Quantitative assessment of Myxobolus cerebralis viability and infective success in the salmonid host
by Crystal Jean Hudson

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Veterinary Molecular Biology
Montana State University
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Abstract:
Whirling disease in trout is caused by a myxozoan parasite, Myxobolus cerebralis. The infectious stages of this parasite move from the skin, through the nerves and into the cartilage of young fish where the destructive effects of the parasite are seen. Very little is known about the immune response or mechanisms of resistance of different salmonids when exposed to M cerebralis. The triactinomyxon (TAM) parasite stage appears to have a limited time when released from the oligochaete (Tubifex tubifex) to infect the salmonid host. Limited data has been obtained regarding the age-related viability of the TAM and its ability to infect fish after release from the worm host.

To quantitatively assess TAM viability and infectivity, scanning electron microscopy was used to count TAM attachments at various time intervals after TAMs were harvested from oligochaete cultures. Both phase-contrast microscopy and vital staining protocol were used to enumerate TAMs and determine viability at increasing TAM age. In addition, sagittal whole fish sections were prepared for histological observation of parasite migration in fish epidermis.

Results with freshly harvested, 24, and 48 hour TAMs documented consistency between TAM attachment, phase-contrast, vital staining, and sporoplasm migration data which indicated a significant reduction in TAM viability and infectivity over time. At 72 hours post-harvest, phase-contrast microscopy and the vital staining protocol documented 38% and 39% parasite viability, but TAM attachment and sporoplasm migration data indicated few attachments or infective stages were present in the epidermis. These results suggest visual observation of TAM morphology may indicate viable parasites at increasing TAM age, but actual infectivity may be dramatically reduced in the first 48 hours after release from oligochaetes. Results also suggest this brief period of peak TAM viability may be the most susceptible to disruption of the M. cerebralis lifecycle for prevention of disease expression in early salmonid life stages.

QUANTITATIVE ASSESSMENT OF *MYXOBOLUS CEREBRALIS* VIABILITY AND INFECTIVE SUCCESS IN THE SALMONID HOST

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A thesis submitted in partial fulfillment

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MONTANA STATE UNIVERSITY-BOZEMAN
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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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Abstract

Whirling disease in trout is caused by a myxozoan parasite, *Myxobolus cerebralis*. The infectious stages of this parasite move from the skin, through the nerves and into the cartilage of young fish where the destructive effects of the parasite are seen. Very little is known about the immune response or mechanisms of resistance of different salmonids when exposed to *M. cerebralis*. The triactinomyxon (TAM) parasite stage appears to have a limited time when released from the oligochaete (*Tubifex tubifex*) to infect the salmonid host. Limited data has been obtained regarding the age-related viability of the TAM and its ability to infect fish after release from the worm host.

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Results with freshly harvested, 24, and 48 hour TAMs documented consistency between TAM attachment, phase-contrast, vital staining, and sporoplasm migration data which indicated a significant reduction in TAM viability and infectivity over time. At 72 hours post-harvest, phase-contrast microscopy and the vital staining protocol documented 38% and 39% parasite viability, but TAM attachment and sporoplasm migration data indicated few attachments or infective stages were present in the epidermis. These results suggest visual observation of TAM morphology may indicate viable parasites at increasing TAM age, but actual infectivity may be dramatically reduced in the first 48 hours after release from oligochaetes. Results also suggest this brief period of peak TAM viability may be the most susceptible to disruption of the *M. cerebralis* lifecycle for prevention of disease expression in early salmonid life stages.

INTRODUCTION

Salmonid whirling disease was discovered in Europe in 1893. The causative agent was identified as the protozoan parasite *Myxobolus cerebralis* (*Mc*). It is believed the parasite originally developed in association with brown trout, *Salmo trutta,* in central Europe and Asia and was non-pathogenic. Hofer initially reported the disease in rainbow trout, *Onchorynchus mykiss,* in Germany. The infected fish had recently been imported from the United States. Since 1893, *Mc* has been shown to infect numerous salmonid species, (Hoffman 1990; Hedrick 1998). Over time, the parasite has spread worldwide to over 21 countries due to the stocking of infected fish, discarding non-consumable fish carcasses, or from avian droppings (Hoffman 1990; Taylor and Lott 1978). Whirling disease was first diagnosed in the US in Pennsylvania in 1958. Circumstantial evidence strongly suggests the origin of the disease in this country to be from imported frozen European rainbow trout. At approximately the same time, whirling disease was also found in Nevada. Importations of European fish ceased and no further positive sites were reported until 1961. However, whirling disease has now been confirmed in 22 other states. It has been surmised that the spread of whirling disease was largely due to three major vectors: (1) transfer of live fish; (2) movements of infected fish in streams; and (3) parasite transfer from the feces of fish eating birds. Whirling disease is currently one of the most serious threats to wild and captive salmonids throughout the country (Rognlie and Knapp 1998), and has been associated with significant rainbow trout population declines in both Colorado and Montana (Vincent 1996).

Myxobolus cerebralis - Taxonomy, Life Cycle and Parasitic Characteristics

Myxobolus cerebralis possesses unique phenotypic and genotypic characteristics when compared with other Myxozoan parasites. Myxozoans are a diverse group of multicellular organisms, although they were previously considered members of the Protozoa, more recent comparisons of ribosomal and Hox genes suggest relationships with the Bilateria or the Cnidaria (Smothers et al. 1994; Siddall et al. 1995; Schlegel et al. 1996; Anderson et al. 1998; Kent et al. 2001). The distinct structural features of *Mc* strongly suggest a close similarity to the Cnidaria. The Cnidaria utilize differentiated cells with extrusive filaments (cnidocysts) that are capable of trapping or attaching to their host or prey (Siddall et al. 1995). *Mc* similarly utilizes extrusion of polar filaments for attaching to the salmonid and oligochaete hosts. It falls within the order Bivalvulidae, suborder Platysporina, and finally the genus *Myxobolus* (Kent et al. 2001). Genetically based studies have documented a branching by *Mc,* separating it from other *Myxobolus* species infecting fish. Several *Mc* isolates have been studied genetically and the lack of variation between the isolates from diverse geographic regions appears to validate the theory that the parasite was recently introduced to North America (Andree et al. 1999). Wolf and Markiw (1984) originally described a model for a two host life cycle of *Mc* which included an obligate oligochaete, *Tubifex tubifex* (Figure 1). Sequential development of *Mc* in both the salmonid and the oligochaete hosts have been further illustrated through intensive studies conducted by researchers from the University of Munich (El-Matbouli 1998) (Figure 2).

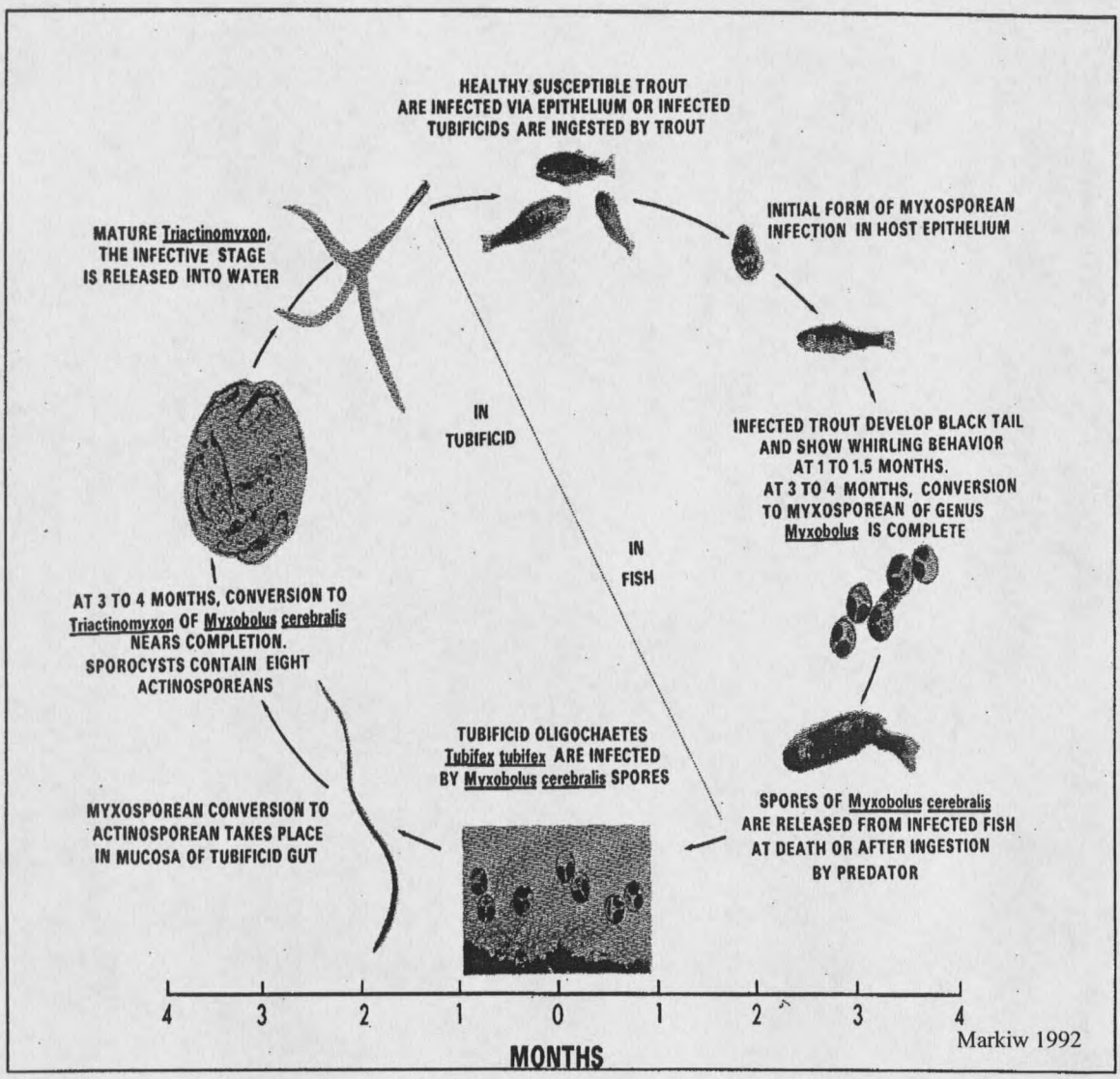
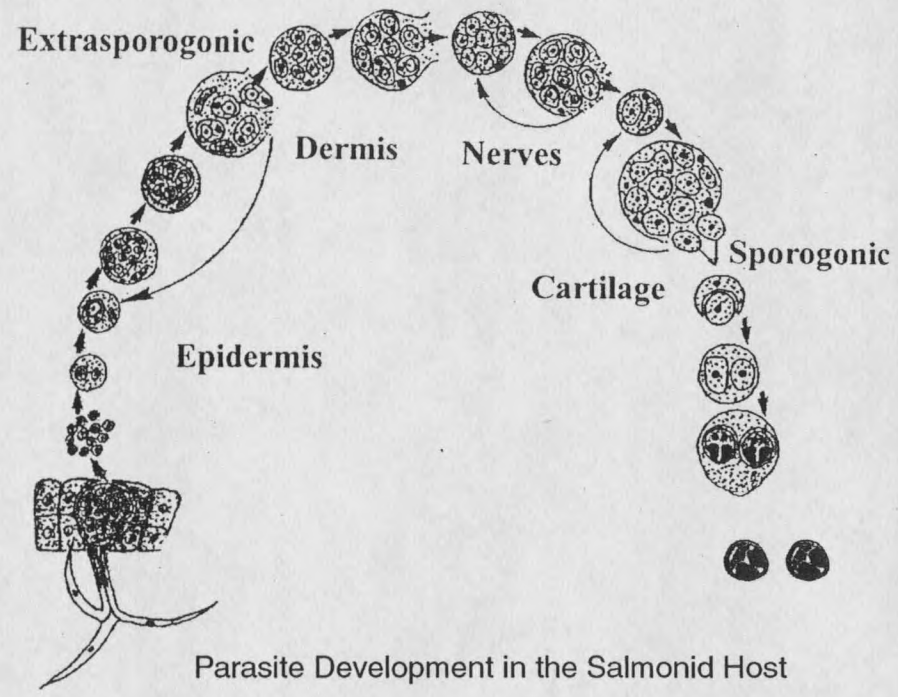
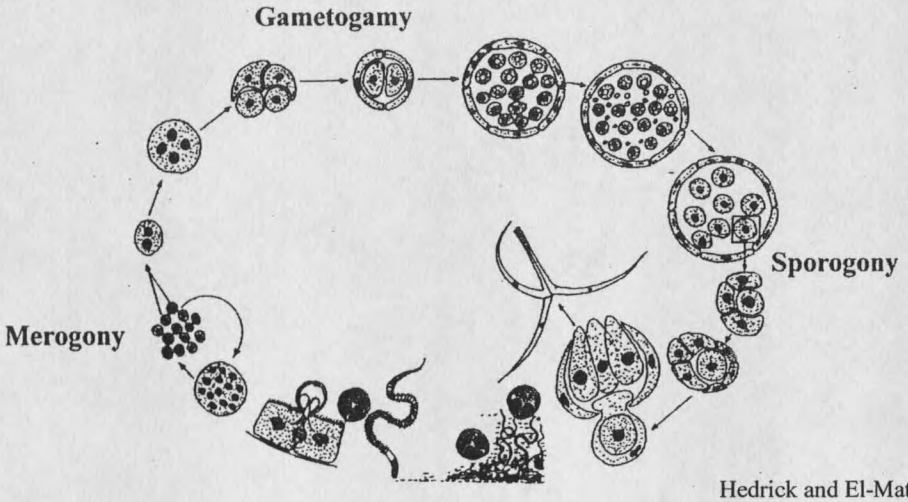


Figure 1. Two host life cycle of *Myxobolus cerebralis* describing salmonid and oligochaete host sequential development. (illustration from Markiw 1992).



Parasite Development in the Salmonid Host



Hedrick and El-Matbouli 2001

Parasite Development in the Oligochaete Host

Figure 2. Life cycle stages of *Myxobolus cerebralis* in the salmonid and oligochaete hosts. (illustration from 2001 Whirling Disease Symposium)

The initial development of *Mc* in the salmonid host begins with parasite attachment and penetration of the host by the triactinomyxon stage of the parasite. Triactinomyxons (TAMs) are released into the water column by the oligochaete worm *T. tubifex*, and may remain viable for periods of 6 to 15 ds at water temperatures of 7-15^0 C (Markiw 1992; El-Matbouli et al. 1999). Movement of TAMs is primarily a function of drifting in the water column and is facilitated by the buoyancy of the TAM structure. The TAM must locate a salmonid host to continue the parasites life cycle. The waterborne TAM consists of an epistyle containing 3 polar capsules, each with a polar filament and a sporoplasm aggregate with 64 germ cells (34 μm in height). The associated stylus is approximately 134 μm long and the 3 rays or processes are approximately 193 μm each in length (El-Matbouli and Hoffman 1998) (Figure 3). The salmonid phase of the life cycle of *Mc* begins with parasite attachment and penetration of the host by the TAM. TAM attachment causes significant epidermal damage by three mechanisms: (1) extrusion of polar filaments, (2) migration of the sporoplasm between cells, and (3) intracellular development and release of parasite daughter cells from infected host cells.

Evidently, distinct chemical and mechanical stimuli must be present on the salmonid host to facilitate firing of polar filaments for TAM attachment (El-Matbouli et al. 1999). TAMs do not extrude their polar filaments in the presence of non-living salmonid hosts, indicating that both mechanical and chemical stimuli are necessary for infection. Invading sporoplasm packets have a dense coat of villi combined with preformed proteases which facilitates entry between cells in the epidermis (Speer 2000).

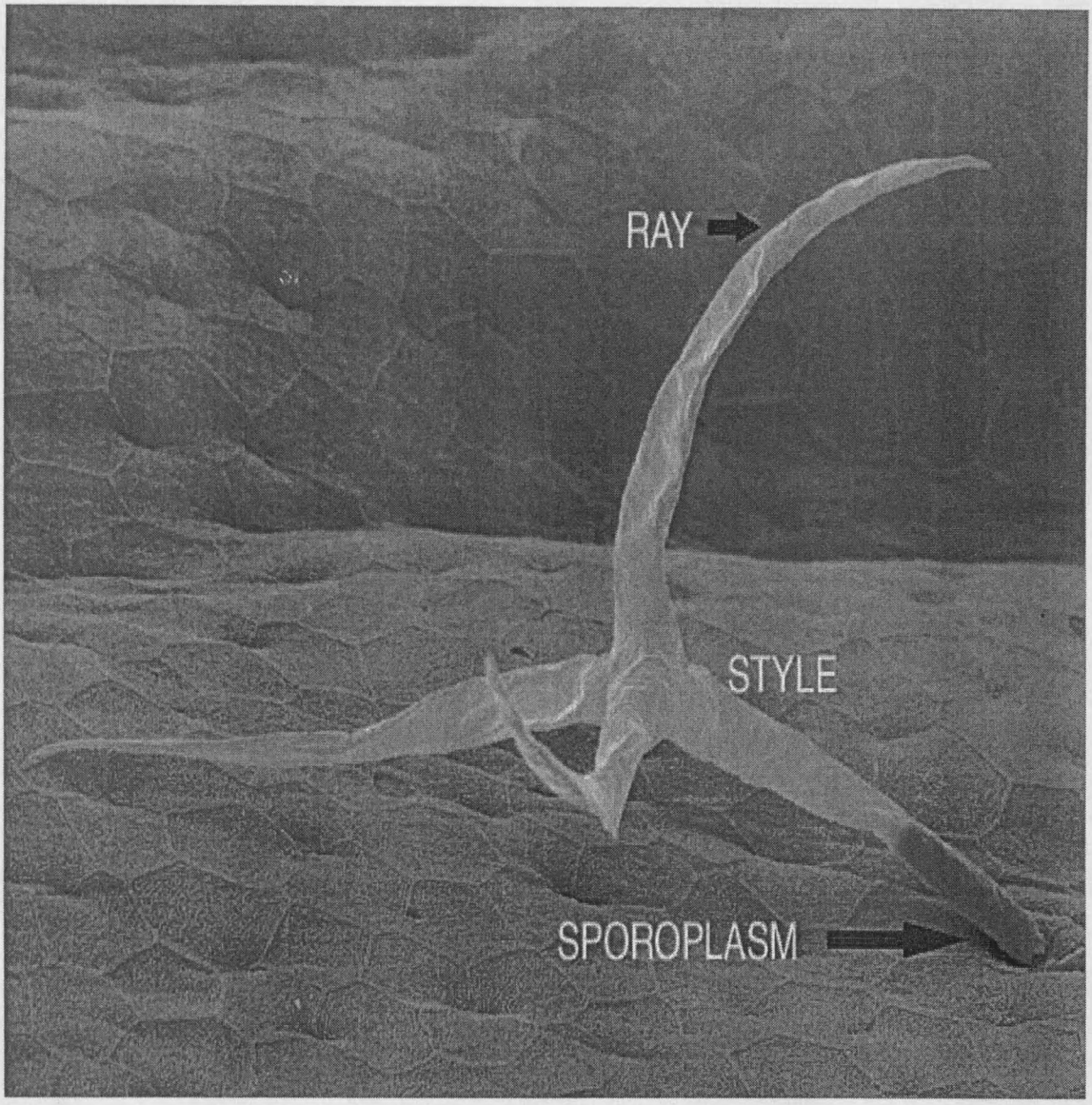


Figure 3. *Myxobolus cerebralis* triactinomyxon attaching to epidermal surface of the salmonid host. (Scanning Electron Micrograph 1278X).

Parasite attachment and penetration occurs within seconds after contact between the TAM and the fish host. Within 5 min post-exposure, sporoplasms are separated from the style and are found in the epidermis of the fish (Figure 4). At 1 h after penetration of the epidermis, the sporoplasm (consisting of germ cells) penetrates into epidermal cells (El-Matbouli 1995).

At 2 h, post-exposure, most germ cells within the sporoplasm have undergone mitotic division and begin to migrate into deeper tissues away from the epidermis. At 24 h, few parasites remain in the epidermis, and at 4 to 24 d post-exposure, parasites are found primarily in nervous tissue, which they use to migrate to areas of host cartilage, where further parasite replication occurs.

Parasite development in the cartilage appears to be temperature dependent. At 16-17^0 C, parasites appear as early as 20 d post-exposure, while as many as 35 d may be required at temperatures of 12-13^{0}C (Halliday 1973). When parasites reach fish cartilage, they digest the cartilage matrix and chondrocytes, which then signifies the end of the presporogonic stage. Sporogony or sporogenesis begins which results in the development of mature spores (Lom and Dykova 1992), which are elliptically shaped, approximately 10 um in diameter, and may exceed several million in number per fish (Figure 5). Spore development requires 52-121 d, depending on water temperature (Halliday 1973). Spores remain embedded in the fish skeleton even after cartilage ossifies into bone. Parasite spores can only be liberated from the fish host by fish death and decay, or by predation by other fish, birds, or fish eating animals.

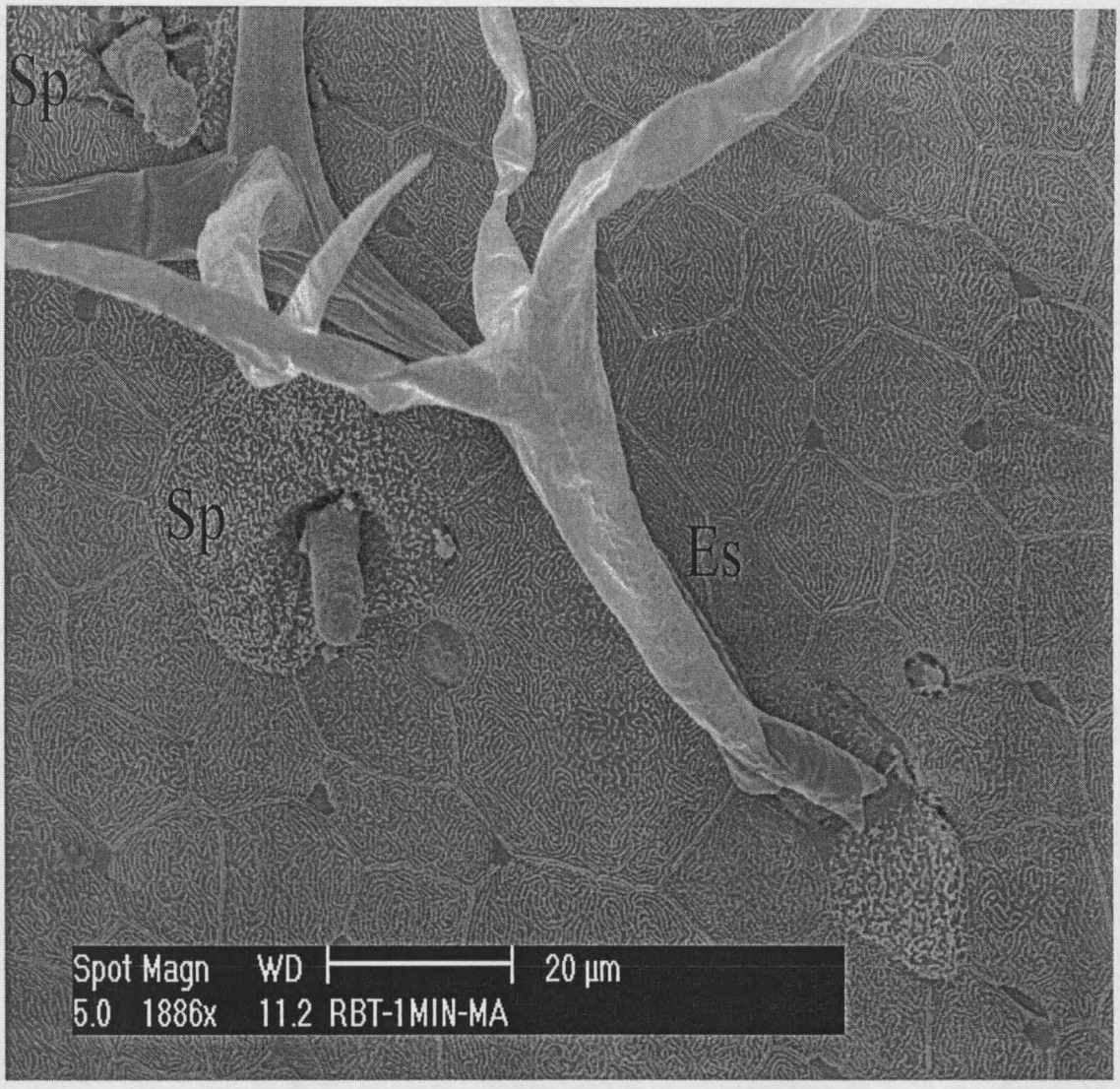


Figure 4. Scanning electron micrograph of TAMs and sporoplasms of *Mc* attached to and invading mucous epidermal cells of rainbow trout
Note: epistylus (Es) of one TAM and two sporoplasms (Sp) devoid of the remainder of the TAM.

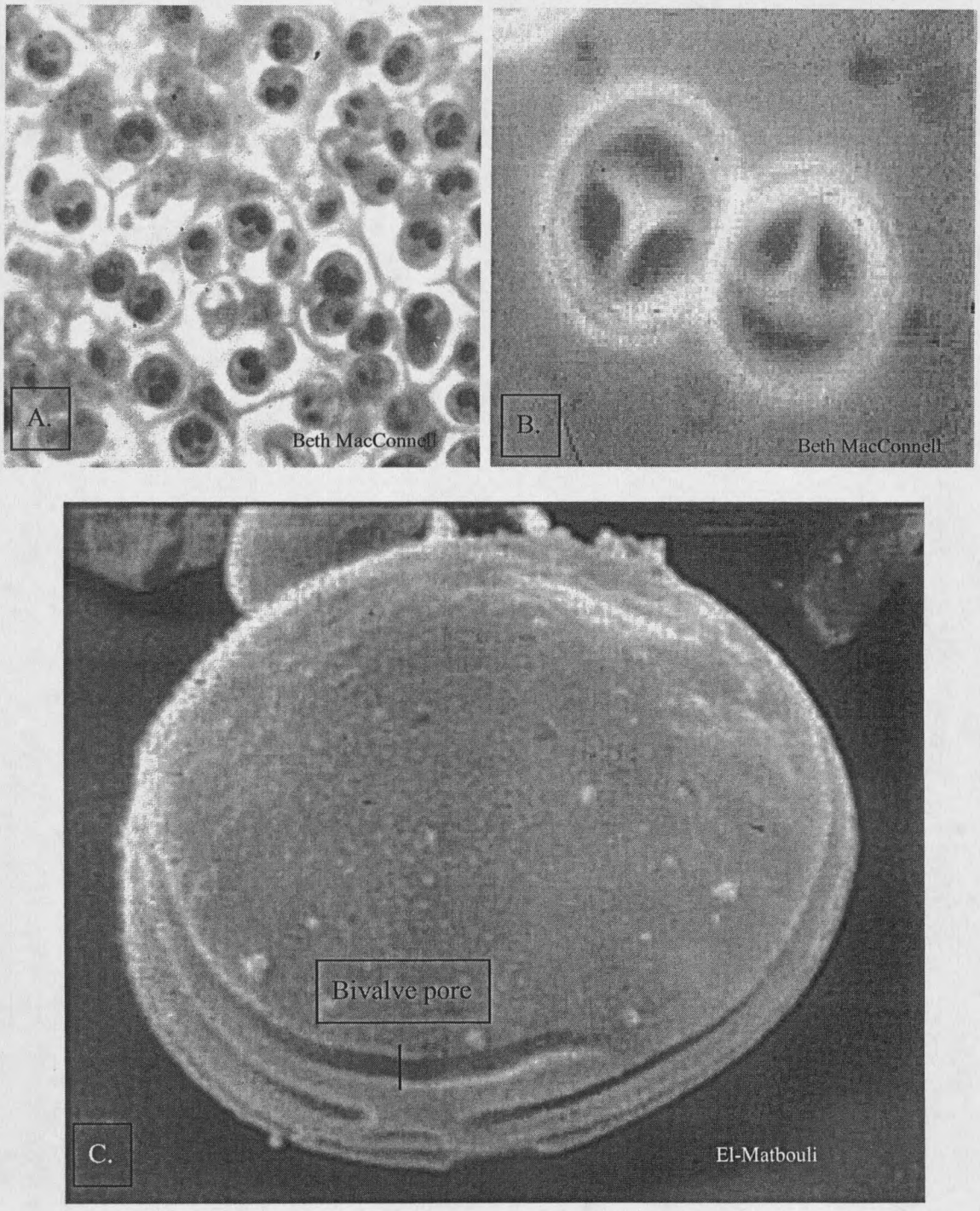


Figure 5. (A and B): *M. cerebralis* myxospores stained preparations (Photos courtesy of Beth MacConnell). (C) Myxospore of *Mc* viewed with scanning electron microscopy. (Courtesy of Dr. Mansour El-Matbouli, University of Munich).

Viable spores can pass through the alimentary tract of birds (El-Matbouli and Hoffman 1991). Spores released in the environment, may remain viable for periods as long as 12 years and can withstand extreme physical and environmental parameters (Schaperclaus 1954; Lom and Dykova 1995).

Mature spores released from salmonid hosts are ingested by aquatic oligochaetes, however, continuation of the *Mc* life cycle is dependent upon ingestion by *T. tubifex* as an obligate host (Figure 6). Similar to the TAM stage, ingested spores extrude polar filaments that facilitate attachment to the mucosal lining of the intestine. Upon polar filament extrusion, spore valves open, and the binucleate sporoplasm migrates between the mucosal epithelium. Further parasite development occurs in the lining of the oligochaete just beneath the epithelium. Similar to sporogonic development in the salmonid hosts, parasite development in the oligochaete is temperature dependent. Temperatures lower than 17^0 C delay development, whereas temperatures above 30^0 C may completely arrest the process. Within the oligochaete, *Mc* undergoes a sequence of developmental phases, consisting of merogony, gametogony, and sporogony. At 17^0 C, merogony occurs 5 to 25 d after ingestion and consists of several cycles of binucleate amoeboid cells dividing into daughter cells. Gametogony occurs 25 to 46 d post ingestion, which produces pansporocysts through multiple divisions of binucleate cells. Pansporocysts consist of somatic cells surrounding two generative cells. Sporogony begins at about 50 d post ingestion when gametocytes fuse to form zygotes. Eventually, zygotes develop into triactinomyxon spores, which may reach full development at 90 d

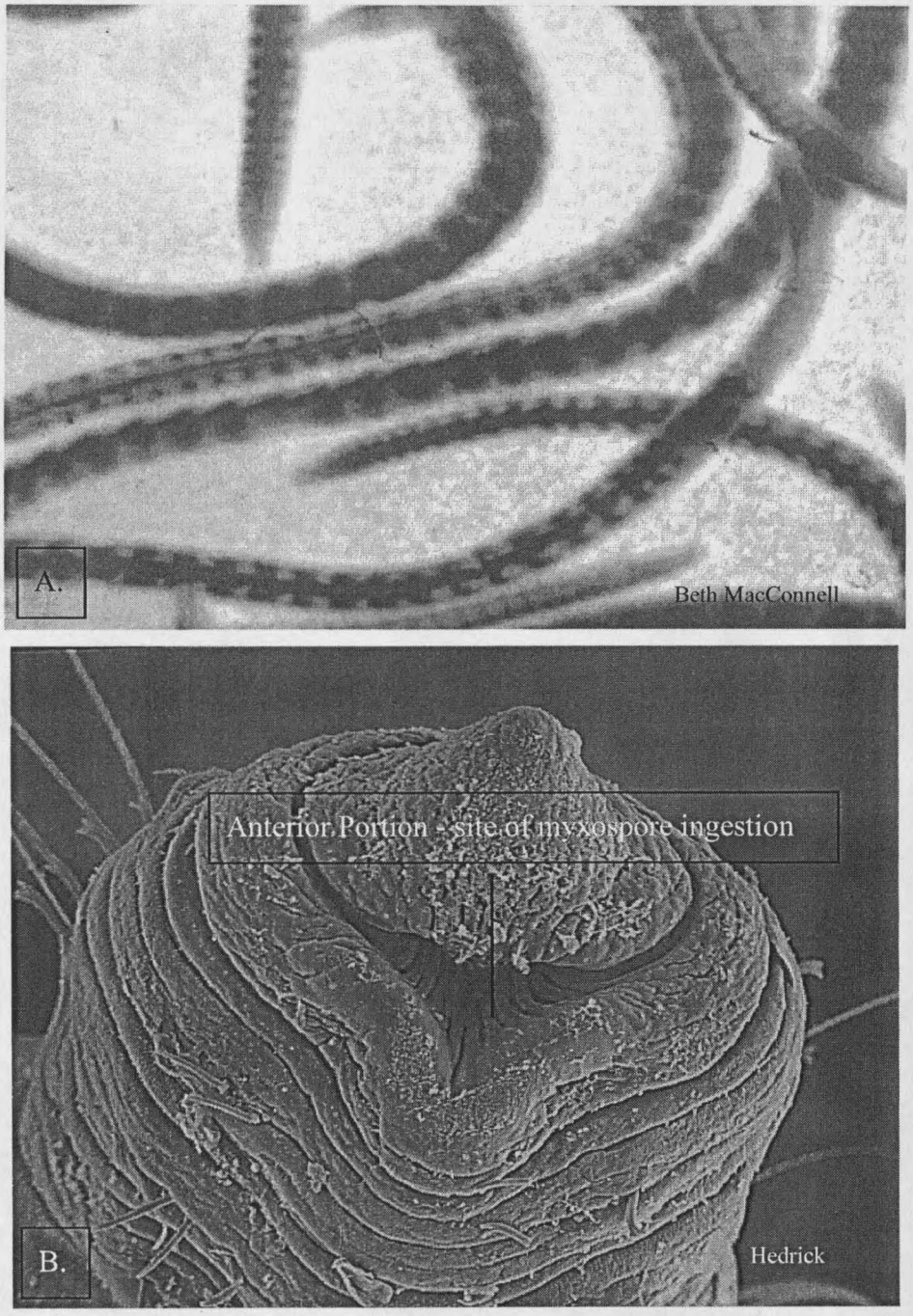


Figure 6 . The oligochaete host for *Myxobolus cerebralis*: *Tubifex tubifex.*
(A) Red segmented worm clusters (Photo courtesy of Beth MacConnell).
(B) SEM micrograph of mouth portion of oligochaete. (SEM micrograph courtesy of Ron Hedrick, UC Davis)

(El-Matbouli 1995). TAMs are released into the oligochaete lumen and passed through the anus into the aquatic environment. This event signals the completion of the two-host life cycle as the TAMs are immediately capable of attaching to the fish host and initiating a new cycle. A single oligochaete may remain infected for life and continually release TAMs (Markiw 1986).

The level and severity of *Mc* infection has been reported to be dose and temperature dependent (Markiw 1992; El-Matbouli et al. 1999; Hedrick et al. 1999). At 15° C an optimal relationship appears to exist between *T. tubifex* and *Mc*. At 25° C development and release of mature TAMs are hindered and production ceases after 96 h. At 5° C TAM maturation occurs, but at a much slower rate (El-Matbouli et al. 1999). *M. cerebralis* infection severity is also reported to be dependent on fish age at first exposure and TAM dosage. Larval fish (<9 weeks of age) consist mostly of cartilage, are more susceptible to infection by *Mc* because the parasite prefers to develop in cartilaginous tissue. With increasing age, the fish becomes less susceptible to *Mc* as cartilage ossifies to bone and the fish develops a greater level of immunocompetence. Therefore, in general terms, the older a fish is before it is exposed to *Mc*, the greater its chance of survival (Hedrick et al. 1998; Molnar 1991; Hoffman and Byrne 1974).

In general, disease severity increases with increasing parasite dose. Clinical whirling disease and mortality were observed in small, 2 d old rainbow trout exposed to low doses of TAMs (10 TAMs/fish), however, 2 month old rainbow trout exposed to the same dose produced no myxospores. Exposure studies with 2 month old rainbow trout

documented an increasing number of myxospores per fish at 5 months post-exposure with increasing TAM dosages (Markiw 1991, 1992).

Susceptibility to infection with *Mc* varies among many salmonid species and also among strains and individual fish within a population. It may be measured by prevalence of infection, appearance of clinical signs, severity of infection, and myxospore counts. Among the *Oncorhynchus spp.*, inland trout are highly susceptible to whirling disease, but susceptibility is variable in anadromous salmonids. Coho salmon are much more resistant to whirling disease than steelhead, sockeye, or chinook salmon (Hedrick et al. 2001). The greatest variation has been observed in *Salvelinus spp.* where brook trout are susceptible, bull trout are partially resistant, and lake trout are resistant (O'Grodnick 1979; Hedrick et al. 1999). Susceptibility is determined by a variety of factors which include: genetic variation between species or strains, fish age, fish size, parasite dose, immune response, and water temperature. Recent observations indicate a German strain of rainbow trout may possess a higher degree of resistance to *Mc*. Preliminary investigations by Hedrick et al. (2002) confirmed the Hofer strain is considerably more resistant than any rainbow trout strains tested previously. Various immune parameters and resistance mechanisms are currently being studied in the Hofer strain.

Fish severely infected with Mc may develop clinical signs that prompted the description of "whirling disease". Clinical signs of an *Mc* infection include whirling behavior, a blackened tail, shortened snout and operculum, exopthalmia, and scoliosis, all of which are due to the direct and indirect effects of the parasite developing within

cartilaginous tissue. Trophozoites of *Mc* digest cartilage and destroy normal architecture necessary for proper bone formation, leading to skeletal deformities (Figures 7 and 8.) Recent investigations indicate that developing parasites in the skull and vertebrae results in constriction of the brainstem and spinal cord, which in turn disrupts the neural control of swimming, causing the radical whirling or tail chasing behavior (Hedrick et al. 1998).

The study of *Mc* reveals a very complex parasite whose evolution into a serious fish pathogen has had severe economic and ecological consequences in North America. The parasite has the potential to cause significant damage in both the salmonid and oligochaete host. Management biologists in several states have monitored salmonid population declines associated with the presence of *Mc*. The complexity of the parasite life cycle and environmental variables provide a difficult task for parasite management.

Prior to discovery of the *Mc* life cycle, control measures generally involved total removal of the infected fish and the spores from most hatchery facilities. In many instances, hatchery facilities were totally depopulated and disinfected prior to continuance of production programs. With the knowledge that the parasite uses two hosts to complete its life cycle, hatchery managers can now use various practices to manage this disease. It is common practice to minimize the use of earthen or gravel bottom ponds in *Mc* infected areas when culturing susceptible species, thereby eliminating the aquatic oligochaete habitat. In addition, with knowledge of the impacts of the parasite on young fish, it is now become an important management strategy to provide older fish for stocking into known *Mc* positive waters in order to minimize the development of the

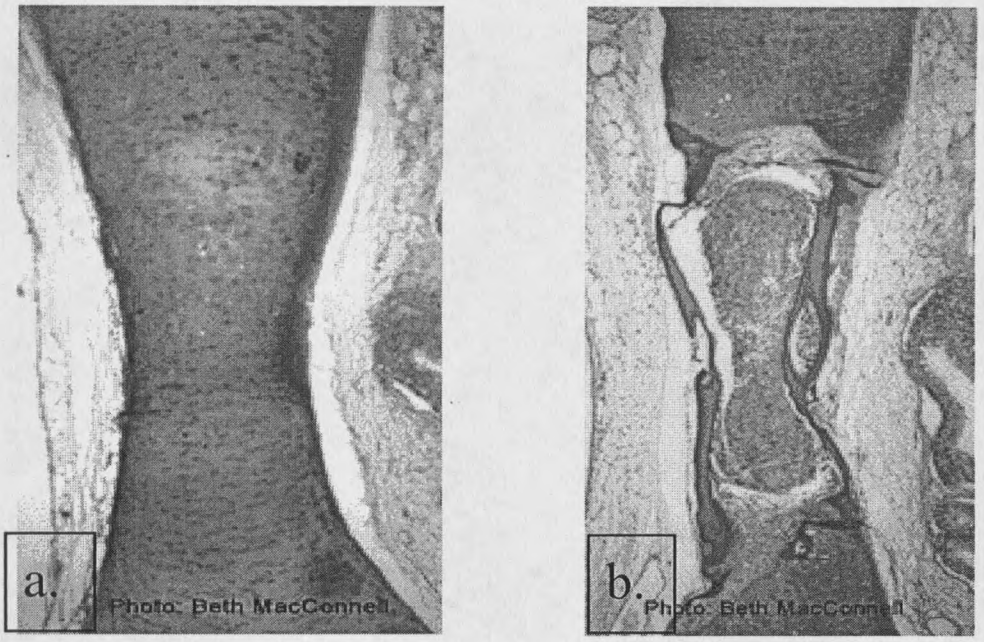


Figure 7. (a) Normal cartilage in rainbow trout nares. (b) Cartilage infected with *M. cerebralis*.

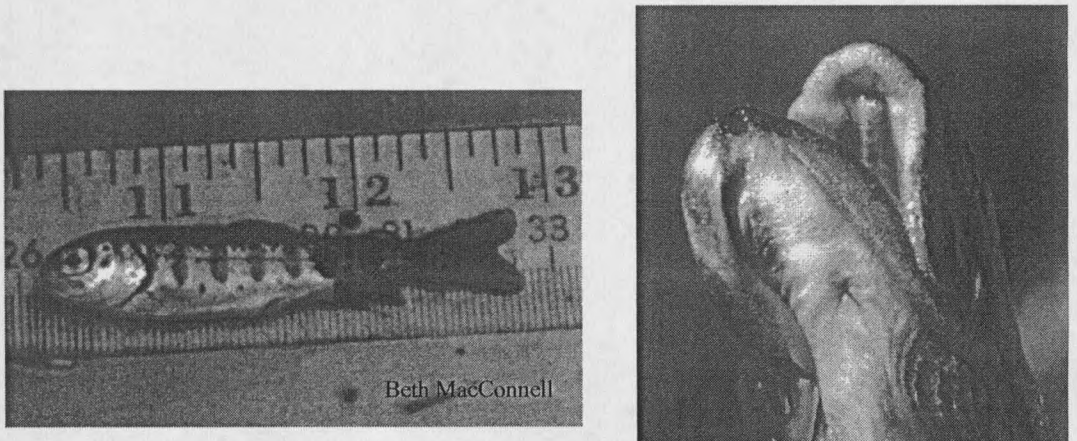


Figure 8. (a) Blackened caudal area in rainbow trout infected with *M. cerebralis*. (b) Jaw deformity in rainbow trout infected with *M. cerebralis*. (Photos courtesy of Beth MacConnell)

disease. The life cycle of the parasite makes vertical transmission of the parasite unlikely as spores are not known to be actively shed from live fish, and the parasite must first pass through the appropriate oligochaete to produce TAMs that are infectious to salmonids. Fish management strategies for whirling disease-infected rivers are focusing on managing fish development as well as parasite development. It may be preferable to use fish species that spawn and rear in *Mc*-free habitats and only later migrate to infected waters.

Statement of Research and Project Objectives

Numerous whirling disease investigations have concentrated on the oligochaete host, evaluating various parameters including genetics, life cycle, environment, and temperature (Wolf et al. 1986; Hamilton and Canning 1988; El-Matbouli and Hoffman 1998; El-Matbouli et al. 1999; Antonio et al. 1999). Other studies involving the fish host have concentrated on age susceptibility, species susceptibility, and parasite exposure levels in attempts to find the weakest link in the parasite's life cycle (Halliday 1974; Baldwin et al. 2000; Thompson et al. 1999).

As with many other pathogens, immunological responses to *Mc* often occur too late to combat infection and prevent disease. A number of factors appear to play a role in the initiation and management of an *Mc* infection . Soon after attachment and penetration, *Mc* begins to multiply in the epidermis before it begins to migrate and multiply in other tissues. Some parasites appear to spontaneously degenerate in the skin of the fish, which may be due to a humoral response, but this has yet to be determined.

After migrating through the fish integument, *Mc* enters the peripheral nerves, which appear normal with no tissue reaction. By using peripheral nerves to migrate to cartilaginous tissue in the cephalic region of the fish, *Mc* is rapidly sequestered in nervous tissue, thereby avoiding any obvious humoral or cellular immune response. Only the lysis of cartilage elicits a cellular response in the salmonid host, which is generally an intense inflammatory reaction. Specific major histocompatibility (MHC) class I or II alleles that confer resistance or protection against *Mc* have yet to be identified in trout.

Initial parasite/host interactions at the level of the epidermis/dermis may be critical in determining why certain salmonid species are more resistant than others. Also, more research is needed to determine the mechanisms involved in the lack of a significant immune response during early stages of infection. Because early parasite stages in the salmonid host are likely the most vulnerable to disruption by control methods, this study focused on the initial parasite/host interactions which are still not well understood.

During the 2000 National Whirling Disease Symposium, leading researchers on whirling disease identified several major areas of investigation that were considered critical to improving our understanding of the parasite/salmonid host interaction. These areas include analysis of TAM viability and attachment in different salmonid species, possible interactions of lectins and carbohydrate structures involved in parasite attachment, and studies of differing immune response to *Mc* between species. This project identified three specific objectives which serve to address those areas of whirling disease investigations currently considered important to the national research effort.

Project Hypothesis

This study hypothesizes that TAM viability and infectivity decreases with increasing TAM age based on release from the oligochaete host. It is also assumed that methods to enumerate TAMs and determine parasite viability will positively correlate with the decrease in TAM infectivity over time.

Study Objectives

TAM Attachment

One of the high priority research areas included placing an emphasis on mechanisms involved in attachment and penetration of the parasite into the salmonid host. This research would require 1) developing a better understanding of the infective process, and 2) determining the mechanical and chemical mechanisms involved in parasite attachment, migration, and multiplication.

Parasite attachment to and its initial interaction with the salmonid host require a combination of TAM viability studies and quantitative measures of TAM attachment. To address these objectives, scanning electron microscopy and photomicroscopy of histological preparations were used to visualize and quantify TAM attachment and penetration in larval trout.

Techniques/Methods to Enumerate TAMs and Measure Viability

The National Whirling Disease Steering Committee and the National Whirling Disease Foundation have identified parasite viability as a key issue in research on

whirling disease. Numerous research results and conclusions have been based on widely used procedures that have not been validated. TAM viability has generally been a major unknown variable in all applied research investigations. Two previous research efforts on TAM viability and longevity, after release from the oligochaete, documented significantly different time periods for peak viability. Markiw (1992) recorded a 3 d peak viability period for TAMs after release from the worm host. However, El-Matbouli (1999) indicated TAMs could remain 60% viable for 15 d. These conflicting results suggested additional research was needed to document the period of peak viability for the TAM life stage of *Mc.*

Essentially all parasite/salmonid host research projects require the use of a method to determine TAM numbers and viability for controlled experimental dosages. Current applied research relies on methods that assume controlled TAM dosages provide an equal number of TAM attachments for each fish in a controlled experiment. An objective of this project is to provide information necessary to validate current techniques. This will be accomplished using a vital staining protocol and phase-contrast microscopy to measure apparent TAM viability and parasite attachment to determine actual infectivity. Phase-contrast microscopy and vital staining procedures will provide quantitative information of total TAMs harvested from oligochaetes combined with percent viable and non-viable parasites, which will then be compared to the ability of the TAMs to attach and ultimately infect fish.

TAM Age, Viability and Infective Success

A final major objective of this project is to provide information with biological significance with regard to resource management issues. In natural stream systems, researchers and managers have documented the occurrence of point source infection with regard to the whirling disease parasite (Vincent 1996). Briefly, dramatic variation in *Mc* infection and severity may occur throughout a stream's entire reach. This is presumably due to discrete, localized populations of oligochaetes and subsequent seasonal releases of TAMs concurrent with salmonid fry emergence. TAM counts, viability, and sporoplasm infection observations will attempt to confirm the period of peak parasite viability and demonstrate this possible effect on the development of whirling disease in stream environments. This will be accomplished in the laboratory with the observation of viability at increasing TAM ages and subsequent parasite attachment quantitative data.

The results of this investigation may assist further research to study specific mechanisms of attachment and penetration of *Mc* including the identification of chemical and mechanical stimuli present at the time of infection. Although this research will be carried out under controlled laboratory conditions, it is designed to provide applicable information necessary to understand the interaction of the fish host and the parasite in natural systems. Project design was initiated to both complement current whirling disease research and provide research results with biological significance and value to fishery managers to ultimately protect fishery resources.

EXPERIMENTAL METHODS

Establishment and Maintenance of Aquatic Oligochaete Cultures

Oligochaete populations were obtained from Mt. Whitney State Fish Hatchery in Bishop, California. Substrate was collected from the hatchery and the substrate/oligochaete mixture was maintained in 2 L aquaria with aerated 15^0 C water. Microscopic examination of worm morphology revealed that approximately 90% of oligochaetes collected were *Tubifex tubifex*. The remaining 10% were identified as *Limnodrilus hoffmeisteri*. The mud substrate collection was poured through a 500 μm mesh screen, with mud and debris washed away by swirling in a shallow pan. *T. tubifex* cultures formed clusters which were easily observed when substrate was rinsed. The mass of worms was then rinsed repeatedly and hand-picked to remove organic matter. The resulting collections were almost pure oligochaetes. Individual cultures were initiated by placing 20 g of mixed oligochaetes in a 9 X 13" plastic container. Containers were filled with unfiltered, non-chlorinated well water to a depth of 2 to 3 inches. Two inches of autoclaved aquarium sand was placed on the bottom of each container. Aeration was provided by pump-driven air stones and partial water changes made one to three times a week depending on the density of the culture and water turbidity. Cultures were loosely covered with plastic lids to prevent water loss and maintained at 15^0C in a climate controlled low-temperature incubator with a photoperiod of 12 h of light and 12 h of darkness. Oligochaete cultures were fed crumbled spirulina discs and Algamac-2000,

a dried algae preparation, once a week. However, additional feed was added to cultures if no pieces of spirulina were visible on the sand.

Salmonid Exposure to *Myxobolus cerebralis* and Diagnostic Protocols

Salmonid Infection Protocol

Rainbow trout *(Onchorynchus mykiss)* were experimentally infected with the TAMs of *Myxobolus cerebralis.* At five weeks post-hatch, 50 rainbow trout fry were exposed to 1,000 TAMs per fish for two h at 11^0 C. Fish were held in 10 gallon experimental aquaria at 12^0 C for 11 months and monitored weekly for clinical signs of disease and mortality. At five months post-exposure, fish were tested for the presence of Mc. Fish were euthanized using Tricaine Methane Sulfonate (MS-222). Cranial elements were extracted from fish heads. Polymerase Chain Reaction (PCR) was used to test for the presence of *Mc* by a nested protocol designed by researchers at the University of California at Davis (Andree 1997). Microscopic examination of histological preparations from the cranial region was also used to determine the presence of mature *Mc* spores and associated pathological lesions.

Spore Extraction

Spores were extracted from fish tissues using the plankton centrifuge method as described in Suggested Procedures for Pathogen Detection in Finfish and Shellfish (Thoesen 1994; O'Grodnick 1975; Markiw and Wolf 1974).

This method was used on whole fish heads sampled at 5 months post-exposure to concentrate spores extracted from cranial cartilage. Five heads were processed at each sampling time. Fish heads were heated in a 95^0 C water bath for 20 min and cartilage and bone separated from flesh. A Waring blender was used to grind 5 fish pooled samples. The sample was passed through a chlorine disinfected urinary calculi filter, added to a separatory funnel, and allowed to flow at a consistent pace into a plankton centrifuge cup. After each sample, the separatory funnel was rinsed three times with tap water (~ 20 mL) to flush down residual sample material into the plankton centrifuge cup. Spores were recovered by scraping the walls of the plankton centrifuge cup with a rubber spatula and then rinsing the spatula and cup with 5 to 20 mL of water. The spore suspension was pipetted into a labeled specimen cup and the final volume recorded to facilitate enumeration of total spores from sub-sample calculation. After each use, the plankton centrifuge, blender and calculi filter were disinfected in 10% bleach (5.25% sodium hypochloride) for 10 min. A 50 μl aliquot of myxospore suspension was placed on a microscope slide and spores enumerated counting all fields at 40X. This procedure was performed 3 times and the total spore counts averaged.

Polymerase Chain Reaction Nested Protocol

Polymerase Chain Reaction (PCR) was used to test for the presence of a 415 base pair (bp) sequence specific for *Mc.* At 5 months post-exposure to *Mc*, fish were euthanized and their heads removed. Each head was placed in screw capped test tube, covered with tap water, and heated in a water bath at 95^0 C for 15 min. The fish head

was placed on polypropylene cutting mat and defleshed by using a scalpel and forceps. Protocol for the Qiagen Dneasy Tissue Kit was used for DNA preparation. Briefly, bone and cartilage were placed in a 1.7 mL microcentrifuge tube, tissue lysis buffer (ATL) added to at a 1:1 (v:w) and proteinase K solution equivalent to 1/10 volume of the buffer. Sample suspension was homogenized by a vortex mixer and incubated at 55^0C for 60 min with occasional agitation. The sample suspension was centrifuged at 16,000 x g for 5 min and a 200 μl aqueous supernatant added to microcentrifuge tubes. Twenty microliters of Rnase A was added, homogenized with a vortex mixer, and incubated at room temperature for 2 min. To obtain purified DNA, the Qiagen Dneasy protocol for rodent tail DNA extraction was followed. Known *Mc* positive and negative control tissues were used in the assay. DNA was amplified using the nested protocol developed by Andree et. al. (1997) by which extracted cranial cartilage samples were amplified using Sigma brand Tr 5-16 and Tr 3-16 primers for the first round on a Perk and Elmer thermocycler. The second round amplification of *Mc* DNA was conducted using Sigma brand Tr 5-17 and Tr 3-17 primers. Amplified samples of fish cranial cartilage were loaded onto a 1.5% agarose gel consisting of 24 wells, and electrophoresed at 80 volts for 35 min. The gel was removed, stained with ethidium bromide solution (6.4 X) for 15 to 20 min, de-stained in water for 5-60 min, visualized on a UV transilluminator for DNA bands. Known positive samples, negative controls and experimental samples were compared for banding at the 413 bp location on the second round of the nested protocol.

Oligochaete Exposure to *Myxobolus cerebralis*

Upon confirmation of salmonid *Mc* infection by PCR, 6 fish from experimental aquaria were euthanized using MS-222 and placed whole in a heated water bath at 95^{0}C for 30 min, then fish cartilage and skeleton were separated from tissue. Water in oligochaete culture containers was drained to about ½ inch above the substrate, 2 infected fish were placed in each culture container (20 g oligochaetes) and incubated overnight at 15^0 C, after which, culture water was replaced and aeration continued. *Mc* myxospores extracted by the plankton centrifuge method were also used to infect oligochaete cultures. Spores from 2 fish were added directly to each 20 g oligochaete culture unit. The level of exposure for a 20g oligochaete culture was approximately 10^6 myxospores. TAMs were generally detectable at 90 to 120 d post-infection.

Harvesting TAMs from Oligochaete Cultures

TAM collections were made twice a week from known infected oligochaete cultures. Water was siphoned from oligochaete culture units into a 1 gallon bucket and allowed to sit for 15 min to reduce particulate matter suspended in the water column, and then poured through a 20 μm Nitex mesh filter. A 50-100 mL concentrated suspension of TAMs was created by flushing parasites from Nitex filters with well water into a collection jar. TAMs were maintained at 15^0 C for immediate fish exposures or maintained for exposure intervals, with slight aeration via aquarium grade air pump and compressed sand air stone.

Quantitative TAM Assessment

Enumeration of TAMs using Phase-Contrast Microscopy

TAMs were maintained at 15^0 C or refrigerated prior to the enumeration protocol. A 50 μl aliquot of the TAM suspension was placed on a microscope slide, coverslipped, and examined with a Nikon Opti-Phot II Phase-Contrast Microscope at 20X magnification. All fields were examined on each prepared microscope slide. Viable, non-viable, and viable/not infectious TAMs were observed and recorded separately. Viable TAMs were considered those with sporoplasms contained within an enveloping cell and located at the apical end of the TAM. Polar capsules containing intact polar filaments were not used as an indication of viability, due to their unstable nature when heated during microscopy. Non-viable TAMs were observed as either empty TAM shells and valves or sporoplasm packet germ cells were dispersed throughout the style. Viable/not infectious TAMs were those parasites retaining an intact sporoplasm packet, but located distant from the polar capsule end (Figure 9). The total number of viable TAMs was determined, and this number multiplied by 20 to quantify TAMs/mL. The procedure was repeated three times and the number of TAMs/mL averaged. The total number of TAMs collected from the worm cultures was calculated by multiplying the mean number of TAMs/mL by the total ml of TAM suspension:

TAMs/mL = Total Number Counted (in 50 μl) x 20 x TAM Water Volume

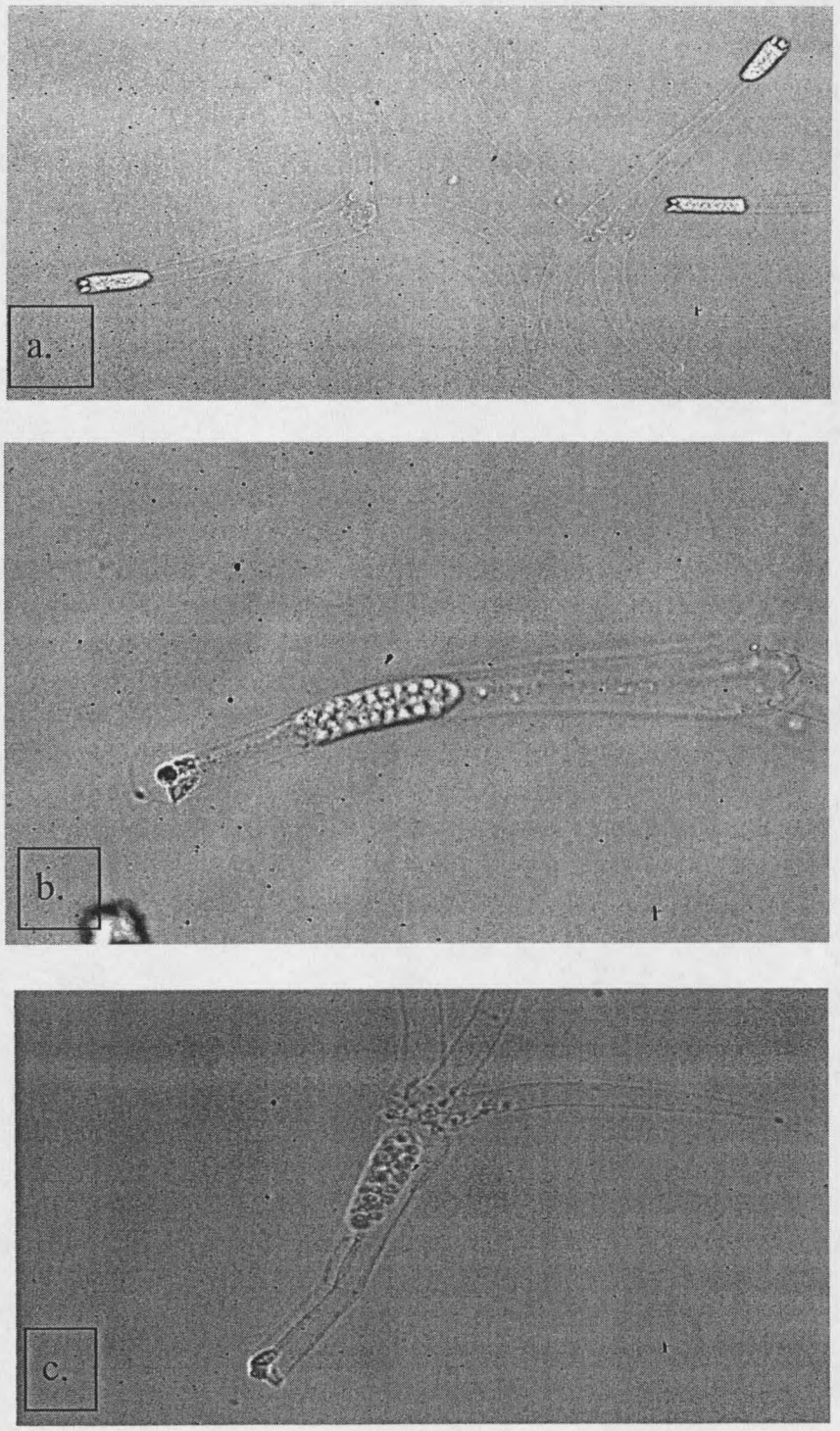


Figure 9. Phase-contrast microscopy micrographs of *M. cerebralis* (a) viable, (b) non-viable, and (c) viable/not infectious TAMs.

TAM Vital Staining Protocol

TAM vital staining used a combination of fluorescein diacetate (FDA) and propidium iodide (PI). Working solutions were prepared fresh each time TAMs were harvested. First, 0.05 mL of FDA stock solution (5 mg/mL) dissolved in acetone and stored at -20^{0}C) was added to 8.3 mL of well water. An aqueous solution of PI at a concentration of 2mg/100 mL was kept at -20^{0}C in 2 mL aliquots. In a 2 mL micro-centrifuge tube, 25 μl FDA (30 μg/ml), 25 μl PI (20 μg/ml), and 50 μl TAM suspension was combined. The sample tube was gently rotated for 5 min and then diluted with 200 μl of a 20% (weight/volume) aqueous solution of bovine albumin. Stained TAM suspension was held in glass tubes or incubated on slides in a refrigerated compartment at 4^{0}C. Slides were held in humid chambers to prevent drying for 2-3 weeks. When slides could be viewed immediately after staining, the albumin step was eliminated. The slides were viewed with epifluorescence microscopy at 25X magnification. Fluorescing epispores of TAMs containing sporoplasms were considered viable if they illuminated bright green and non-viable if they illuminated red (Markiw 1992) (Jones 1985) (Figure 10).

The FDA and PI vital staining protocol combines two stains with differing properties. Propidium iodide is a non-permeant dye that can penetrate the membranes of dying/dead cells. It intercalates into the major groove of the DNA and produces a bright red fluorescence. Fluorescein Diacetate is a non-polar, non-fluorescent fluorescein analogue which can pass through the cell membrane. Intracellular esterases cleave off the diacetate group producing the brightly green fluorescein which accumulates in cells.