassays (Milbrink, 1980; Brinkhurst, 1966; Lang, 1985; Widerholm, 1976). Oligochaetes have

been used as indicators of organic loading in Finland (Sarkka, 1994), Sweden (Wiederholm, 1987), Norway (Holtan, 1990), Canada (Reynoldson, 1994), Britain and the United States (Brinkhurst, 1965; 1969; 1974). Tubificids occur in all large rivers in Britain used by power stations for condenser cooling water (Langford, 1971) and are particularly abundant in polluted reaches (Aston, 1973).

Although *T. tubifex* is the only confirmed oligochaete host for *M. cerebralis* various additional oligochaetes are under investigation as possible intermediate hosts. The ecological requirements of several oligochaete species, including *T. tubifex*, do not seem to differ much from one part of the world to another (Milbrink, 1980). Factors affecting oligochaete abundances include food availability, sediment composition, sediment accumulation, water flow, and water quality (Lazim and Learner, 1987; Sarkka, 1994; Aston, 1973; Finogenova and Lobasheva, 1987; Sloreid, 1994; Lang 1989). The primary food source of tubificids is thought to be bacteria (Brinkhurst and Chua 1969; Monikov 1972; Mc Murtry et al., 1983) which are associated with organic portions of sediments (Dutka et al., 1974). Abundance and species composition have correlated well with levels of organic enrichment and it has been shown that oligochaetes selectively feed on organically rich sediment (Brinkhurst, 1969; Mozely and Howmiller, 1977; Howmiller and Scott, 1977, Brinkhurst and Austin, 1979). A significant positive correlation (P= 0.05) was obtained between tubificid distribution and the distribution of leaf litter (Lazim and Learner, 1987).

However, there are instances when tubificid abundances have not correlated well with the organic carbon content of sediments (Della and Groce, 1955; Plildzinskas, 1978; Mc Murty et al., 1983). This may occur if the action of sediment recycling by tubificids

dilutes food resources or if increased deposits of sediment suppresses the concentration of organic carbon (Robbins et al., 1989).

Klahr 1981 and White et al. (1987) showed that the depth of tubificid feeding (and mixing) is directly related to their abundance. Such results imply that abundance might be correlated with the organic carbon flux calculated as the product of the mass sedimentation rate and the organic carbon content of near-surface sediments (Robbins et al., 1989). Large scale surveys can be biased if sampling design is not adapted to include areas of small-scale patches of sediment accumulation (Lang, 1989). These data suggest sediment accumulations as well as organic content are important factors influencing oligochaete habitat specificity.

Tubificids have been shown to exhibit preferential response to different substrate particle size (Mc Murty, 1982; Lazim and Learner, 1987). In laboratory studies in the absence of conditioned leaves, significantly more oligochaetes were found in the slit-clay mixture than in any of the coarser substrates available. The next most commonly chosen substratum was coarse sand (600 mm- 850 mm), the coarsest material used in the study (Lazim and Learner, 1987). *T. tubifex* did not discriminate between silt-clay and coarse sand ($P > 0.05$) and more *Tubifex* were found in these substrates than in either fine or medium sand. Interestingly, observations of oligochaete behavior indicated that the oligochaetes attempted to burrow into the fine and medium sand but found penetration difficult (Reynoldson, 1994; Lang, 1989; Sarkka, 1994).

Oligochaetes respond to changes in physical environment, such as water velocity and human activity. A study by Sloried (1994) of oligochaete responses to changes in water flow caused by hydroelectric power development showed that oligochaete

abundance increased from 6,096/ m^2 in 1988 to 100,051/ m^2 in 1989 and 154,345/ m^2 in 1990 below the dam. Reduced water flow from 1988 to 1989 and 1990 caused reduced disturbance of the bottom sediments and is considered the main reason for the change in species composition and abundances (Sloreid, 1994). Additionally, human activities on water bodies such as boating, swimming and fishing, have been shown to increase numbers and biomass of oligochaetes up to hundreds of thousands of individuals per 1 m^2 (Finogenova, 1987). This fact may be explained when an increase in pollution, organic inputs or bank erosion occurs which may contribute to favorable oligochaete habitat conditions. These facts suggest that rivers which are regulated and sustain heavy recreational use, such as the Madison River, may become suitable for oligochaete colonization over a period of time. Additionally, heavily pressured rivers used for recreation in nonendemic whirling disease areas, may be at risk of setting up ideal conditions for the oligochaete intermediate host and thereby may be a contributing factor in the spread of whirling disease.

In light of the foregoing, a short study was performed on the environmental conditions along Willow Creek, a known *M. cerebralis* positive area (see appendix B). Descriptions of oligochaete site locations and sample collections were made, although this was not the principal focus of the thesis work. The main research objectives involved preliminary *M. cerebralis* polymerase chain reaction (PCR) diagnostic testing on subsets of oligochaetes collected from Willow Creek, development of an improved host DNA processing method, and PCR testing of rainbow trout and *M. cerebralis* myxospores.

CHAPTER 2

PRELIMINARY TESTING AND VALIDATION OF A POLYMERASE CHAIN REACTION ASSAY USED TO DETECT MONTANA STRAINS OF MYXOBOLUS CEREBRALIS IN NATURALLY INFECTED INTERMEDIATE HOSTS

Introduction

Wolf and Markiw (1983) were the first researchers to demonstrate in laboratory transmission studies that the *M. cerebralis* life cycle involved two separate hosts, one an aquatic oligochaete and the other a salmonid fish. They established that the salmonid host released a myxosporean (myxospore) while the oligochaete released an actinosporean. They designated the actinosporean stage as *Triactinomyxon gryrosalmo* and proved that this stage, infective only to fish, was released from the aquatic oligochaete *Tubifex tubifex* (Markiw and Wolf 1986). Furthermore, Wolf and Markiw (1984) strongly suggested that myxosporeans and actinosporeans were not separate classes in the phylum Myxozoa, but were actually alternating life cycle stages of a single organism. However, controversy remained surrounding the suggestion that the two phases of *M. cerebralis* were indeed the same organism. Further investigations by some researchers were unable to duplicate their results (Hamilton, 1987). Additional challenges came from citing the disparity between the 1,200 species of known myxozoans versus the 39 described actinosporeans. If each myxozoan had an alternating actinosporean life stage, researchers believed more actinosporeans should have been known (Lom, 1987).

To resolve this controversy, Andree et al. (1997) analyzed the 18S rRNA gene sequences of both alternate stages of *M. cerebralis*. A conserved region of the 18S rRNA gene of *M. cerebralis* spores from fish was compared to the same segment of the actinosporean gene. Sequence data indicated the two genes were 99.8% similar (Andree et al., 1997) confirming the hypothesis of Wolf and Markiw (1984) that the triactinomyxon released from the aquatic oligochaete was an alternate form of the myxozoan *M. cerebralis*. Since Willow Creek was a confirmed whirling disease transmission area, our first laboratory objective involved sorting the oligochaetes collected from several sites along Willow Creek. Our second objective required the preparation of oligochaetes for polymerase chain reaction (PCR) detection of *M. cerebralis* using the methods developed by Andree (1997), including duplicating his results under similar conditions. Our final objective involved preparing oligochaete samples for future taxonomic identification by an aquatic invertebrate specialist.

Materials and Methods

Oligochaete Processing

After oligochaetes were transported from the field site to the laboratory they were prepared for identification and DNA analysis. The first phase in processing involved sorting the oligochaetes from each individual site. A small amount (150-200 ml) of distilled water and substrate were placed in a shallow pan and oligochaetes were hand picked using fine forceps. Oligochaetes were each placed on a coverslip and dissected behind the 10th segment using an entomology "0" specimen pin or dissection needle. The coverslip and pin were discarded after each dissection to prevent cross contamination

of parasite DNA. The posterior half of the specimen was placed in a 0.6 ml microcentrifuge tube containing a DNA lysis solution (see next section) for use in PCR while the corresponding anterior half was placed in a small glass screw top identification vial containing non-buffered formalin. Depending on the available oligochaete numbers from each habitat site, the anterior ends of 20 oligochaetes and a separate batch of 10-25 oligochaetes were placed in individual glass screw-top vials filled with non-buffered formalin. Each vial contained an identification sheet with an accession number, site number, date, collector name, location, and specimen number. This method allowed for future identification of the oligochaete species that corresponded to a positive PCR parasite sample. This would thereby determine any new intermediate host species as well as confirm the role of *T. tubifex* as a host for *M. cerebralis*.

Oligochaetes from different geographic locations were maintained in laboratory cultures under similar conditions. Oligochaetes were obtained from Colorado (CO), Willow Creek (WC), MT, Lake Whitney Hatchery (LWH), CA, and the Great Lakes (GL). LWH and WC oligochaetes originate from a known *M. cerebralis* positive transmission area, GL oligochaetes from a known negative area for whirling disease, and oligochaetes from CO possessed an unknown parasite load. These oligochaetes were used as controls for the initial PCR testing.

Oligochaete DNA Preparation. Preparation of each oligochaete for use in the PCR reaction followed the procedure described by Andree et al. (1997) with the exception of using one half of an oligochaete instead of a whole one. Oligochaete DNA preparation for use in the PCR reaction consisted of digesting one half of one oligochaete

or a batch of 10-25 oligochaetes in either 100 or 500 µl, respectively, of buffer (100Mn NaCl, 10 mM Tris pH 7.6, 10 mM EDTA, 0.2% SDS, 0.2 mg/ml proteinase K) and incubating at 37° C for 16 hr. Equal volumes of phenol and chloroform were then added to the digested samples and rocked by hand for 10 min. After centrifugation for 10 min at 5220 x g in a microcentrifuge, the upper aqueous phase was removed and transferred to a clean 0.6 ml microcentrifuge tube. In the clean tube containing the aqueous phase, the same extraction procedure was performed a second time. After centrifugation for 10 min at 5220 x g in a microcentrifuge, an equal volume of isoamyl alcohol/chloroform (24:1) was added to the transferred aqueous phase. The solution was then rocked back and forth, centrifuged for 10 min at 5220 x g, and the upper aqueous phase was removed. To precipitate the DNA from the transferred aqueous phase, two equal volumes of 100% cold ethanol were added and the solution hand rocked for 10 min. After centrifugation for 10 min at 16,000 x g in a microcentrifuge, the pelleted DNA was washed once in 70% ethanol and air-dried for 15 min prior to resuspension in TE buffer (10 mM Tris pH 8.0, 1 mM EDTA). DNA concentration was determined by spectrophotometry.

PCR Testing of Oligochaetes

A nested PCR amplification method was used to amplify the target myxosporean 18S rDNA. The nested PCR procedure involved two rounds of PCR amplification. The first set of primers (Tr5-3 and Tr3-1) amplified a specific target sequence of parasite DNA which was used as a DNA template in the second round. Two µl of first round product was amplified in the second round by a second set of primers (Tr5-6 and Tr3-6) to obtain the 410 bp *M. cerebralis* diagnostic fragment. First-generation primer sequences were obtained from Dr. Ron Hedrick,

Department of Medicine and Epidemiology, School of Veterinary Medicine, University of California, Davis, CA.

First-Generation Primer Sequences:

Tr5-3 5'-CGTGAGACTGCGGACGGCTCAG-3'

Tr3-1 5'-CGGTGTGTACAAAGGGCAGGGAC-3'

Tr5-6 5'-GGCAGCGTTAAAACTGTCTCACG-3'

Tr3-6 5'-CCTCACAGTCTCTCCATGACAC-3'

In the first round, primer Tr 5-3 and primer Tr 3-1 were used. In the second round, primer Tr 5-6 and primer Tr 3-6 were used. Positive controls consisted of *M. cerebralis*-infected *T. tubifex* and rainbow trout DNA preparations obtained from U.C. Davis. Negative controls consisted of using no DNA in the reaction. Later, noninfected *M. cerebralis* fish were used for negative controls (see chapter 4). The 18S rDNA fragment was amplified under the following reaction conditions in a Gene Amp 2400 PCR apparatus (Perkin Elmer).

Total PCR reaction volume was 50 µl and contained 1X PCR buffer (10 mM Tris-HCL, pH 8.3, 50 mM KCL, 1.5 mM $MgCl_2$), 400 µM dNTP's, 40 pmol of each primer and 2 U Taq polymerase (Perkin Elmer). The first-round thermal cycle was: an initial PCR denaturation step at 95° C for 5 min, followed by 40 cycles of [95° C- 1 min., 45° C- 2 min., 72° C- 4.5 min.], followed by an extended elongation step at 72° C for 10 min. The samples were then maintained at 4° C until removed from the PCR machine. Long term storage was at -20° C. Second-round conditions were similar to the first round with the exception of the amount of starting template (2 µl of the first round product) and the second round primers were used (Tr 5-6 and Tr 3-6). Second round PCR products were

electrophoresed for 50 min at 95 volts on a 1.2 % agarose gel, stained with ethidium bromide, and DNA bands were visualized by short wavelength ultraviolet light.

Results

Initial PCR testing involved duplicating amplification of the 410 bp *M. cerebralis* fragment using the positive controls obtained from U.C. Davis. Results indicated that ~150 ng of parasite DNA was successfully amplified using the methods described by Andree et al. (1997). Band fragments of 410 bp and occasionally a 510 bp were amplified from the DNA preparation. The 510 bp band, which is occasionally seen, may be due to carry over of the first-round primer Tr 5-3 reacting with the second-round primer Tr 3-6, according to Andree (1997, pers. commun.). However, this may also be due to a false positive resulting from a cross-reaction of the first generation primers with at least one other myxozoan species (Andree, pers. commun., 1997).

Subsequent PCR testing involved oligochaetes from different geographic locations. Individual WC, LWH, and GL oligochaetes were processed using the methods described earlier. After the final precipitation of DNA with 70% ethanol, no visible DNA pellet was seen. Upon subsequent spectrophotometry very low to negative 260 OD values (-1.191 to 0.020) were obtained, indicating small amounts or no DNA present in the samples. Subsequent PCR results using these samples were negative (data not shown) except for the positive DNA control preparation.

Discussion

The 18S rDNA segments of *M. cerebralis* contained in the positive controls were amplified using the reaction conditions of Andree et al. (1997). However, I was unable to obtain similar positive results with WC or LWH oligochaetes using the DNA preparation method described earlier. The low spectrophotometer readings combined with the lack of positive PCR amplification indicate that neither *M. cerebralis* nor oligochaete DNA was present after the DNA preparation step. Possible explanations for this observation include 1) a smaller amount of initial DNA was present in our samples since only the posterior halves of individual oligochaetes were used versus whole oligochaetes used by the Davis group. Also, TAM development within the gut of the oligochaete may favor the anterior portion of the oligochaete digestive system, rather than the posterior half, which was used in PCR tests. 2) Loss of DNA may have occurred during the numerous phenol and chloroform extractions. For example, the DNA may not have been completely aspirated with the upper aqueous phase. This combined with reduced initial DNA amounts might have resulted in a cumulative loss of available parasite DNA for use in the PCR reaction. 3) DNA degradation may have occurred during preparation or storage resulting in no PCR amplification. This, however, would not explain the low spectrophotometer readings. 4) Finally, the oligochaetes may not have been infected with *M. cerebralis*. Because of the difficulties associated with recovering DNA, an improved method for DNA preparation and recovery from oligochaete samples was developed (see chapter 3).

CHAPTER 3

DEVELOPMENT OF AN IMPROVED METHOD FOR DNA PROCESSING FOR USE IN THE PCR DETECTION OF MYXOBOLUS CEREBRALIS INFECTED HOSTS

Introduction

In May 1997, a cooperative National Wild Fish Health Survey was established by the U. S. Fish and Wildlife Service (USFWS) to detect wild fish pathogens, including *M. cerebralis* (anonymous, 1997). All USFWS fish hatcheries conduct routine whirling disease testing. In 1996, an estimated 5,000 fish were evaluated for the presence of *M. cerebralis* in the 6-state central western region alone (Hudson, pers. comm., 1997). Because of the wild fish health survey and routine hatchery testing, thousands of fish in the U. S. will require PCR testing for *M. cerebralis*. Additionally, several facilities are using PCR to test feral oligochaetes for the presence of *M. cerebralis*. PCR testing of oligochaetes will likely be used to identify potential disease transmission sites as well as for early detection of whirling disease in various river systems.

In the definitive host, the current USFWS whirling disease test involves an initial pepsin/trypsin (P/T) digestion (Markiw, 1980) performed on fish heads to detect *M. cerebralis* spores microscopically followed by PCR confirmation of positive spore samples (anonymous, 1997). The process involves dissecting the fish head into two halves. One half is P/T digested and the other half is preserved and archived for microscopic examination. Because researchers have had difficulty in obtaining adequate amounts of *M. cerebralis* DNA from P/T isolated myxospores (Andree et al., 1997), the

half set aside for microscopic examination frequently had to be combined with the P/T digest half in order to have sufficient material for PCR testing. This procedure wastes time and results in the destruction of the tissues set aside for microscopic examination.

Phenol-chloroform/ethanol DNA preparation procedures have routinely been used to process fish heads for use in the PCR (Andree et al., 1997). The chemicals used in this method are expensive and hazardous, requiring special handling and disposal. The numerous phenol/chloroform extractions are also time consuming, requiring several hours. Since the amount of myxospore DNA is relatively small, there is a risk of losing the parasite DNA during the extraction process (Gaillard and Strauss, 1989). Because of these drawbacks to the phenol/chloroform method our laboratory recently modified a DNA detection procedure for use in PCR detection of *M. cerebralis* without the use of organic solvents.

Our laboratory objectives included development of a DNA lysis buffer for use on oligochaete and salmonid host tissues that releases *M. cerebralis* DNA for subsequent PCR amplification. Secondly, PCR parameters were optimized using the latest PCR primers (F_2 generation) and a new thermal profile. Lastly, a method to release the DNA from myxospores and immature trophozoites from cartilage of *M. cerebralis* positive fish heads, without the use of organic solvents, was developed. To meet these objectives and to complement the current USFWS *M. cerebralis* identification protocol, we used *M. cerebralis* myxospores isolated using the P/T digestion.

Materials and Methods

DNA Preparation

DNA preparations of oligochaete and fish tissues involve an initial tissue dissection step followed by mechanical disruption and subsequent chemical digestion of tissue. The exact DNA processing procedure varies slightly depending on the type of tissue being prepared for PCR. For example, oligochaete tissue was easier to digest than fish cartilage or myxospores.

All types of tissue digestions (oligochaete, fish and myxospore) were performed using a detergent lysis buffer (1.5 mM $MgCl_2$, 20mM Tris pH 8.0, 20 mM KCL, 0.5% Tween-20, 0.5% IGEPAL CA-630 [Sigma], 0.2 mg/ml (for oligochaetes) or 1.0 mg/ml (for fish and myxospores). Proteinase-K was added immediately prior to incubation in a 57^0 C water bath for 4 hr.

Initially, several geographic isolates of oligochaetes were PCR tested using the new lysis buffer to determine if the lysis solution would allow amplification of *M. cerebralis* DNA in infected oligochaetes. Later, PCR testing was performed on *M. cerebralis* infected rainbow trout and isolated myxospores.

Oligochaete DNA Processing. Willow Creek (WC), Lake Whitney Hatchery (LWH), and Great Lakes (GL) oligochaetes were used to test the new lysis buffer. LWH and WC oligochaetes originated from known *M. cerebralis* positive transmission sites; GL oligochaetes were from a known negative area for whirling disease. GL oligochaetes were used as a negative control and *M. cerebralis* positive LWH oligochaete DNA (processed at U.C. Davis) was used as a positive control. To determine if the new lysis

buffer would allow amplification of the target DNA sequence, negative GL oligochaetes were spiked with 1 µl purified *M. cerebralis* DNA. At this point, a short study was done to find an improved way to isolate oligochaetes from benthic substrates by using a sucrose flotation method (see appendix B). The sucrose flotation method was found to isolate specimens faster than hand picking and oligochaetes collected from WC, LWH and GL were isolated using the sucrose flotation method prior to DNA processing. The posterior half of an oligochaete was placed in a 0.6 ml microcentrifuge containing 100 µl of lysis buffer. Next, a disposable Kontes Pellet Pestle (Fischer Scientific) was used to mechanically disrupt the oligochaete and mix the digestion solution. A single addition of a 2 µl aliquot of 10 mg/ml proteinase-K was added to each microcentrifuge tube immediately before incubation at 37 ° C overnight or 50 ° C for 4 hr. The sample was then heated to 100 ° C for 10 min to inactivate the action of proteinase-K and to degrade any contaminating proteins. The sample was stored at -20 ° C and tested later by PCR.

Rainbow Trout DNA Processing. Rainbow trout heads were obtained from Leadville, Colorado, a known whirling disease infected hatchery. The heads were approximately 5 to 6 1/2 cm long and were obtained from a rainbow trout lot suspected of being infected with *M. cerebralis*. Fish heads were placed on an acrylic cutting block covered with an aluminum foil sheet. Two methods of isolating cartilage from the fish heads were used; dissection of uncooked heads or "rolling" of cooked heads. The first method involved dissecting fish heads with a disposable scalpel to isolate potential spore containing sites. The lower jaw, all soft tissue, gill filaments and skin were removed and discarded in a biohazard container. The remaining gill arches and cranial cartilage were

placed in a 50 ml polyethylene vial for use in the P/T digest followed by a second digestion using the lysis buffer or a single digestion with lysis buffer alone. The second method involved placing fish heads in sealed plastic bags and cooking them in a rocking water bath at 57^0 C for approximately 10 min to soften the fleshy tissue surrounding the cartilage which facilitated removal. Each fish head was placed on a stack of several folded paper towels. A wooden tongue depressor was used to scrape away external flesh while the underlying flesh was then pulled over the surface of the paper towel. This caused the softened tissues to adhere to the paper leaving clean defleshed bones and cartilage. All bones, gill arches and cartilage surrounding the brain were collected and placed in a 50 ml polyethylene vial. This method proved to be quicker and easier than dissection of uncooked fish heads.

These two methods for isolating fish cartilage were compared to determine if any PCR amplification differences existed between them. In the first set of experiments (sets A and B), 10 uncooked fish heads were dissected using a disposable scalpel blade and the isolated cartilage was minced into small fragments approximately 2-5 mm^3. Subsequently, the minced fragments were ground with a disposable Kontes Pestle in 1 ml tubes containing 500 µl lysis buffer until a slurry was formed. The contents then were transferred to 15 ml polyethylene vials and 4.5 ml lysis buffer were added to each vial. In order to facilitate disruption of the tough myxospore coat, and release of DNA, vials were microwaved at maximum power (700 watts) for 1 min, using short bursts and then set aside and allowed to cool. A 250 µl aliquot of 20 mg/ml of proteinase-K was added to the 5 ml samples. The samples were digested in a water bath as described below.

Set A consisted of 5 vials containing single fish heads (FH #1-FH #5) which were placed in a 57^0 C rocking water bath overnight. Set B consisted of 5 vials containing single fish heads (4H-FH #6 through 4H-FH #10) which were treated in the same manner as set A except the digestion time was shortened to 4 hr. Upon completion of digestion, all vials were heated to 95^0 C for 10 min to inactivate the proteinase-K and stored at 4^0 C until tested by PCR.

In the second set of experiments (sets C and D), the fish heads were first defleshed using the cooking and rolling method and P/T digested prior to being processed with the new lysis buffer. Since the USFWS protocol for identification of *M. cerebralis* positive lots recommends 5 fish head pools to be P/T digested, I also pooled 5 fish heads to obtain P/T isolated myxospores and subsequently processed them using the new lysis buffer digestion. This experiment determined if positive PCR results could be obtained using P/T isolated myxospores, thus eliminating the need to destroy archived heads.

Initially, all samples were cooked in a 57^0 C water bath for 10 -15 min. then P/T digested as described in the USFWS wild fish health survey (anonymous, 1997). Each fish cartilage sample was disrupted using a P/T digest blender containing approximately 30-50 ml 1X HBSS. The cartilage was blended for approximately 3 min or until fine particles (5-10 mm) were seen in the bottom of the blender. The contents were then transferred to a 50 ml polyethylene vial and centrifuged at 1875 x g for 10 min. The supernatant was aspirated and discarded, and the remaining pellet resuspended in 5 ml 70% ethanol and briefly agitated on a Vortex style mixer. At this point, the number of spores was determined for each fish sample by removing approximately 125 µl of

solution and counting on a 0.1 mm deep Reichart/Neubauer hemacytometer. The samples were then centrifuged at 1875 x g for 5 min, the ethanol supernatant was aspirated from the pellet, and the pellet microwaved as described for previous sample sets.

All samples in sets C and D were defleshed and P/T digested prior to incubation with the lysis solution. Set C contained two vials of 5 fish head pools (SP-#1 and SP-#2). SP-#1 was digested with 10 ml lysis buffer under conditions similar to set A (overnight digestion); SP-#2 was treated similar to SP-#1 except that the sample was digested for 4 hr. Set D consisted of 3 vials (SP-#3, SP-#4 and SP-#5), containing single fish heads. SP-#3 was processed similarly to SP-#1. Samples SP-#4 and SP-#5 were treated similarly as SP-#3 except they were digested for 1 hr in the lysis buffer. Set E contained 2 fish heads (CFH-#11 and CFH-#12) which were not P/T digested but were cooked for 10-15 minutes prior to being processed in the same manner as set A. After digestion was completed, all samples were heated to 95^0 C for 10 min and stored at 4^0 C until tested by PCR.

Table 3. *M. cerebralis* spore counts for selected Leadville rainbow trout samples.

Fish Sample	# of Fish	Digest Time	Spore Count/ Head
SP-#1	5	Overnight	6, 000
SP-#2	5	4 h	25, 800
SP-#3	1	4 h	15, 800
SP-#4	1	1 h	2, 500
SP-#5	1	1 h	0

Myxospore DNA Processing. Myxospores originating from the Clark Fork river (CF) were obtained from the USFWS Bozeman Fish Health laboratory (lot #7-245 1A and 1B) by the P/T digestion method. A staff technician verified positive identification of *M. cerebralis* myxospores. Myxospores were processed for PCR amplification of *M. cerebralis* DNA by either the phenol/chloroform extraction method or the new lysis buffer method.

The CF myxospore vial, containing approximately 15 ml of a 70% ethanol/myxospore solution, was agitated on a Vortex style mixer for approximately 20 sec and then divided in half with one half being placed in each of the two vials (labeled LB and CH). After centrifugation at 1875 x g for 10 min, the ethanol supernatant was removed leaving approximately a 3 ml pellet in each vial. The vials were then microwaved for 1 min, using short bursts, to avoid overheating and loss of contents. After the samples were heated, the microwave was wiped down with a 25 % Clorox solution (1.3% sodium hypochlorite) in order to avoid contamination of future samples. Approximately 5 ml lysis buffer containing 0.5 mg/ml proteinase-K was added to the LB sample which was then incubated in a Robbins Scientific model 2000 microhybridization oven at 57^0 C for 4 hr. After the samples were heated to 95^0 C for 10 min to inactivate the proteinase-K, they were stored at 4^0C.

An equal volume (3 ml) of phenol/chloroform isoamyl alcohol (25:24:1) was added to the CH sample, gently inverted by hand for 10 min, and centrifuged at 1875 x g for 10 min. The upper aqueous phase containing DNA was removed and placed in a clean 0.6 ml centrifuge tube. The aqueous phase at this point looked clean so no additional extractions were performed and the sample was again divided in half (labeled

CH#1 and CH#2). The DNA was precipitated in CH #1 by adding 1/10 volume 3 M sodium acetate pH 7.0 and 2.5 volumes cold 100% ethanol, mixing by hand for 5 min, and centrifuging at 14,000 rpm in a microcentrifuge for 10 min. The supernatant was removed and 1 ml 70% ethanol was added to wash the DNA pellet. After centrifugation at 14,000 rpm for 10 min and removal of the supernatant, the DNA pellet was air dried for 20 min. A linear polyacrylamide DNA carrier was added to CH #2, as described by Gaillard and Strauss (1990). Both samples were resuspended in 100 µl TE buffer and stored at 4^0 C.

PCR Testing

More than 150 PCR experiments were performed on isolated myxospores, oligochaetes, and fish heads. A revised nested-PCR approach was used to amplify the 18S rDNA parasite target sequence. A new thermal profile and second-generation primer sequences were obtained from Dr. Ron Hedrick, Department of Medicine and Epidemiology, School of Veterinary Medicine, University of California, Davis, CA.

Second-Generation Primer Sequences:

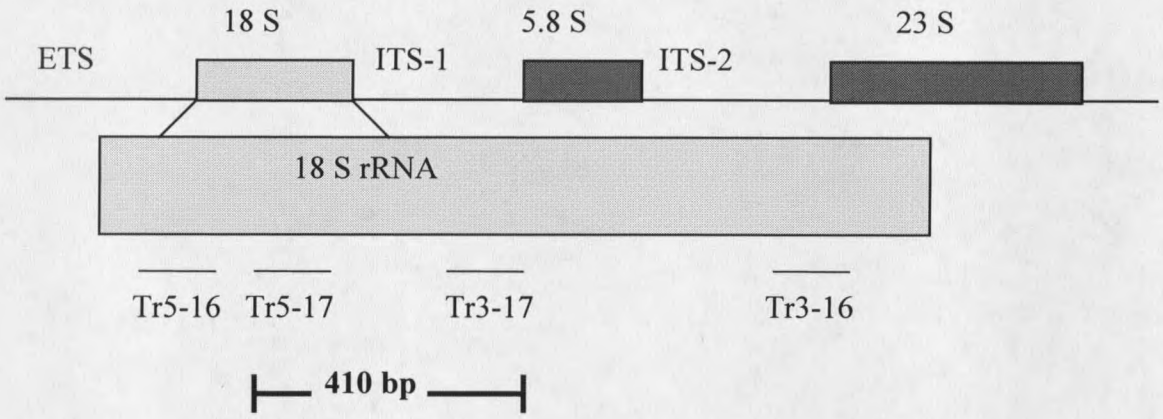
Tr 3-16 5'-GAATCGCCGAAACAATCATCGAGCTA-3'

Tr 5-16 5'-GCATTGGTTTACGCTGATGTAGCGA-3'

Tr 3-17 5'-GGCACACTACTCCAACACTGAATTTG-3'

Tr 5-17 5'-GCCCTATTAACTAGTTGGTAGTATAGAAGC-3'

In the first round, primers Tr 3-16 and Tr 5-16 were used. In the second round, primers Tr 3-17 and Tr 5-17 were used. The primers are oriented along the gene encoding for the 18S rRNA as follows:



An updated thermal cycle profile was also used which had been optimized for use with the second-generation primers. The first round thermal cycle consisted of an initial PCR denaturation step at 94° C for 5 min., followed by 34 cycles of [94° C- 1 min., 65° C- 2 min., 72° C- 2 min.], followed by an extended elongation step at 72° C for 5 min. Samples were then stored at 4° C. Second round conditions were similar to the first round, except for the use of 2 µl of the first round product as a starting template and the use of second round primers (Tr 3-17 and Tr 5-17). Ten µl of second round PCR products were electrophoresed for 50 min at 95 volts on a 1.2 % agarose gel, stained with ethidium bromide, and DNA bands visualized under short wavelength ultraviolet light.

Oligochaete PCR Testing. Oligochaete PCR reaction conditions and thermal cycle profiles were similar to those described previously (chapter 2), except that they were digested with the new lysis buffer. Seven µl of digested sample were used directly

as a DNA template in the first round of amplification, without further purification with organic solvents.

M. cerebralis Myxospore and Fish PCR Testing. PCR testing was performed at the USFWS Bozeman Fish Health Laboratory using rainbow trout from the Leadville Hatchery or myxospores from the Clark Fork river. After digestion with the new lysis buffer as described previously, PCR testing of *M. cerebralis* myxospores and *M. cerebralis*-infected fish was completed using the updated thermal profile and second-generation primers. The PCR reaction conditions were similar to the oligochaete parameters, except that Fischer Biotech Taq polymerase and Fischer Biotech 1X PCR Buffer A (containing 10 mM Tris-HCL pH 8.3, 50 mM KCL, 1.5 mM $MgCl_2$) were used.

Results

Oligochaete PCR Results

WC, LWH, and GL oligochaetes were PCR tested using the new lysis buffer digestion method as described earlier (Fig.8). *M. cerebralis* oligochaete preparations from LWH amplified a 410 bp diagnostic fragment (lanes 5, 8). Negative GL oligochaete controls did not amplify any DNA fragment (lanes 3, 6 and 9). GL oligochaetes, processed using the new lysis buffer, to which 1 µl of *M. cerebralis* DNA was added, showed amplification of a 410 bp diagnostic fragment (lanes 10 and 12). WC oligochaetes (lanes 4, 7 and 11) were positive for *M. cerebralis* as indicated by the 410 bp amplified fragment. , the new lysis buffer solution allows amplification of *M. cerebralis* DNA in a background of oligochaete DNA.

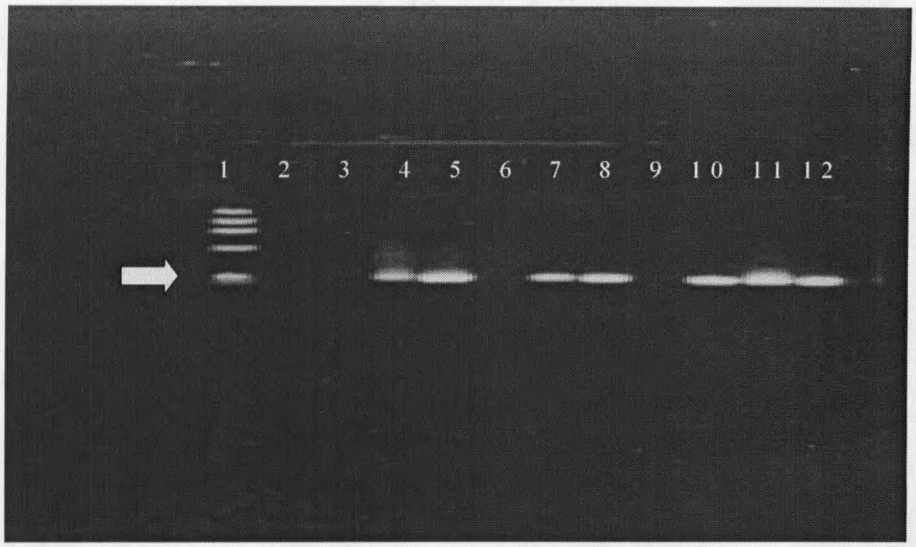


Figure 8. PCR products visualized on an ethidium bromide stained 0.8% agarose gel. Lane 1 contains molecular weight markers (arrow = 300 bp); lane 2 is vacant; lanes 3, 6, and 9 contain negative control GL oligochaetes; lanes 4, 7 and 11 contain positive *M. cerebralis* infected WC oligochaetes; lanes 5 and 8 contain positive *M. cerebralis* DNA preparations of LWH oligochaetes; lanes 10 and 12 contain GL oligochaetes with 1 µl of *M. cerebralis* DNA added to the lysis solution.

M. cerebralis Myxospore PCR Results

CF *M. cerebralis* myxospores, isolated by P/T digestion, were PCR tested using the new lysis buffer method described previously (Fig. 9). Lanes 1 and 11 are molecular weight markers; lane 2 contains a 410 bp amplified fragment of *M. cerebralis* myxospores digested with the new lysis buffer; lanes 3, 6, and 9 contain *M. cerebralis* prepared DNA positive controls; lanes 4, 7 and 10 contain *M. cerebralis* noninfected rainbow trout negative controls; lane 5 shows a faint 500 bp band amplified using 7 µl of phenol/chloroform purified myxospores with a linear polyacrylamide DNA carrier; lane 8 shows no amplification of myxospores using 7 µl of phenol/chloroform purified DNA alone.

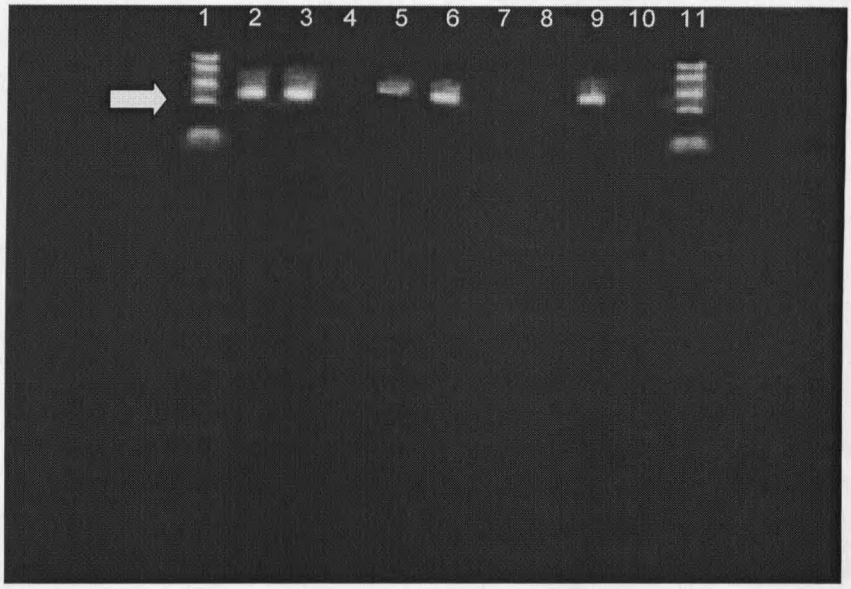


Figure 9. PCR products visualized on an ethidium bromide stained 1.2% agarose gel. Lanes 1 and 11 contain molecular weight markers (arrow = 300bp). Lane 2 contains *M. cerebralis* myxospore DNA digested with the new lysis buffer. Lanes 3, 6, and 9 contain positive *M. cerebralis* controls. Lanes 4, 7, and 10 contain negative controls, Lane 5 contains *M. cerebralis* myxospore DNA processed using a phenol/chloroform extraction and precipitated using a linear polyacrylamide carrier. Lane 8 is negative for PCR amplification of phenol/chloroform extracted *M. cerebralis* myxospore DNA.

PCR Results of Fish Experiments

Set A-E fish heads from Leadville, CO were PCR tested as described in the materials and methods (Fig. 16). In the top row, lanes 1 and 16 are molecular weight markers; lanes 2, 3, 11 and 12 are noninfected rainbow trout negative controls; lanes 4 and 5 contain 410 bp amplified *M. cerebralis* fragments from set C (SP#1 and SP#2, respectively); lane 6 contains a 410 bp amplified *M. cerebralis* fragment from set D (SP#3); lanes 7 and 8 show no amplification of a *M. cerebralis* fragment from set D (SP#4 and SP#5, respectively); lane 9 contains a 410 bp amplified *M. cerebralis* fragment from set E (CFH #11); lane 10 is a *M. cerebralis* prepared DNA positive control; lanes

13, 14 and 15 show faint 410 bp amplified *M. cerebralis* bands (blue arrows) from set A (FH#1, FH#2 and FH#3, respectively). In the bottom row, lanes 1 and 14 contain molecular weight markers; lanes 2, 3, 6, 7 and 8 are noninfected rainbow trout negative controls; lanes 4 and 5 show no amplification of a *M. cerebralis* fragment from set A (FH#4 and FH#5, respectively); lanes 9, 10, 11, 12 and 13 show no amplification of a *M. cerebralis* fragment from set B (4H-FH#6 to 4H-FH#10, respectively).

When processing fish heads, the cooking and rolling method resulted in the least amount of residual fish tissue that adhered to bones and cartilage. This reduced the amount of background fish DNA, which appeared to enhance PCR amplification of myxospore DNA (Fig 10). Samples CF#11 and CF#12, which were processed using the cooking and rolling method followed by lysis buffer digestion, produced strong amplification of a *M. cerebralis* fragment

The *M. cerebralis* DNA in infected oligochaete positive controls was successfully amplified using the new lysis buffer solution. Additionally, oligochaetes treated with the new lysis buffer that had *M. cerebralis* DNA added to the solution amplified a 410 bp diagnostic band. These results indicate that *M. cerebralis* DNA may be amplified in a background of oligochaete DNA without interference, under the processing conditions described earlier.

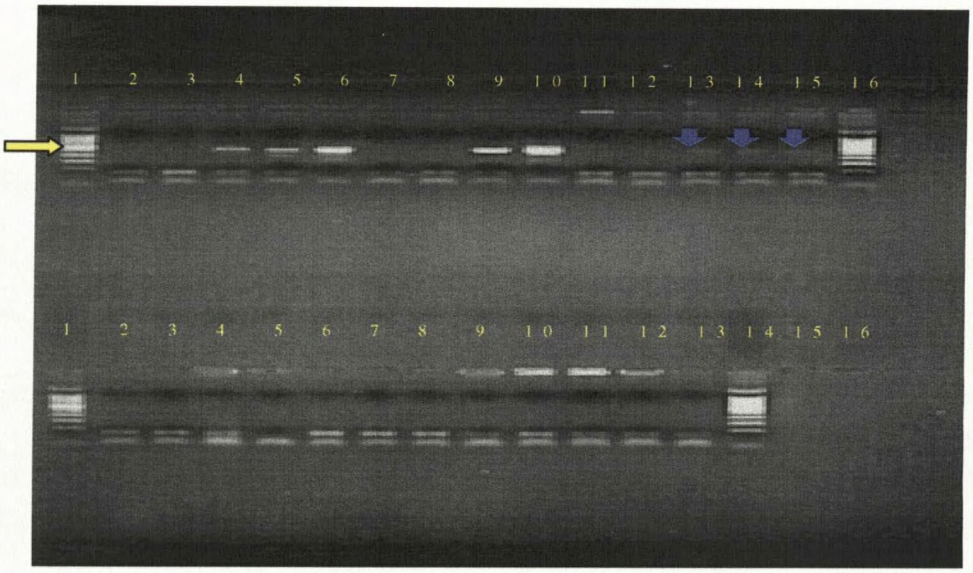


Figure 10. PCR products visualized on an ethidium bromide stained 1.2% agarose gel. **Top row**: lanes 1 and 16 contain molecular weight markers (arrow=400 bp); lanes 2, 3, 11 and 12 are negative controls; lanes 4 and 5 contain positive *M. cerebralis* samples from set C (SP#1 and SP#2, respectively); lane 6 contains a 410 bp *M. cerebralis* fragment from set D (SP#3); lanes 7 and 8 show no amplification of a *M. cerebralis* fragment from set D (SP#4 and SP#5, respectively); lane 9 contains a positive *M. cerebralis* fragment from set E (CFH #11); lane 10 is a positive *M. cerebralis* control, lanes 13, 14 and 15 contain faint 410 bp *M. cerebralis* bands (blue arrows) from set A (FH#1, FH#2, and FH#3, respectively). **Bottom row**: lanes 1 and 14 contain molecular weight markers; lanes 2, 3, 6, 7, and 8 contain negative controls; lanes 4 and 5 show no amplification of *M. cerebralis* fragments from set A (FH#4 and FH#5, respectively); lanes 9, 10, 11, 12 and 13 show no amplification of *M. cerebralis* fragments from set B (4H-FH#6 to 4H-FH#10, respectively)

Discussion

Previously, several researchers have successfully used a lysis detergent DNA digestion procedure for PCR detection of unpurified DNA in transgenic mice (Hanley and Merlie, 1991; Higuchi, 1989; Longmire, et al., 1987). In the present study, modification of this DNA digestion method was applied successfully to the PCR amplification of *M. cerebralis* DNA in both fish and oligochaete hosts. There are several advantages to using this technique. For example, processing time is reduced substantially, typically only 4 hr

this technique. For example, processing time is reduced substantially, typically only 4 hr (instead of 12 hr) of digestion time are needed before the DNA is ready for molecular analysis. Also, no hazardous organic solvents or extractions are used in preparing the tissue, which also minimizes the loss of DNA. In fact, except for the ethanol/polyacrylamide precipitation step used in fish, the whole digestion procedure is carried out in one vial which also reduces the chance of cross-contamination of DNA between samples.

The P/T digestion of cartilage acts as a mechanical tool to disrupt the cartilage, which releases myxospores for microscopic identification, but does little to increase the successful PCR amplification of a sample. In fact, due to the numerous supernatant extractions performed during this procedure, *M. cerebralis* DNA from immature trophozoites or disrupted myxospores is lost. P/T digestion is not necessary and, in fact, may be detrimental when confirming positive *M. cerebralis* microscopic samples with PCR. Thus, the lysis solution appears superior to the pepsin/trypsin solution when performing the mechanical disruption step. In addition, the use of a small microblender would ensure the DNA does not become too dilute for successful PCR amplification. The lysis solution method has the advantage that it may be microscopically examined for intact myxospores and may be used directly in the PCR reaction. This would eliminate the loss of *M. cerebralis* DNA, save time and resources.

We included a linear polyacrylamide DNA carrier in the ethanol DNA precipitation step to increase the amount of myxospore DNA recovered by the phenol/chloroform extraction process. The addition of a linear polyacrylamide DNA carrier has been shown to precipitate picogram amounts of DNA when normally the DNA

would be completely lost (Gaillard and Strauss, 1990). Addition of the linear polyacrylamide appeared to improve the recovery of small amounts of myxospore DNA. The use of linear polyacrylamide may be an option for researchers to use after initial digestion with the lysis buffer in fish with light myxospore loads to improve *M. cerebralis* DNA recovery. This may have been helpful in obtaining positive *M. cerebralis* amplification of sample SP#4, which had a light spore load of 2,500 yet did not amplify any DNA fragments (Fig. 10). However, this sample was digested only for 1 hr and may have needed more digestion time.

Based on the findings from Fig. 10, it appears that prior cooking of fish heads and an overnight or 4 hr digestion time using the new lysis buffer protocol is able to produce successful amplification of myxospore DNA (lanes 6 and 9). Also, the new lysis buffer protocol succeeded in amplifying myxospore DNA when used following the P/T digest (lanes 4, 5). It appears the removal of fleshy tissue residues from the cartilage is important for enhanced PCR amplification as demonstrated by the negative results of suspected *M. cerebralis* positive fish heads which were not defleshed prior to PCR testing (set A and B). Finally, this method is relatively inexpensive and easy to perform and provides the first successful protocol that may be used directly from the P/T digest of *M. cerebralis* spores. I propose that the USFWS consider replacing the P/T digest with the lysis buffer solution to minimize loss of *M. cerebralis* DNA for PCR confirmation.

CHAPTER 4

SUMMARY

Although whirling disease was first described in 1903 by Hofer, today's researchers have an incomplete understanding of the environmental parameters involved in disease transmission. Currently, new methods are being developed for the detection and diagnosis of Whirling Disease in definitive as well as intermediate hosts. To gain further understanding in both these areas, some of my research focused on describing intermediate host locations along Willow Creek, a known positive Whirling Disease area, and developing an improved DNA processing technique for use in PCR detection of Whirling Disease.

The Willow Creek study area provided several examples of oligochaete colony locations comprised of varied microhabitats. My observations on oligochaete colony locations and environmental preferences showed a dynamic relationship between colony location, numbers of oligochaetes present, and the snow-melt phenomenon that occurs in Willow Creek. Oligochaete colonies and increased sediment deposition appeared in the delta area after runoff. No oligochaete colonies had been detected in the delta area prior to runoff. In addition, upstream colonies were displaced or significantly reduced in numbers during runoff. Interestingly, as water velocities declined, colony numbers showed an increase in most locations. This may be attributed to upstream displacements multiplying or hatching of buried oligochaete cocoons.

Positive oligochaete colonies were observed in areas that consisted of black silt mixed with fine organic debris or a sandy/silt mixture. Often, positive locations appeared to be protected from high water scouring. These areas, including eddy formations, oxbows and downstream locations along bars, also showed depositions of organic matter.

These observations suggest similar colony dynamics may occur in rivers that are snow-melt fed similar to Willow Creek. Thus, several samples should be taken from each site at different times of the year (i.e., Fall and early Summer) to obtain realistic oligochaete numbers.

The second part of my research focused on testing and improving a PCR diagnostic method for use in detecting Montana strains of *Myxobolus cerebralis*. Drs. R. Hedrick and K. Andree originally developed the PCR test at U.C. Davis, California. Willow Creek oligochaetes were tested for the presence of *M. cerebralis* using first-generation primers, obtained from Dr. Hedrick, and successful amplification of the *M. cerebralis* 18S rDNA occurred in several of the Willow Creek oligochaetes. Positive controls consisted of *M. cerebralis*-infected DNA preparations obtained form U. C. Davis. Noninfected *M. cerebralis* rainbow trout, or oligochaetes, were used as negative controls. An amplified 410 bp fragment indicated the presence of *M. cerebralis*. However, according to Andree (pers. commun., 1996), the first generation primers were known to cross-react with at least one other species of myxozoan; therefore, a second-generation set of more specific primers was developed and provided by Andree for use in subsequent PCR testing.

However, when the second-generation primers and a revised nested-PCR profile were tested on known *M. cerebralis* infected oligochaetes using the DNA preparation method described by Andree (1997) no amplification was seen. Spectrophotometer readings suggested inadequate DNA amounts for amplification were present in the half-oligochaete samples. Because of this, a modified DNA preparation procedure was developed that eliminated organic extractions and potential DNA loss. This new method resulted in successful amplification the 410 bp fragment of *M. cerebralis* in infected oligochaetes.

Final PCR testing involved using the second-generation primers, new PCR profile and modified DNA preparation procedure on potentially infected oligochaetes and rainbow trout from known whirling disease transmission areas. After mechanical disruption, a detergent lysis solution was used to process the host tissue for DNA release and subsequent amplification. This lysis buffer consisted of a high magnesium solution, which complemented the PCR reaction buffer and did not interfere with PCR amplification. In addition, no extractions or organic solvents were used which may have interfered with PCR amplification. In conclusion, the lysis solution was used successfully to prepare materials from both fish and oligochaete tissues for the amplification of the *M. cerebralis* 18S rDNA diagnostic fragment.

APPENDICES

APPENDIX A

USE OF A SUCROSE OR PERCOLL® FLOTATION METHOD TO ISOLATE OLIGOCHAETES FROM BENTHIC SUBSTRATES

Introduction

Tubifex tubifex is often found in various mixed benthic substrates, including fine organic sediments, sand, silt and fine organic sediments (Brinkhurst, 1966; Della, 1955; Lazim, 1987). Since *T. tubifex* may be found in murky areas containing moss and leaf debris, (Robbins, 1989; Lazim, 1987) isolation and enumeration of individual oligochaetes from the soil is often a tedious and labor intensive process. Hand sorting using forceps, a method used to collect oligochaetes from soil, is often unable to accurately determine the number pf organisms present in the substrate sample. Immature oligochaetes are small and difficult to pick up using forceps and adults may not be detected at all if hidden or trapped in fine leafy debris.

To solve these difficulties, a sucrose or Percoll ® density flotation technique was used to separate oligochaetes from different substrate types commonly found in stream beds. This modified isolation technique provided a time saving method to isolate and enumerate oligochaetes from various oligochaete habitat substrates (Jenderekjian, 1994; Johnson, 1993).

Materials and Methods

Oligochaete Isolation

Approximately 45 ml of a mixture containing sediment and oligochaetes, was dispersed into a 250 ml glass beaker containing 100 ml of an aqueous 30% sucrose or 30% Percoll (Sigma Chemical) solution. The substrate/solution was gently stirred with a polyethylene bulb pipette or tongue depressor. Within approximately 20 seconds, oligochaetes floated to the top while the sediment and debris sank to the bottom. Oligochaetes were collected by aspiration using a polyethylene bulb pipette. For optimal survivability, oligochaetes were collected from the flotation solution within 5 min and immediately placed in a beaker containing approximately 100 ml distilled water. A second gentle agitation of the flotation solution was performed and any remaining oligochaetes were collected as described above. The distilled water was changed in the collection beaker after all oligochaetes had been added to eliminate any remaining sucrose or Percoll, which prevented osmotic rupture of oligochaetes (Fig. 11)

Figure 11. Photograph demonstrating oligochaetes floating in a 30% sucrose solution (left)and a collection beaker filled with distilled water containing oligochaetes (right).

An experiment using the floatation method described above was employed to determine the percent of oligochaete recovery with two common substrate types. Two river bottom soil types, sandy and fine silt mixed with organic debris, were used as model substrates. Both sucrose and Percoll flotation solutions (30%) were tested, however, no significant differences were observed using either solution (data not shown). Therefore, given that sucrose is much less expensive than Percoll, the experiment used a 30% sucrose flotation solution exclusively.

Two separate oligochaete groups were placed in each substrate type to compare recovery rates. The first group consisted of 20 randomly selected oligochaetes; the second, 100 randomly selected oligochaetes. Three soil volume amounts, 10, 15, and 20 ml, of each soil type were used with both oligochaete groups. Average recovery percentages were calculated for each experiment.

In experiment A, 20 oligochaetes were placed sequentially in three vials containing 10, 15 and 20 ml of fine organic soil. Experiment B was similar to experiment A, except sandy soil was used as a test substrate. In experiment C, 100 oligochaetes were added to three vials sequentially containing 10, 15 and 20 ml of fine organic soil. Experiment D was similar to experiment C, except sandy soil was usedAfter adding distilled water to bring the total volume up to ~ 45 ml in each vial, the vials were treated as previously described for mixing and collection.

Results

Recovery rates were similar for each experiment (Fig. 12). However, there were slightly fewer oligochaetes recovered when the ratio of oligochaetes to flotation solution exceeded 1:3. In Fig. 12, note the decline in recovery of oligochaetes in all experiment sets when using 20 ml of substrate to 45 ml of flotation solution. Regardless of the amount of oligochaetes used (20 or 100), there was a slight decline in the total numbers of oligochaetes recovered (represented by the black bars).

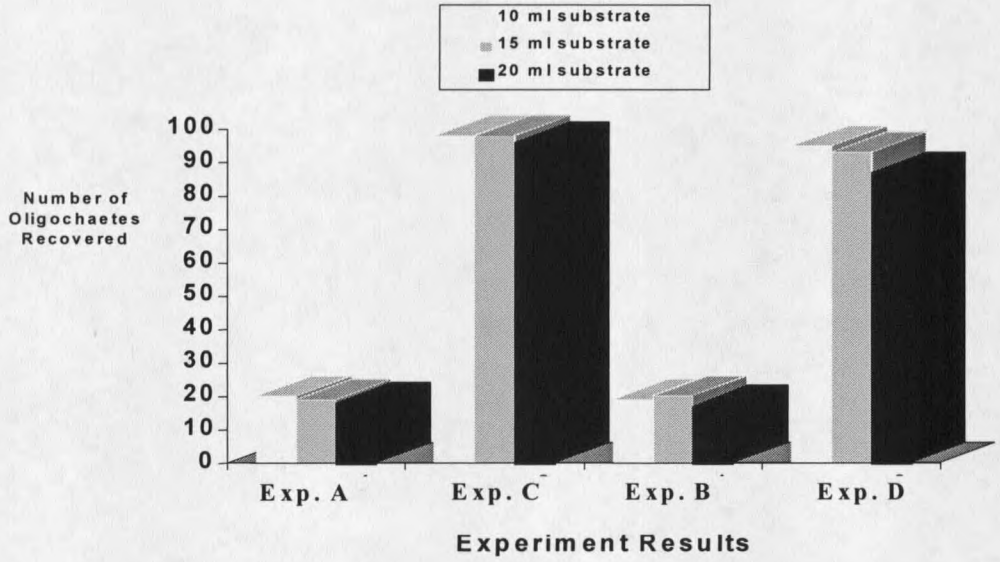


Figure 12. Recovery rates for experiments A, B, C, and D are shown on the graph. The white, gray, and black bars represent 10, 15 and 20 ml of substrate, respectively. Experiments A and B used 20 oligochaetes; experiments C and D used 100 oligochaetes.

In experiments A and B, mean recovery rates of 95.0% and 86.6% were obtained for 20 oligochaetes in organic and sandy soil, respectively. In experiment C and D, mean recovery rates of 95.6% and 91.0% were obtained for 100 oligochaetes in organic and sandy soil, respectively.

Discussion

The sucrose or Percoll flotation method showed that oligochaetes (including immature oligochaetes) can be quickly and easily isolated and purified as compared to hand picking of oligochaetes with forceps. Occasionally, when found in organic debris, adult oligochaetes may get trapped within leafy material as it sinks, thereby making flotation to the top difficult. Oligochaetes that get caught on the bottom of the beaker in such material can be displaced from entangling debris by gently stirring with a bulb pipette or tongue depressor. Once freed from heavier debris, they float to the top and are easily aspirated and collected. This flotation method gave similar results with different substrates, but caution should be used to avoid using too much substrate with inadequate volumes of flotation solution since the oligochaetes may become trapped in the sediment. Regardless of the substrate type, the substrate mixture should be agitated at least twice to completely loosen the oligochaetes from the heavier substrate material. The solution/substrate mixture may be sieved and the flotation solution recycled.

This flotation method can be used successfully with various substrates and eliminates the tedious process of hand sorting individual oligochaetes. As an added benefit, the sucrose flotation method has been shown not to interfere with PCR testing. Therefore, after the oligochaetes are isolated using this method, they are ready for DNA digestion and subsequent PCR analysis.

APPENDIX B

OLIGOCHAETE COMMUNITY DISTRIBUTION ALONG WILLOW CREEK, A KNOWN WHIRLING DISEASE POSITIVE TRANSMISSION AREA

Introduction

Willow Creek is a meandering snow-melt fed stream that contains De Smet rainbow (*Oncorynchus mykiss*) and brown trout (*Salmo trutta*). It has been confirmed positive for whirling disease by the Montana Fish, Wildlife and Parks Department (MFWP) and is located in Madison County near Harrison, Montana. In conjunction with an on-site MFWP whirling disease seasonality and fish exposure experiment, we undertook a study of oligochaete distribution and potential disease transmission factors along Willow Creek. Our goal was to provide information regarding oligochaete distribution and infectivity rates over a period of time along Willow Creek, which would be subsequently compared to the MFWP experiment findings. This included collecting and testing oligochaetes for *M. cerebralis* using PCR (see chapter 2). The results may provide a better understanding of whirling disease transmission dynamics between the intermediate and definitive host, including seasonality and transmission factors.

The Willow Creek study site is located above Willow Creek Reservoir in an agriculturally rich area. Crop and livestock productions contribute organic inputs into the water system. Flow rates fluctuate depending on the time of year, with average flow <100 cubic feet per second (cfs) and peak rates up to 500 cfs during runoff (USGS Willow Creek gauging station, 1996). It is a small stream which has a drainage of 83.80 square miles and lies at 45^0 43.358' N longitude by 111^0 44.387' W latitude. The study site is

approximately 48 miles from Marsh Lab, Bozeman, Montana, and is approximately 4750 feet in elevation (USGS, 1996). Substrates in the stream consist of a mixture of coarse rocks, sandy point bars, and varied organic and mud depositions. A mixture of these substrate types were found in various locations and recorded per site visit (see table 4).

Materials and Methods

Willow Creek was examined for potential oligochaete colony sites based on substrate type, amount of sediment deposition, water flow, and preliminary oligochaete test samples. A total of 33 sites were located along Willow Creek, a known whirling disease positive transmission area (Fig.18). Physical descriptions of substrate type, area of collection site and abundance of oligochaetes over time were recorded for each sampling site. On-site locations were labeled with numbered colored flags for future identification. Oligochaete collections were taken from each site on a periodic basis from February to September 1996.

Two different methods were used for benthic sampling of oligochaetes. The first was a kicknet, which was dragged along the river bottom of a designated area. This method proved difficult during periods of high water, since the increased water velocity made it difficult to control the kicknet. Quantitative estimates of how much soil was actually being sampled and the concentration of oligochaetes in a given area also proved difficult to determine using this method. Therefore, two individual samples were taken from each site location exclusively using an Eckman dredge benthic sampler beginning on May 9, 1996. The Eckman is a spring-loaded 12 x 12 inch bottom sampler that

approximately the top 2-20 cm of substratum depending on the substrate type (sand, soft silt, or gravel). Positive oligochaete sites were sampled on a regular basis throughout the research period to study seasonality and temporal distribution factors of transmission.

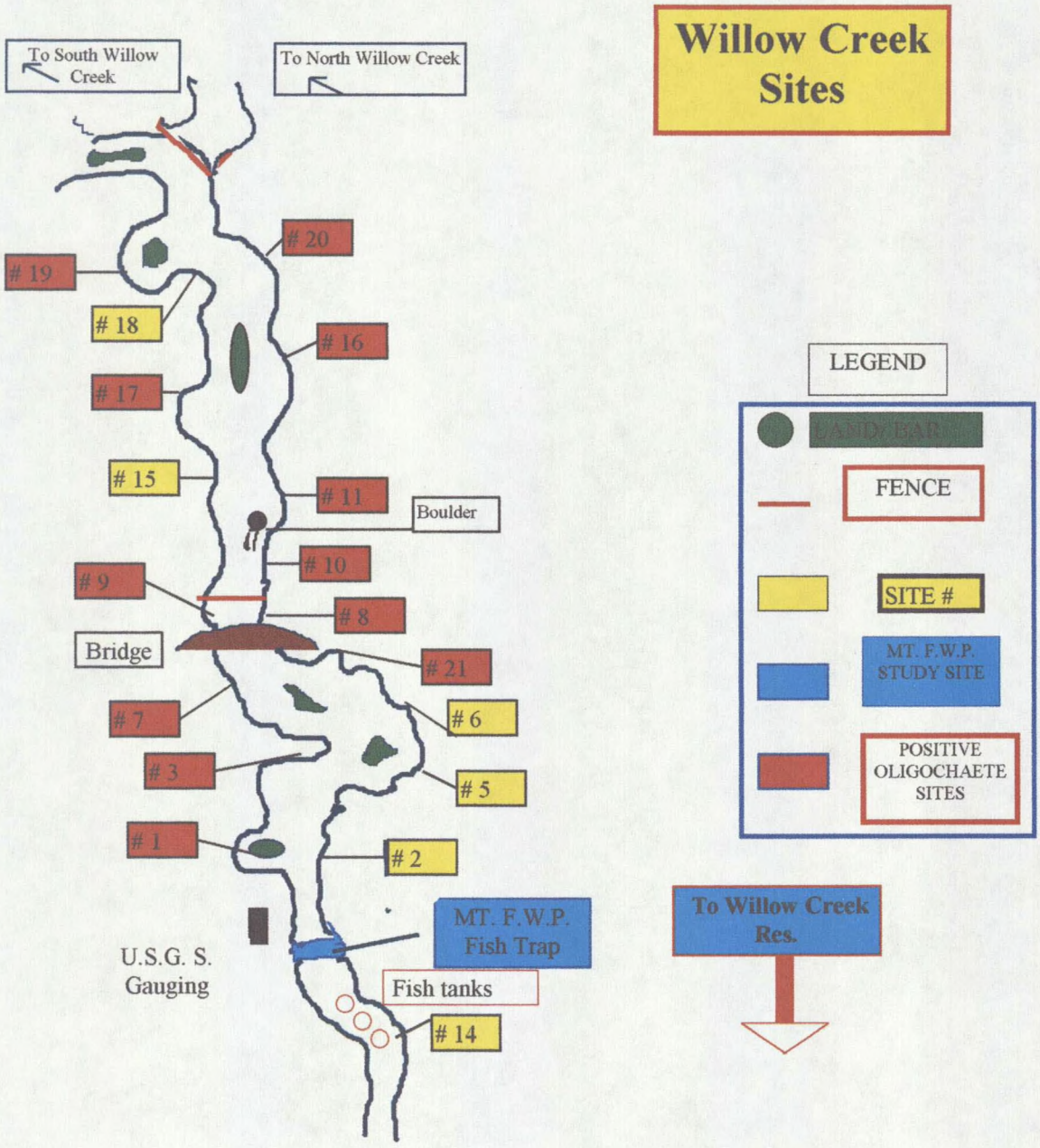


Figure 13. Schematic map depicting oligochaete collection sites along the Willow Creek study area.