Invertebrate aspects of whirling disease
by Richard Irving Stevens

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Fish and Wildlife Management
Montana State University

Abstract:
I examined invertebrate aspects of salmonid whirling disease by experimentally -infecting several species of aquatic oligochaetes with the causative parasite, Myxobolus cerebralis. The primary goal of this research was to achieve a greater understanding of the role of the oligochaete host in regulating disease severity in salmonids. Only the oligochaete species Tubifex tubifex propagated the triactinomyxon stage of the parasite; no triactinomyxons were produced by the species Limnodrilus hoffmeisteri and Ilyodrilus templetoni. A variant of T. tubifex from the Mount Whitney Fish Hatchery in California produced significantly greater numbers of triactinomyxons than a variant from the Madison River in Montana, which in turn produced significantly higher numbers than a variant from a tributary of the Gallatin River, Montana. All three variants suffered significant reproductive depression when exposed to the parasite. Parasite myxospore doses at levels of 50, 500, and 1,000 myxospores per worm had no significant effect upon the total numbers of triactinomyxons released by the oligochaetes. Controlled temperatures of 15°C and 8deg; C did not significantly affect total triactinomyxon production. Reproductive success was highest for the Madison River variant at 15°C, and for the Gallatin River variant at 8° C, irrespective of exposure to M. cerebralis. Reproduction within the California variant was not significantly different between temperatures. The variability in the worm response to parasitic infection may offer at least a partial explanation for the variability in disease severity seen in wild salmonid populations.

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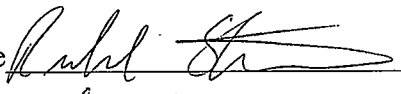
Signature 

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure Page

ABSTRACT

I examined invertebrate aspects of salmonid whirling disease by experimentally infecting several species of aquatic oligochaetes with the causative parasite, *Myxobolus cerebralis*. The primary goal of this research was to achieve a greater understanding of the role of the oligochaete host in regulating disease severity in salmonids. Only the oligochaete species *Tubifex tubifex* propagated the triactinomyxon stage of the parasite; no triactinomyxons were produced by the species *Limnodrilus hoffmeisteri* and *Ilyodrilus templetoni*. A variant of *T. tubifex* from the Mount Whitney Fish Hatchery in California produced significantly greater numbers of triactinomyxons than a variant from the Madison River in Montana, which in turn produced significantly higher numbers than a variant from a tributary of the Gallatin River, Montana. All three variants suffered significant reproductive depression when exposed to the parasite. Parasite myxospore doses at levels of 50, 500, and 1,000 myxospores per worm had no significant effect upon the total numbers of triactinomyxons released by the oligochaetes. Controlled temperatures of 15° C and 8° C did not significantly affect total triactinomyxon production. Reproductive success was highest for the Madison River variant at 15° C, and for the Gallatin River variant at 8° C, irrespective of exposure to *M. cerebralis*. Reproduction within the California variant was not significantly different between temperatures. The variability in the worm response to parasitic infection may offer at least a partial explanation for the variability in disease severity seen in wild salmonid populations.

INTRODUCTION

Whirling disease is the common name given the pathological condition suffered by several species of salmonid fishes infected with the parasite *Myxobolus cerebralis* (Hedrick et al. 1998). The name derives from the rapid circular swimming pattern observed in some infected individuals. This aberrant swimming pattern results from damage to the central nervous system of the fish, particularly in the cartilage of the cranial region (El-Matbouli et al. 1995, Rose et al. 2000).

Myxobolus cerebralis is believed to be a native of Eurasia, and a relative newcomer to North America (Hoffman 1990). This history is supported by the fact that the brown trout (*Salmo trutta*), also a native of Eurasia, seldom develops disease symptoms following exposure (Hedrick et al. 1999). The parasite was initially thought to pose a threat only to fish hatcheries and aquaculture facilities, (Lom 1987), but in 1994 *M. cerebralis* was identified as the cause of a catastrophic decline in the abundance of rainbow trout (*Oncorhynchus mykiss*) in the Madison River of Montana (Vincent 1996). The parasite has also been linked to fish population declines in other western states, particularly Colorado (Nehring and Walker 1996). Concern over economically and biologically important salmonid fisheries, particularly in the western United States, has led to an increasing research effort, of which this project is a part, aimed at whirling disease and the complex ecology of *M. cerebralis*.

BACKGROUND

Myxobolus cerebralis has a complex, two-host life cycle in which an actinosporean transmission stage alternates with a myxosporean transmission stage (Wolf and Markiw 1984, El-Matbouli and Hoffman 1989). The different morphologies of the two transmission stages had previously misled taxonomists into classifying them as separate species (Kent et al. 1994). Earlier research into whirling disease was often characterized by a failure to infect fish with *M. cerebralis* myxospores extracted from infected fish (Hoffman and Putz 1971). The discovery that only the actinosporean phase of the parasite, the triactinomyxon (TAM), is infectious to fish has led to rapid progress in the study of this and other diseases of fish caused by myxosporeans (Brinkhurst 1996).

The discovery of the two-host life cycle of the parasite led to the identification of the aquatic oligochaete *Tubifex tubifex* as an alternate host capable of producing the TAM life history phase (Wolf et al. 1986). A member of the family Tubificidae, *T. tubifex* is a widely distributed species, best known for its utility as an indicator of water quality owing to its high tolerance of organic pollution (Brinkhurst and Gelder 1991). Abundance of this aquatic oligochaete has typically been found to increase with the concentration of organic nutrients and fine sediments in lakes and rivers (Brinkhurst and Gelder 1991, Lestochova 1994).

Several research efforts have failed to identify other oligochaete species capable of acting as a host for *M. cerebralis* (Wolf et al. 1986). However, similar life cycles,

date suggests that each myxosporean species uses a single oligochaete species as its alternate host, but one recent study reported a myxosporean species capable of developing in two different oligochaete hosts (El Mansy and Molnar 1997).

The identification of aquatic oligochaete species presents a challenge to researchers, and typically requires microscopic examination of fixed specimens (Brinkhurst 1986). The number and type of chaetae as well as the shape and size of the internal sexual organs are the principal morphological structures used to identify most common aquatic oligochaetes, including *T. tubifex* (Brinkhurst 1986).

Aquatic oligochaetes often comprise a significant component of the benthic invertebrate assemblage in both lotic and lentic waters, yet relatively little is known of their ecology. Most species feed by ingesting sediment particles, from which they glean the bacteria that constitute their primary food (Brinkhurst 1970). It is during feeding that the worms ingest the myxospores of *M. cerebralis.*

The myxospores of *M. cerebralis* are small, generally on the order of 8-10 m (Hoffman and Markiw 1977). When viewed with a compound microscope at 400x magnification, myxospores appear slightly ovoid and often display a clear apical point. Two dark polar bodies can be seen in proximity to this apex. In side view, a ridged border between the two shells of the myxospore can be seen.

In the gut lumen of the oligochaete, the myxospore attaches to the gut epithelium by firing filaments from the polar bodies (El-Matbouli and Hoffmann 1998). Sporoplasms then migrate to the inter-cellular spaces in the epithelium and undergo a cycle of sexual and sporogonic reproduction (El-Matbouli and Hoffmann 1998). The entire process of reproduction and multiplication requires between 80 and 120 days. The time required for

development of the TAM stage is dependent on temperature and possibly other environmental factors. When complete, the process results in the release of mature TAMS from the oligochaete. Individual worms may release as many as 8,000 TAMS (Dan Gustafson, Montana State University Ecology Department, personal communication).

The mature TAMS are neutrally buoyant and remain suspended in the water column. TAM structure consists of a central style about 150 m in length, which contains the active sporoplasts, typically 64 in number, surrounded by three radially symmetric processes (El-Matbouli and Hoffmann 1998). Three polar cells crown the style and when a fish contacts the TAM, these polar cells fire filaments that attach, allowing the TAM to inject the sporoplasts into the fish s epidermis (El-Matbouli et al. 1995). The caudal fin and the mouth cavity of the fish are believed to be the most successful areas of entry for the parasite (Hedrick et al. 1998).

The parasite migrates primarily to cartilaginous tissue, including the cranium, via the nerves of the central and peripheral nervous systems, a process that requires about three weeks (El-Matbouli et al. 1995). The parasite then undergoes an asexual reproductive cycle, which results in the formation of numerous mature myxospores. These myxospores are released into the environment when the fish dies or is consumed by either aquatic or terrestrial predators (El-Matbouli and Hoffman 1991). When the myxospores are ingested by *T. tubifex*, the parasite life cycle is complete.

Despite the wide distribution of both *M. cerebralis* and *T. tubifex* in salmonid bearing waters of the United States, declines to the fishery have been documented in only a few areas (Nehring and Walker 1996, Vincent 1996). Even in southwestern Montana, one of

the most affected regions, many waters that have tested positive for the parasite retain high fish populations and appear to have suffered no ill effects to date from the presence of the parasite (Baldwin et al. 1998).

The causes of this spatial variation in disease severity are likely the result of numerous interacting biotic and abiotic factors. A study of epizootiology suggests that the severity of a disease outbreak is in part related to the abundance of the disease-causing organism in a susceptible population (May and Anderson 1979, Hedrick 1998, Reno 1998). Determining the reasons for the varying pattern of salmonid disease severity will provide greater insight into the dynamics of this epizootic, and may suggest actions that will minimize harm to the fishery. The goal of my research was to investigate the relationship between *M. cerebralis* and aquatic oligochaetes, particularly *T. tubifex*. To this end, a series of controlled experiments were conducted at the Wild Trout Research Laboratory on the campus of Montana State University.

My thesis details four experiments I conducted during a two-year period beginning in 1997. My first two experiments investigated the level of susceptibility of several geographic variants of *T. tubifex* to infection with *M. cerebralis*. Experiment three tested the effect of varying the dose of *M. cerebralis* myxospores to which two populations of *T.* tubifex were exposed upon the abundance of TAMS ultimately produced. The final experiment was designed to ascertain the effect of temperature on TAM production, using three geographic variants of *T. tubifex*.

These experiments were based on the premise that the greater the number of TAMS released into a susceptible salmonid population, the greater the severity of disease was likely to be. This assumption has been supported by recent laboratory experimentation at

Montana State University, which demonstrated a correlation between the number of TAMS to which young trout were exposed and the resultant level and severity of infection (Ryce et al.1998).

METHODS

Variant Experiment I

The first experiment was designed to determine whether several geographic variants of *T. tubifex* produced different numbers of TAMS under similar conditions in a laboratory setting. One researcher had previously reported that genetic variants of *T. tubifex* displayed different growth and reproductive rates under similar laboratory conditions (Anlauf 1994). These findings suggest that the response to infection from a parasite, such as TAM production, may also vary among geographic variants. I examined TAM production of three populations of *T. tubifex,* originating from different geographic areas, when worms were challenged with similar doses of myxospores. Any variance found in TAM output can be compared to disease levels in the region of origin.

In order to test for factors affecting TAM abundance, I first established self-sustaining laboratory populations of target aquatic oligochaetes. The oligochaetes used in the following experiments were the progeny of either individuals gathered directly from the field or live samples received from other research laboratories. Mixed species mass cultures were established for oligochaetes from several sites in Montana, including the Gallatin and Madison River drainages, as well as from populations in other western states, the Great Lakes region, and the countries of Spain and Argentina.

Mixed cultures of oligochaetes from various geographic regions were held in 1000-ml beakers containing approximately 10 cm of sand. The beakers were placed in aerated aquaria filled with dechlorinated tap water and held at room temperature in the Wild

were maintained at 15-17... C, although temperatures outside this range did occur occasionally. Water in the aquaria was changed biweekly..

Most aquatic oligochaetes, including those species used in these experiments, subsist upon heterotrophic microbes harvested from the sediments that the worms ingest (Brinkhurst and Gelder 1991). Brinkhurst and other researchers have successfully employed a variety of organic materials as a growth medium for this microbial diet (Kaster 1978, Brinkhurst and Gelder 1991). In the laboratory, either frozen lettuce fragments or dehydrated *Spirulina* discs were fed to the oligochaetes on a weekly basis by pushing the food down into the substrate. Care should be taken not to over feed the cultures, as the biological oxygen demand may rise to the point that the oligochaetes can succumb to anoxia.

For the purposes of this investigation, it was necessary to establish monocultures of specific geographic variants of *T.tubifex*. Because positive identification of species can only be determined by microscopic examination of fixed, mature specimens, an indirect route to the establishment of these cultures was needed. Mature live worms from the various mass cultures were isolated individually in the cells of 12-well culture trays and monitored. The culture trays were held in several cm of water in 10-gallon glass aquaria and covered from direct light. The small capacity of the wells required an exchange of fresh water approximately every three days. Small fragments of food were added to the wells when no undigested food was visible.

Worms were maintained and monitored until reproductive cocoons or live young were observed within the individual well. (See Brinkhurst and Gelder 1991 for a discussion of the reproductive cycle). Following reproduction, the mature worms were euthanized in a

dilute ethanol solution and fixed in 10% formalin. The fixed worms were then slide mounted and identified using the key of Brinkhurst (1986).

The offspring of the identified worms were used to found pure cultures of *T. tubifex* from various geographic regions. Monocultures were maintained in a manner similar to mixed cultures. Monocultures of the following geographic variants of *T. tubifex* were established:

> Mount Whitney Fish Hatchery, California (CA) (from R. Hedrick, University of California, Davis)
>
> A pond tributary to the Gallatin River, Montana (GR) (from E. MacConnell, U. S. Fish and Wildlife Service, Bozeman, MT)
>
> Madison River, Montana (MR) (From B. Kerans, Montana State University, Bozeman, Montana)
>
> Willow Creek, Jefferson River, Montana (WC) (from M. Rognlie, Montana State University, Bozeman, Montana.)
>
> Great Lakes (GL) (from T. Reynoldson, National Water Research Institute, Burlington, Ontario, Canada)
>
> Spain (SP) (from T. Reynoldson, National Water Research Institute, Burlington, Ontario, Canada)

All worms were collected from substrate in the respective water bodies. The CA worms were chosen because they have a history of producing TAMS in laboratory experiments, and the hatchery was the site of a serious whirling disease outbreak (Modin 1998). The GR worms were collected from a spring pond on the grounds of the Bozeman Fish Technology Center, which is located on a tributary to the Gallatin River. The Gallatin had never tested positive for the parasite prior to the start of these experiments. The worms from the Madison River (MR) and Willow Creek (WC) were chosen because both had suffered large declines in rainbow trout populations that had been attributed to

whirling disease. The Great Lakes (GL) worms were chosen because they had demonstrated resistance to the parasite in other experiments (Katherine Beauchamp, University of California-Davis, personal communication). The Spanish (SP) variant was chosen in hopes of broadening the geographic range covered by this experiment, and given the European origin of both the parasite and the worms, the SP variant was expected to produce TAMS.

In addition to the cultures of *T. tubifex*, I also established pure cultures of two other common aquatic oligochaete species from worms collected from the Gallatin River and Madison River drainages, respectively. The additional species were *Limnodrilus hoffmeisteri* (LH) and *Ilyodrilus templetoni* (IT) (from D. Gustafson). Attempts to establish a laboratory population of a third common species, *Rhyacodrilus sp*, were unsuccessful.

The variants tested in this experiment were the Great Lakes (GL), the Spanish (SP), and the Willow Creek (WC). Six replicates of each variant were selected in groups of 100 oligochaetes that were picked from the monocultures, resulting in 18 replicate groups. Three groups of 100 oligochaetes were randomly assigned as positive replicates from each variant, destined for exposure to *M. cerebralis* myxospores, while the remaining three groups per variant were assigned as negative controls and not exposed to myxospores. All groups were wet weighed by placing each 100-worm group in plastic weighing dishes that had been tared. Excess water was blotted off the worms using a paper towel. Weights were recorded to the nearest tenth of a gram. After being weighed, each group of 100 worms was held in aquaria water for 24 hours without substrate or food to stimulate hunger. This experiment was begun on December 1, 1997.

Following the period of fasting, the groups were transferred to individual plastic food storage containers measuring 14cm x 7cm x 5cm. Each container held approximately 2 cm of autoclaved sand as substrate. Positive replicates were inoculated with 3,000 *M. cerebralis* myxospores per worm. All myxospores were collected from a single source, young cutthroat trout (*Oncorhynchus clarki*), which had been exposed to the parasite in the wild and held for three months in aquaria in the Wild Trout Laboratory. The fish were surplus from another experiment, and were provided by the researchers, Matt Clow and Dr. Calvin Kaya, Montana State University. All fish were euthanized with MS 222 (tricaine methanesulfonate) and utilized the same day. All myxospores were extracted using the plankton centrifuge method (O'Grodnick 1975) and quantified by replicate hemacytometer counts. Myxospore viability was tested using the methylene blue method (Hoffman and Markiw 1977), which indicated about 90% of the myxospores were viable.

The containers were filled with de-chlorinated tap water, fitted with lids, and aerated. The containers were assigned random positions on two adjoining shelves in the Wild Trout Laboratory at Montana State University, and black plastic sheeting was draped in front of them to minimize light infiltration.

All experimental populations were fed frozen lettuce on a weekly basis. The water was exchanged weekly, and beginning sixty days post-exposure was examined for the presence of TAMS. The water was siphoned from each container using a bulb syringe and filtered through a 20- m mesh. The mesh was then back-flushed into 50-ml plastic tubes using de-ionized water to a volume of 10-ml. Each tube was stirred gently and two 20- l aliquots were withdrawn using a pipetter. The two filtered aliquots from each replicate were placed on glass slides and covered with 22-mm cover slips. The slides

were examined for TAMS with a compound microscope at 100x magnification. Each aliquot was scanned in its entirety. Small imperfections in the cover slip edges were used as visual markers to ensure that no part of the slide was missed or counted twice.

Sub-samples of worms from all *T. tubifex variants* were twice chosen to test for presence of *M. cerebralis* DNA using the polymerase chain reaction (PCR). The first test was conducted three weeks post-exposure, and a second round was conducted six weeks later. All PCR was done at the US Fish and Wildlife Service Fish Health Laboratory in Bozeman, Montana, under the supervision of laboratory personnel. The PCR methodology followed one developed at the University of California at Davis (Andree et al. 1998).

This experiment was concluded on June 30, 1998, approximately seven months after it began. All worms in each replicate were individually counted.

Variant Experiment II

The goals of the second experiment were similar to the first; i.e., to test for significant variation in the response of several geographic variants of *T. tubifex* to infection with *M. cerebralis* myxospores. The primary measures of response were TAM production and impacts to oligochaete survival and reproduction. In addition, this experiment tested two additional oligochaete species for the ability to propagate *M. cerebralis*.

Three different geographic variants of *T. tubifex* were chosen for this experiment. The CA group was chosen because it had been used successfully on numerous occasions to produce TAMS in other laboratories and was believed to be the most susceptible group

among the cultures available in our laboratory. The MR group was selected because of the history of severe whirling disease impacts to the fishery in the Madison River (Vincent 1996). Conversely, the GR group was tested because the Gallatin River had, at the start of the experiment, never tested positive for the presence of *M. cerebralis,* leading to speculation that this variant might be resistant to the parasite.

The two additional species tested were *Limnodrilus hoffmeisteri* (LH) and *Ilyodrilus templetoni* (IT). Both are among the species most commonly encountered with *T. tubifex,* (Brinkhurst 1970), and were viewed as potential candidates to act as hosts for *M. cerebralis* (see Variant Experiment I for a description of methods used to establish laboratory populations).

Six groups of 100 oligochaetes each were selected from each of the five test populations, giving 30 experimental replicates. Prior to exposure, all worms were held for 48 hours in water without food or substrate. Each species or variant group was randomly selected as a positive treatment replicate or negative control, thus resulting in three replicates per group designated for exposure to the parasite.

Following the 48 hour holding period, each 100-oligochaete group was placed in a plastic food container measuring 9cm x 9cm x 5cm, each containing approximately 1cm of autoclaved sand substrate. The containers were filled with tap water from the Wild Trout Laboratory, from which chlorine and trace metals were filtered.

Separate bulb pipettes were used to inoculate each positive treatment replicate with an aliquot of myxospore extract calculated to contain approximately 10^5 *M. cerebralis* myxospores, a dose of 1,000 myxospores per worm. The myxospores used in this experiment were extracted from several sources, and were mixed together prior to use.

The spores were obtained from rainbow trout exposed in Willow Creek, Montana, and held in a living stream at the Wild Trout Laboratory; from rainbow trout experimentally infected at the Wild Trout Laboratory and held in a living stream; and from rainbow trout captured in the Missouri River near Great Falls, Montana, supplied by the Montana Fish, Wildlife, and Parks Department. The Missouri River fish were received and held frozen at the Wild Trout Laboratory until used in this experiment, whereas the remaining fish were euthanized with MS 222 (tricaine methanesulfonate) and utilized the same day. All myxospores were extracted as in the previous experiment.

Negative controls were inoculated with an equivalent aliquot of an emulsion obtained by processing *M. cerebralis*- free fish. This was done to insure that nutrient levels in all groups were initially equivalent. Replicates were assigned random locations on two adjacent shelves and individually aerated with aquarium air pumps. This experiment was conducted under conditions of ambient light and temperature in the oligochaete section of the Wild Trout Laboratory.

This experiment was begun on May 29, 1998. All replicate populations were fed small quantities of dehydrated *Spirulina* beginning three days post-exposure and continuing weekly thereafter. The *Spirulina* was in the form of discs about the size of a dime. Each replicate would receive a small fragment of a disc, approximately 3 mm^2, at each feeding. All replicates received weekly changes of water. Water temperature in the replicates was monitored weekly and consistently found to range from 13¡ to 17¡ C. Water was changed using a suction-bulb pipetter, and negative replicates were processed first to minimize the risk of contamination with *M. cerebralis*.

Beginning forty-five days post-exposure, live oligochaetes from the positive replicates were examined microscopically for visual indication of parasite infection. The method was developed by Dr. Monsour El-Matbouli and demonstrated by him during a visit to the laboratory in July 1998. Individual worms were placed on glass slides upon which two 22-mm cover slips had been placed approximately 15 mm apart from one another. The live worm was placed between the two cover slips, and a third cover slip was placed over the worm, resting on the two lower cover slips. This method restricted the movements of the worm without causing injury. Five individual worms were examined at 400x magnification from each positive treatment group. This visualization technique was repeated at 60 days post-exposure.

Beginning sixty days post-exposure, all water drawn from the 30 experimental replicates was processed and examined for the presence of TAMS. The water was again filtered through 20- m mesh filters to trap any TAMS. The filters were back-flushed with deionized water into 50-ml plastic tubes and sealed with screw tops. Two 20- l aliquots of the filtrate were searched for TAMS, as described above in the methods section of the first experiment. All TAMs were enumerated. The mean of the two counts was extrapolated to calculate the total number of TAMS in each replicate during each time period. At the end of the experiment, I summed the TAM production on each day for each replicate to calculate total TAM production for each replicate. I used analysis of variance (ANOVA) followed by Tukey s Honest Significant Difference test (Tukey s HSD) to compare total TAM production among groups of *T. tubifex*.

At the conclusion of this experiment on October 27, 1998, all oligochaetes in all replicates were counted, using the method described in the previous experiment. Ten

individual worms from the three *M. cerebralis*-exposed variants of *T. tubifex* were shipped live to Dr. Charlotte Rasmussen at the Western Fisheries Research Center at the University of Washington in Seattle for genetic analysis. To determine the degree of genetic similarity between and among oligochaete groups, she used PCR to amplify two areas of the oligochaete genome, Random Amplified Polymorphic DNA s (RAPDs) and the internal transcribed spacer region 1 (ITS-1). RAPDs use a single primer of arbitrary sequence to prime PCR amplification to produce discrete, anonymous but reproducible bands from genomic DNA. Six different RAPD primers were used to assess genetic differences within and between the three experimentally infected geographic variants of *T. tubifex*. Amplification of the ITS-1 locus with two specific primers produces a single amplification product of varying size, which may serve as a valuable species or sub-species marker. Genomic DNA was extracted from the 10 individuals from each of the three *M. cerebralis*-infected variants of *T. tubifex* using the Nucleospin Tissue Kit (Clontech Laboratories, Inc).

Reproductive success was determined for each variant/myxospore treatment combination by calculating the population growth rate of each replicate, calculated as: (ln{final worm abundance} — ln{100; initial worm abundance})/64; the number of days of production. Two-way ANOVA was employed to test for significant reproductive effects between treatments. If the ANOVA was significant, I tested for specific effects by comparing whether daily growth rate of each variant differed between treatments with myxospores and those without myxospores using Student t-tests. Residuals from all ANOVAs were examined for normality using normal probability plots, box plots, and stem and leaf plots. Moreover, residuals were plotted against predicted values to examine

whether variances were homogeneous. All statistical analyses were conducted using the SAS program (SAS Institute 1998-1999).

Dose Experiment

The goal of this experiment was to ascertain whether TAM production varied when two variants of *T. tubifex* were exposed to different doses of *M. cerebralis* myxospores. This information would be useful to laboratory researchers by quantifying the optimal myxospore dose for maximum TAM production. The relationship between myxospore dose and TAM abundance might also assist field researchers in efforts to model disease severity if *M. cerebralis* myxospore densities in wild communities can be determined.

Laboratory experiments designed to produce the TAM life stage of *M.cerebralis* have employed a wide range of myxospore exposure doses (Markiw 1986, El-Matbouli and Hoffmann 1998, Gilbert and Granath Jr. 1998). The choice of spore dose has been largely a random one, predicated in part on the availability of viable myxospores or the pattern of previous experiments. By exposing equal numbers of susceptible oligochaetes to a controlled range of parasite myxospores, my goal was to investigate the relationship between spore dose and TAM production.

The pathology of infectious organisms is often density dependent, i.e., a sufficiently large host population or pathogen load is required to initiate the disease condition (Robinson and Bolen 1989, Reno 1998). By exposing the tubificid host to a range of parasite densities, I attempted to uncover some variation in response (TAM production, reproductive success) that would allow researchers to quantify thresholds of critical host/parasite relative density.

I began this experiment on December 6, 1998, testing the effect of myxospore dose upon TAM production in the CA and MR variants of *T. tubifex*. The experiment was conducted as a double blind, in which I did not know the identity or treatment group of the individual replicates. I picked a total of 1200 oligochaetes from each of the two source populations, which are maintained in mass cultures at the Wild Trout Laboratory at Montana State University (see Variant Experiment I for a description of methods used to establish laboratory populations). The worms were picked in groups of 100, resulting in twelve replicate groups from each variant. Each 100-worm group was wet-weighed and held for 24 hours without food in dechlorinated tap water.

Each replicate was randomly assigned to one of four treatment groups, and received a dose of 0, 50, 500, or 1,000 *M. cerebralis* myxospores per oligochaete on December 7, 1998. Myxospores used in this experiment were extracted from live rainbow trout (*Oncorhynchus mykiss*) that had been exposed to the parasite in the Wild Trout Laboratory and held in a living stream. Myxospores were extracted as in the previous experiments. Each 100-worm group was placed in a 250-ml plastic food container (9 x 9 x 5 cm) to which 40 ml of autoclaved sand substrate and 200 ml of dechlorinated tap water had been added. All replicates were individually aerated with aquarium pumps. Each of the 24 experimental containers were randomly placed on one of two shelves and held under conditions of ambient light and temperature (13-17 ° C). All replicates received a weekly water change and feeding of dehydrated *Spirulina*. Beginning approximately sixty days post-exposure, all water drawn from the replicates was filtered through 20-µm mesh to retain any TAMS present. Water was examined for TAMs and TAMS were enumerated as in the previous experiments.

On May 20, 1999 (164 days post-exposure), all the worms in each replicate were counted and wet weighed. Counting was carried out by sub-sampling portions of the sediment in large metal or plastic pans and hand picking all the *T. tubifex* observed, transferring the worms to small containers of dechlorinated tap water using forceps or suction droppers. This process was repeated until the entire contents of all the individual replicate containers had been processed. After weighing, the oligochaetes were returned to their replicate containers, along with the original sediments.

On June 25, 1999 (199 days post-exposure), this experiment was concluded and all oligochaetes were again counted and weighed as above.

I used two-way ANOVA to test whether the numbers of TAMS produced differed between variants and among myxospore doses, and to determine the significance of any interaction effect across variant and myxospore dose. Data were ln transformed to improve the homogeneity of the variances. Residuals from all ANOVAs were examined for normality using normal probability plots, box plots, and stem and leaf plots. Moreover, residuals were plotted against predicted values to examine whether variances were homogeneous. The number of days required for each replicate to release 50% of its total TAM production (T_{50}) was calculated and difference between variants were tested using a Kruskal-Wallis test. I used ANOVAs followed by Tukey s Pairwise Comparison Tests to determine whether worm weights and numbers differed among spore doses and worm variants at 164 and 199 days post-exposure. All statistical analysis was conducted using the SAS program (SAS Institute 1998=1999).

Temperature Experiment

My goal in this experiment was to examine TAM production among the same three variants of *T. tubifex* tested in Variant Experiment II, at controlled temperatures of 15¡ C and 8¡ C. The highly variable pattern of TAM production observed in Variant Experiment II may offer at least a partial explanation for the patterns of disease severity found in wild fish populations. However, environmental conditions may differentially affect the response of oligochaetes from varying geographic and climactic sources. This experiment tested TAM production at two temperatures to determine whether the TAM production results seen in the earlier experiments would be repeated, at both a similar and a colder temperature. The CA variant originated in an area with a warmer climate than either the MR or GR variants, raising the possibility that these latter variants might be better hosts for the parasite at colder temperatures.

This experiment was begun on February 1, 1999. Twelve replicate groups numbering 100 worms were selected from each of the CA, MR, and GR mass cultures of *T. tubifex*, giving 36 replicates (see Variant Experiment I for a description of methods used to establish laboratory populations).

Half of the replicates from each variant were randomly assigned to the cold temperature treatment (8° C), and half to the warm temperature treatment (15° C). Further randomization assigned half the replicates to the positive treatment (*M. cerebralis* exposed) group and the remaining half to the negative control treatment, resulting in three replicates of each variant per treatment per temperature.

All replicates were held for 24 hours without food or substrate as in previous experiments. Following the holding period each replicate group was wet-weighed and placed in 250 ml plastic food containers to which 40 ml of autoclaved sand had been added as substrate. Replicates were aerated and held in incubators under controlled light conditions with a twelve-hour light/dark cycle.

Myxospores were initially extracted from six euthanized rainbow trout as in previous experiments. This initial myxospore extraction resulted in relatively few *M. cerebralis* myxospores, allowing the positive treatment groups to be inoculated with only 75 *M. cerebralis* myxospores per individual following the fasting period. Negative controls received an equal volume of an emulsion obtained by processing parasite free rainbow trout. The parasite-free emulsion was added to the negative controls to eliminate any potential effect arising from unequal nutrient levels among treatments. Ten days later myxospores were extracted from 12 rainbow trout, allowing an additional 150 myxospores per worm to be added to all positive replicates, while negative controls again received an equal volume of fish emulsion. This resulted in a total dose of 225 *M. cerebralis* myxospores per oligochaete.

All replicates were given weekly water changes beginning one week after the start of the experiment. All replicates were fed small fragments of dehydrated *Spirulina* following the water exchange. Beginning 60 days post-exposure, the water withdrawn from the replicates was filtered through 20 m nytex mesh and examined for the presence of TAMS. TAMS were enumerated as in the previous experiments. Microscopic examination of a small number of randomly selected worms was also begun at this time to look for evidence of *M. cerebralis* infection, as described in the previous experiments

This experiment was concluded on September 11, 1999, at which time all worms were counted.

I used two-way ANOVA to test the significance of total TAM production across variant and temperature treatments. I examined reproductive success employing three-way ANOVAs to test variance in worm numbers at the end of the experiment, with the data ln-transformed to stabilize variances. Any significant interaction effects were examined with Student-t tests in order to further refine the analysis.

I also calculated the number of degree-days required at each temperature for the development and release of TAMS. Degree-days were determined by calculating the number of days required for TAM release multiplied by the temperature, with 0° C used as the baseline temperature.

All statistical analysis was conducted using the SAS program (SAS Institute 1998-1999).

RESULTS

Variant Experiment I

No TAMS were ever observed during this experiment. The experiment continued for approximately 200 days, well beyond the time required for the development of TAMS in other laboratory experiments (Markiw 1986, Gilbert and Granath Jr. 1998). Our method of searching for TAMS was tested and verified using TAM-positive filtrate provided by Elizabeth McConnell of the U.S. Fish and Wildlife Service, and was successful in detecting TAMS.

Results of the PCR analysis were mixed. The first PCR run included both the positive and negative exposure replicates, as well as oligochaetes taken from the original source cultures. The PCR detected *M. cerebralis* DNA in 7 of 9 positive replicates, including all three variants. However, two of the negative controls also tested positive for the parasite genetic material, as well as the GL source culture, indicating contamination during the PCR process. The second PCR analysis was conducted approximately six weeks after the first and again included positive, negative, and source group replicates. In this run, no negative replicates or source culture replicates tested positive. However, only 4 of 9 *M. cerebralis* exposed replicates tested positive at this time.

Enumeration of replicate worm populations at the conclusion of the experiment demonstrated that all groups had successfully reproduced regardless of treatment. Ending populations in all replicates exhibited high reproductive rates as each replicate contained in excess of 1000 worms at the conclusion of the experiment.

Variant Experiment II

No evidence of infection was observed during the visual examination of replicate oligochaetes conducted forty-five days post- exposure. Beginning sixty days post-exposure, I observed what appeared to be clusters of developing parasites within the gut of the *M. cerebralis* exposed worms from the CA group, selected at random from one of the exposed replicates. Several days later the remaining two variants of *T. tubifex* also exhibited visual evidence of infection.

TAMS were first observed in filtrate from the CA and MR variants 84 days post-exposure, and were identified as *M. cerebralis* on the basis of size and morphology. TAM enumeration began 86 days post-exposure and continued for 64 days. TAMS were observed in filtrate from two GR replicates ten days later or 96 days post-exposure. TAMS were not observed in the remaining GR replicate at any time during the experiment.

The CA variant was the most prolific TAM producer followed by the MR and GR variants respectively (Table 1). Variant of *T. tubifex* was a significant predictor of TAM output (ANOVA, $F_{2,6} = 31.88$; p-value = .0006;). The CA variant produced significantly more TAMS than either the MR or GR variants, (Tukey s HSD, p-value< 0.05). Pairwise tests between the MR and GR variant did not find a significant difference in TAM production. Data were not transformed because all normal probability plots, box plots, and stem and leaf plots indicated a good fit with normality and variances were homogeneous.

Table 1. Mean of total TAM production, standard error of mean, average daily TAM production, by *T. tubifex* variant.

T. tubifex Variant	Mean Number of TAMS Produced – 1 Standard Error	Average Daily TAM Production
California (CA)	1,244,741.7 – 149,827.2	19,449
Madison River (MR)	146,300.0 – 145,250.1	2,286
Gallatin River (GR)	1,083.0 – 845.7	17

Total TAM production was remarkably consistent among the CA replicates, whereas the MR replicates displayed the greatest variability among the three groups (Table 1). The GR replicates produced few TAMS (Table 1),

The CA and MR replicates produced TAMS throughout the entire experiment. However, the GR replicates produced TAMS for a much shorter period (Figure 1).

No TAMS were observed in filtrate from the *M. cerebralis* exposed replicates of *L. hoffmeisteri* or *I. templetoni* at any time during the experiment. No TAMS were observed in the negative control replicates of the three species.

As noted above, reproductive success was determined for each variant/myxospore treatment combination by calculating the population growth rate of each replicate, calculated as: (ln {final worm abundance} — ln {100; initial worm abundance})/64,the number of days of production. Among all oligochaete replicates, reproductive success was lower when worms were exposed to *M. cerebralis* than when not exposed (ANOVA, $F_{1,20} = 21.28$, p-value = 0.0002) (Figure 2). Reproductive success differed between *T. tubifex* populations and other tubificid species (ANOVA $F_{4,20} = 9.72$, p-value = 0.0002),

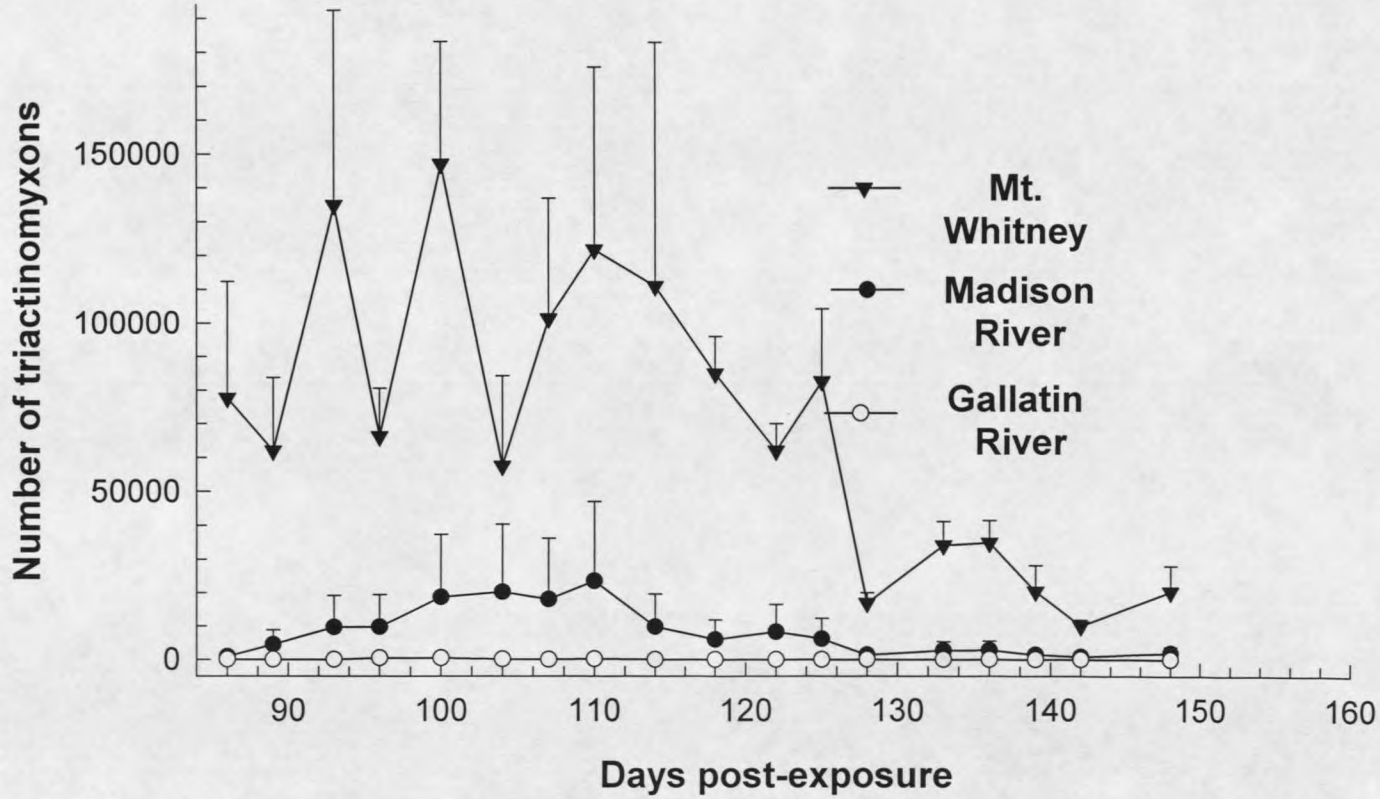

Figure 1. Total TAM production by geographic variant. Error bars indicate one standard error.

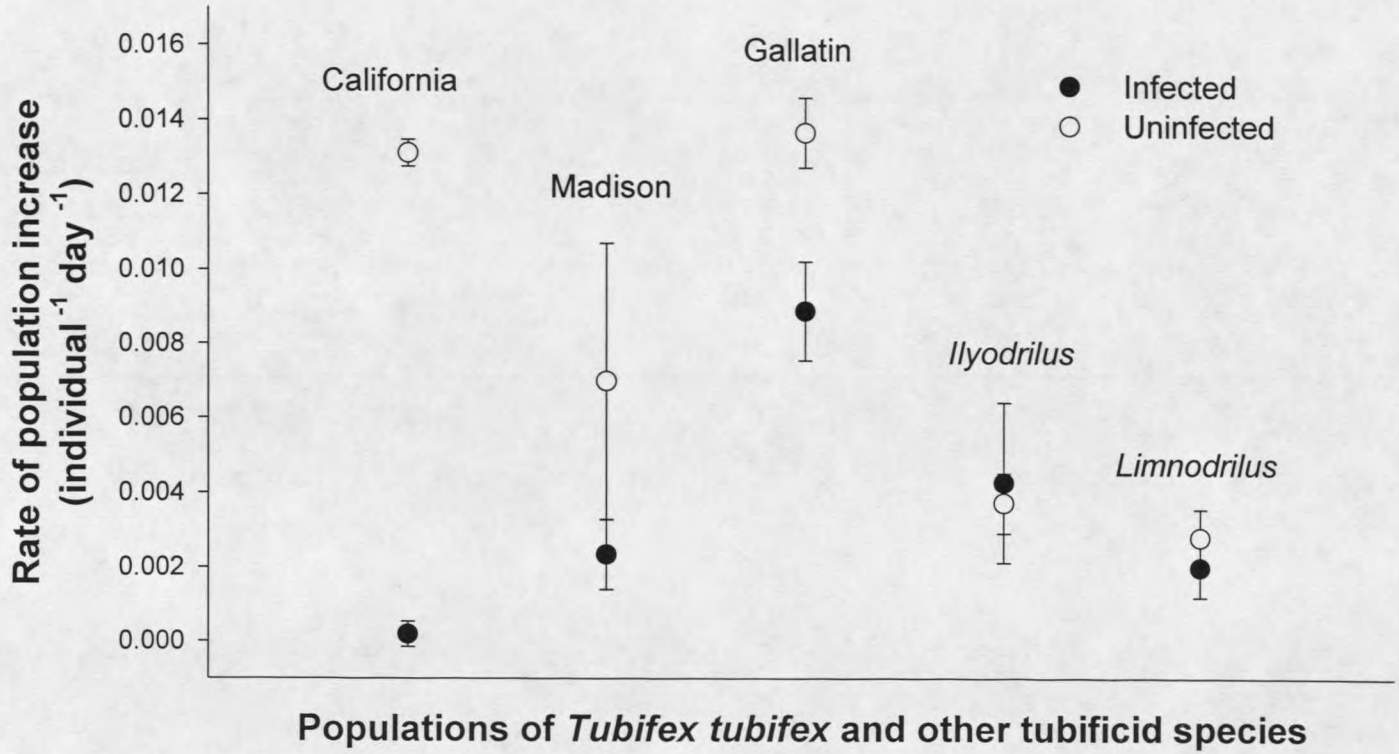

Figure 2. Rate of population increase by geographic variant and *M. cerebralis* exposure. Error bars indicate one standard error.

and populations differed in their response to exposure (ANOVA interaction effect, $F_{4, 20} = 5.74$, p-value = 0.0030). Student *t*-tests used within variants to explore the interaction demonstrated that the degree of reproductive success was significantly lower in *M. cerebralis* exposed populations of the CA ($t_4 = 26.03$, p-value < 0.0001) and GR ($t_4 = 2.94$, p-value = 0.0424) variants, but not the MR variant ($t_4 = 1.21$, p-value = 0.2914). Moreover, rate of population increase was not significantly different between exposed and unexposed populations of *L. hoffmeisteri* and *I. templetoni* (both p-values > 0.1758).

The pattern of reproductive success among the positive replicates for each variant was inversely related to TAM production. Thus, the CA variant, which produced the greatest number of TAMS, had the lowest rate of reproductive success, while the GR variant, which produced the fewest TAMS, enjoyed the greatest reproductive success. The MR variant was intermediate. The remaining two species of oligochaetes did not display any significant reproductive consequence of exposure to *M. cerebralis*.

A comparison of the banding patterns produced with each of the six RAPD primers indicated that the individuals from the CA culture were genetically similar. Likewise, no polymorphic bands were detected in the individual worms from the GR culture, indicating that *T. tubifex* individuals from this group were also genetically similar. However, the RAPD banding patterns produced by the GR individuals differed from those seen in the CA culture. PCR amplification of the individuals from the MR culture using five of the six RAPD primers produced a number of polymorphic bands indicating that genetic variation existed within this culture. A comparison of the banding patterns produced by several of the RAPD primers indicated that the MR culture shared a number

of bands both with the CA culture and the GR culture whereas the CA and GR individuals did not appear to share RAPD banding patterns.

Amplification of the ITS-1 locus resulted in clear size differences between *T. tubifex* individuals from the three different cultures. Amplification of this locus produced an 850 base-pair (bp) product in the replicates from the CA variant. The same locus in the MR variant produced an 800 bp product, while the BFTC replicates produced a product approximately 750 bp in size.

Dose Experiment

TAMS were observed in filtrate from replicates of both variants 87 days post-exposure. The CA variant was the most prolific TAM producer by approximately one order of magnitude (Table 2).

Table 2. Mean total TAM production by variant/spore dose – one standard error.

Variant/Spore Dose	Total TAMS (mean of three replicates) – one standard error
MR 50	31,417 – 3,874
MR 500	29,250 – 11,861
MR 1000	34,750 – 9,148
CA 50	227,667 – 52,747
CA 500	397,667 – 101,014
CA 1000	237,500 – 13,013

Statistical analysis indicated that variant was a significant predictor of TAM production (ANOVA, $F_{1, 12} = 79.16$; p-value = 0.0001). The fit of the log-transformed data with normality appeared good, based upon the normal probability plot, box plot, and

stem and leaf plot of residuals. Total TAM production did not differ among myxospore doses (ANOVA, $F_{2, 12} = 0.04$; p-value = 0.9591), nor was the interaction of dose and variant significant (ANOVA, $F_{2, 12} = 1.18$; p-value = 0.3394). The CA variant produced more TAMS than the MR variant at all dose levels.

Both the CA (Figure 3) and MR (Figure 4) oligochaetes produced TAMS over the entire duration of the experiment. The Kruskal-Wallis test of the time required to release 50% (T_{50}) of the eventual total number of TAMS for each replicate revealed no significant difference among myxospore doses ($\chi^2_5 = 7.733$; p-value = 0.1716). The days required to release 50% of the total TAMS ranged from 113.3 to 149.0 for the CA variant, and 142.7 to 149.7 for the MR variant.

At the beginning of the experiment the MR variant had significantly higher biomass per replicate than the CA variant, (ANOVA, $F_{1, 16} = 75.37$; p-value = 0.0001), indicating that individual MR worms were heavier than CA worms (Table 3). However, total biomass did not differ significantly among dose groups, (ANOVA, $F_{3, 16} = 0.57$, p-value = 0.6426) or with the interaction of dose and variant (ANOVA, $F_{3, 6} = 0.88$, p-value = 0.4706).

At 164 days post-exposure, both variants had significantly higher biomasses, (ANOVA, $F_{3, 16} = 31.79$, p-value = 0.0001) and worm abundances (ANOVA, $F_{3, 16} = 9.99$, p-value = 0.0006) in negative controls than in myxospore positive replicates (Table 3). Tukey s Pairwise Comparisons verified that worm biomasses and abundances were greater in the negative controls than all myxospore positive groups (p-value < 0.05 for all comparisons). There were significant differences between variants in both worm biomass

(ANOVA, $F_{1,16} = 11.82$, p-value = 0.0034) and worm numbers (ANOVA, $F_{1,16} = 6.55$, p-value = 0.0210). The ANOVA found no significant interaction effect upon either parameter (all p-values > 0.1413).

Negative controls again had significantly greater biomass at 199 days post-exposure (ANOVA, $F_{3,16} = 8.60$; p-value = 0.0012) (Table 3). Total abundance of worms was also significantly lower in myxospore positive replicates relative to negative controls at this time (ANOVA, $F_{3,16} = 4.26$; p-value = 0.0216). However, Tukey s Pairwise Comparison found the difference significant only in the 1,000-myxospore dose replicates. Variant was again a significant predictor of total biomass (ANOVA, $F_{1,16} = 20.92$, p-value = 0.0003), but was not significant relative to worm abundance (ANOVA, $F_{1,16} = 0.00$, p-value = 0.9706).

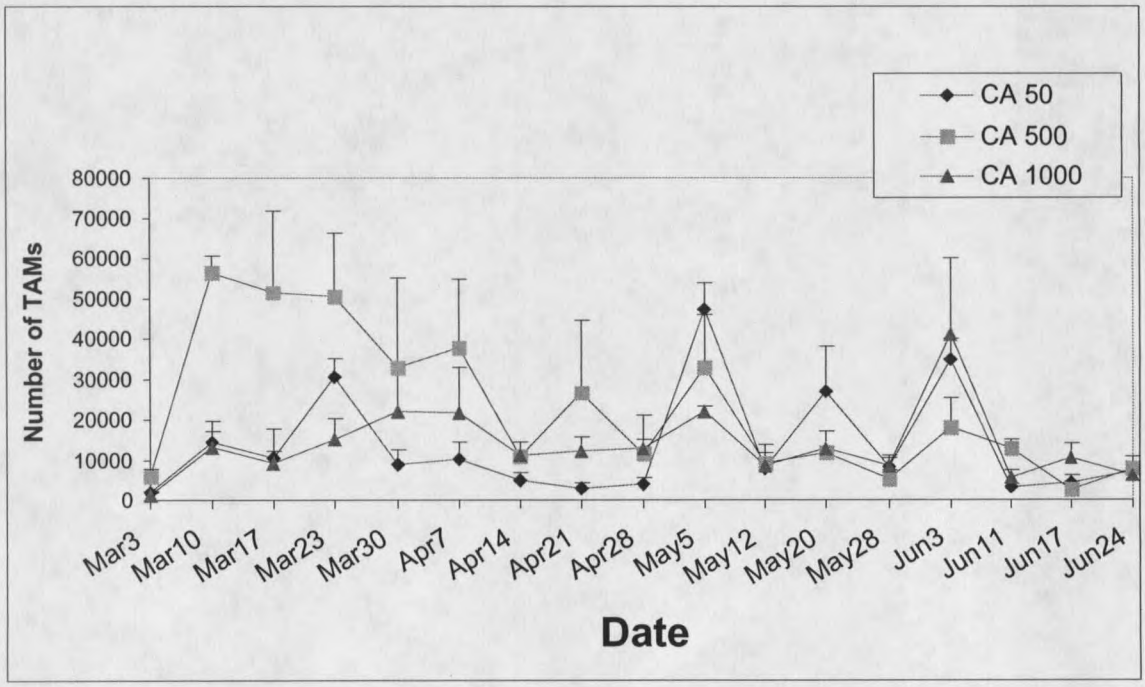

Figure 3. TAM production from CA variant of *T. tubifex* at varying doses of *M.cerebralis* spores. Mean of three replicates plus one standard error.

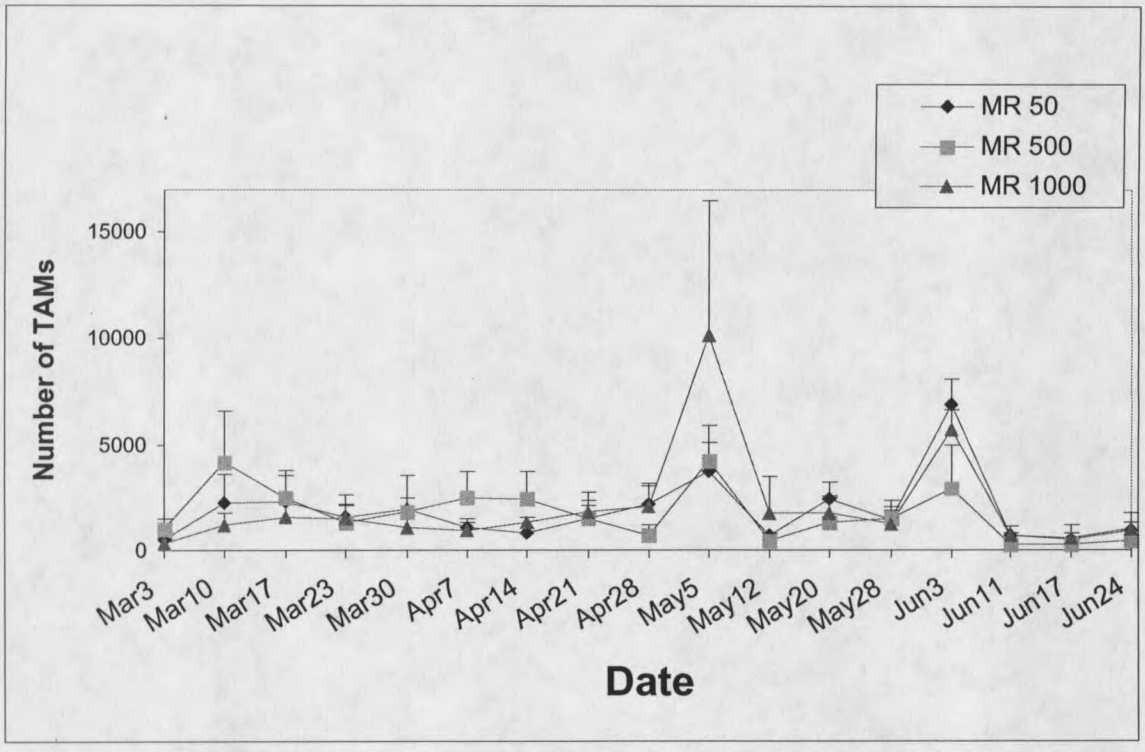

Figure 4. Tam production from MR variant of *T. tubifex* at varying doses of *M. cerebralis* spores. Mean of three replicates plus one standard error.

Table 3. Mean number and weight of worms by variant and dose, plus 1 standard error, 164 and 199 days post-exposure (PE).

Variant/Dose	Initial Mean Wt, mg, ± 1 SE	Mean Number, ± 1 SE, 164 Days PE	Mean Weight, mg, ± 1 SE, 164 Days PE	Mean Number, ± 1 SE, 199 Days PE	Mean Weight, mg, ± 1 SE, 199 Days PE
MR 0	433.33 ± 46.31	200.33 ± 43.68	571.33 ± 86.13	108.33 ± 7.31	251.67 ± 51.72
MR 50	430.00 ± 11.55	83.67 ± 9.91	166.33 ± 18.44	80.33 ± 5.81	142.33 ± 25.86
MR 500	410.00 ± 37.86	83.33 ± 10.04	183.00 ± 29.31	70.33 ± 8.41	108.33 ± 13.87
MR 1000	426.67 ± 6.67	86.00 ± 8.50	217.67 ± 32.59	55.67 ± 13.62	99.00 ± 31.21
CA 0	200.00 ± 35.12	106.33 ± 9.02	649.00 ± 38.97	99.33 ± 16.48	439.67 ± 64.05
CA 50	160.00 ± 37.86	64.67 ± 11.84	228.33 ± 29.63	60.33 ± 17.65	178.67 ± 31.54
CA 500	216.67 ± 16.67	92.67 ± 9.67	325.33 ± 33.07	94.33 ± 8.67	263.33 ± 17.17
CA 1000	200.00 ± 10.00	66.67 ± 6.17	263.33 ± 31.80	61.67 ± 0.88	199.00 ± 36.36

Temperature Experiment

The visual examination of selected worms conducted approximately 70 days post-exposure revealed that worms from the positive CA and MR replicates were infected with developing *M. cerebralis* TAMS. The first TAMS were observed in the filtrate from the three CA and one of the MR positive treatment replicates which had been held at the warm (15¡ C) temperature at 89 days post-exposure. TAM enumeration began two days after the initial observation. The remaining two MR 15° C, *M. cerebralis* exposed replicates began producing TAMS within two weeks of this first TAM enumeration (Figures 5-6). No TAMS were observed in the GR warm positive replicates at any time during the experiment. Likewise, no TAMS were ever observed in any warm negative control replicates during the life of the experiment.

TAMS were first observed in the cold (8¡ C) treatment replicates of the CA variant 170 days post-exposure, and appeared in two of the MR cold positive replicates 14 days later (Figures 7-8). No TAMS were observed in the remaining MR cold positive replicate, or in any of the GR cold positive replicates, during the experiment. No TAMS were ever observed in any negative control 8° C replicates.

Statistical analysis of the relationships between the variables tested and the total TAM production did not produce a clear pattern. Variant was a significant predictor of overall TAM production between the CA and MR variants (the only variants to produce TAMS) (ANOVA, $F_{1,8} = 13.04$; p-value = 0.0069), with the CA variant again the more prolific TAM producer (Table 4). Temperature alone was not a significant predictor of TAM

production, (ANOVA, $F_{1,8} = 2.40$; p-value = 0.1602), nor was the interaction of variant and temperature significant (ANOVA, $F_{1,8} = 0.62$; p-value = 0.4523).

Table 4. Mean TAM production by variant, ± 1 standard error.

Variant	Mean Total TAM production at 15° C ± 1 standard error.	Mean TAM production at 8° C ± 1 standard error.
California (CA)	437,771.0± 95,624.6	245,500.0 ± 113,671.4
Madison River (MR)	75,833.3 ± 69,399.6	13,500.0 ± 13,126.8
Gallatin River (GR)	0	0

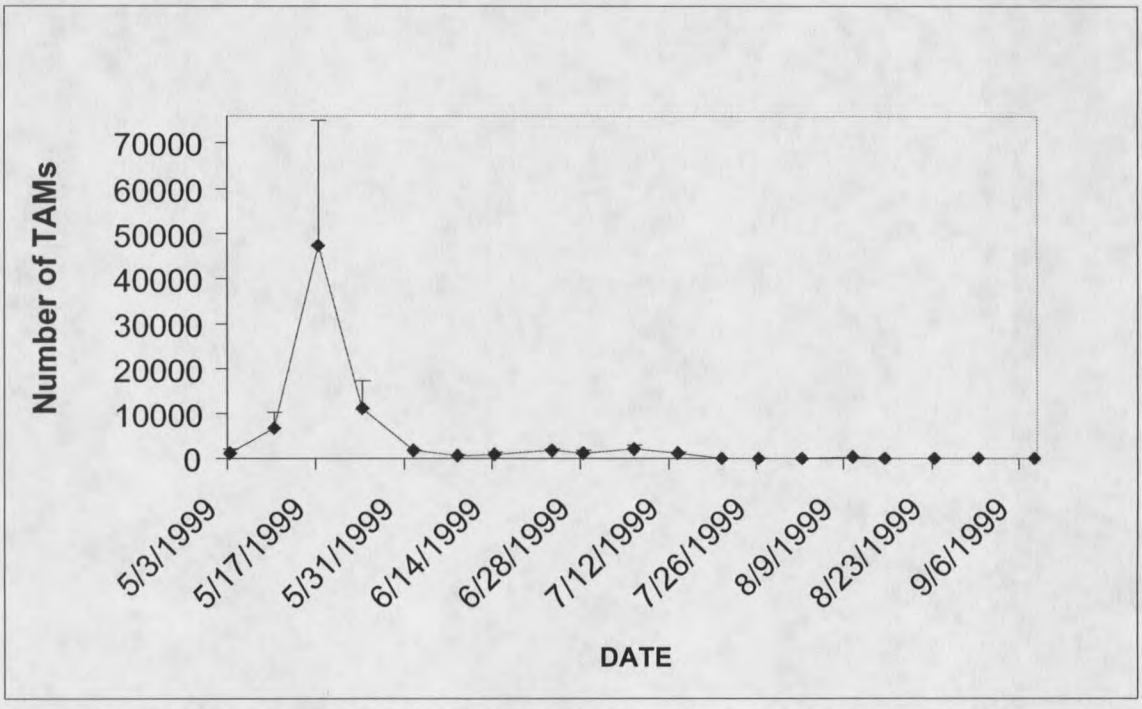

Figure 5. TAM production by Madison River *T. tubifex* at 15¡ C. Mean of three replicates plus one standard error.

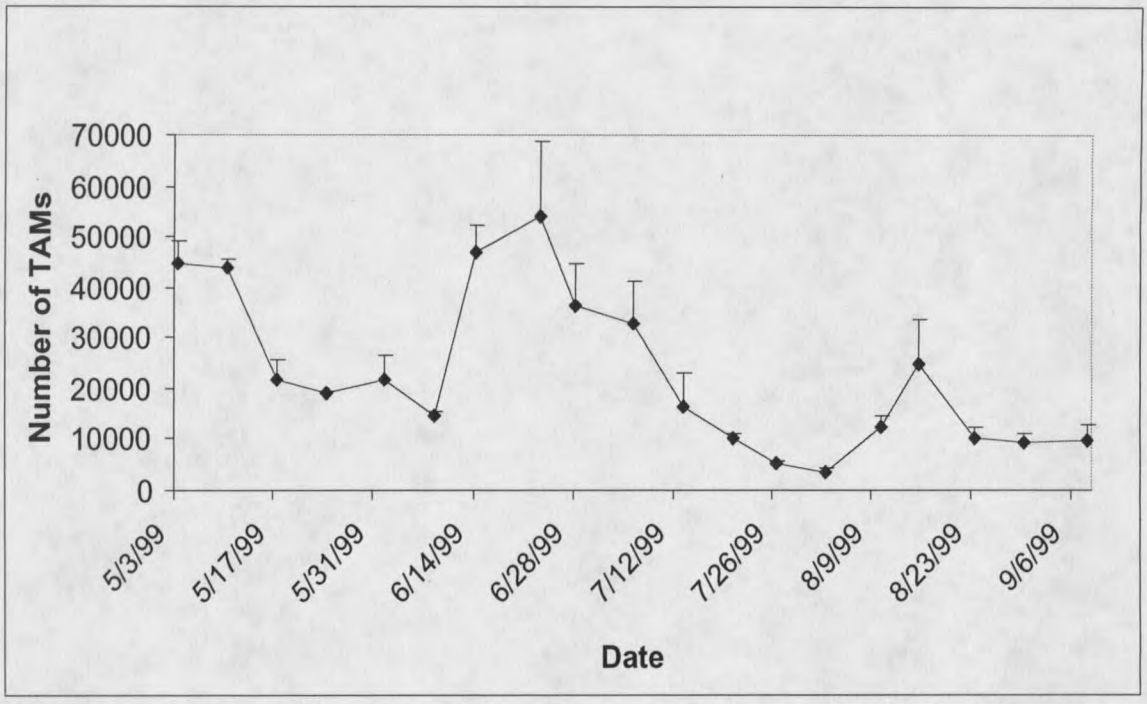

Figure 6. TAM production by California *T. tubifex* at 15¡ C. Mean of three replicates plus one standard error.

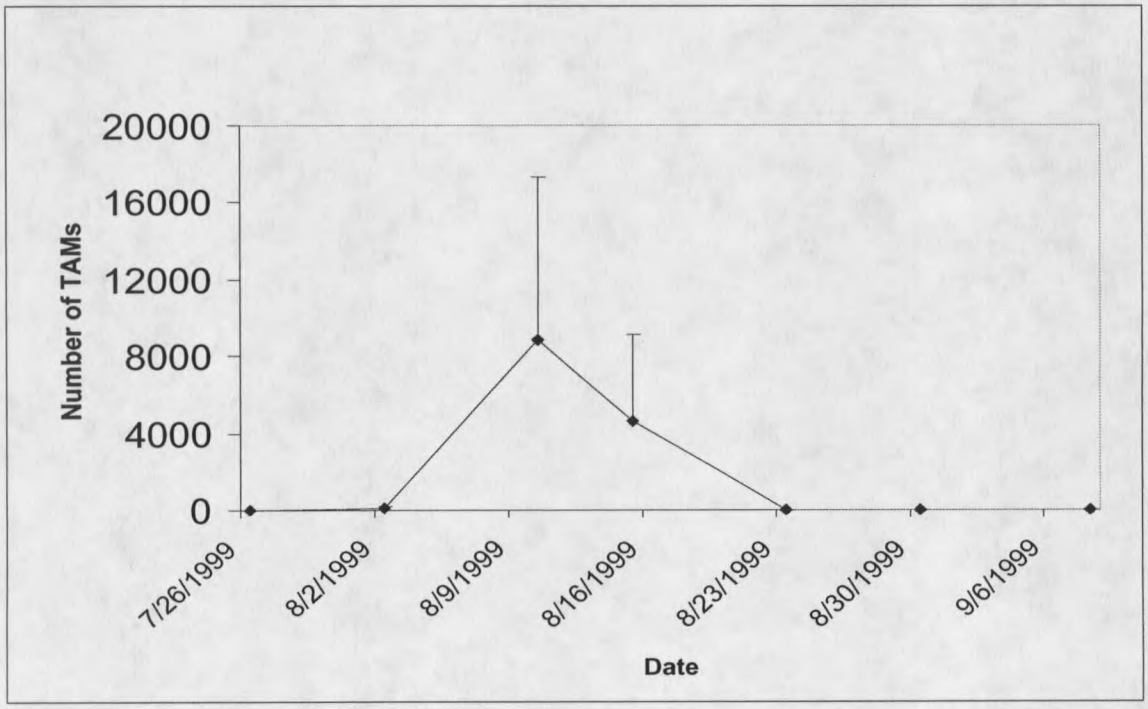

Figure 7. TAM production by Madison River *T. tubifex* at 8° C. Mean of three replicates plus one standard error.

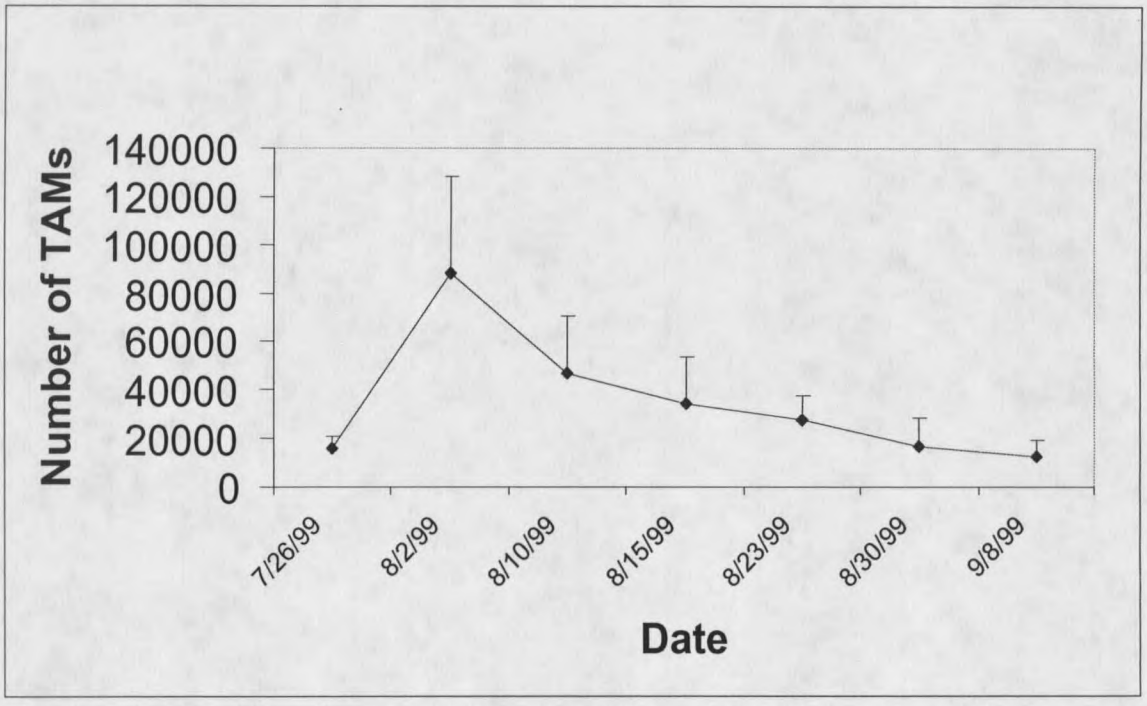

Figure 8. TAM production by California *T. tubifex* at 8¡ C. Mean of three replicates plus one standard error.

Analysis of the ln-transformed worm numbers demonstrated that variant was a significant predictor of reproductive success (ANOVA, $F_{2, 24} = 17.58$; p-value = <0.0001), as was exposure to *M. cerebralis* (ANOVA, $F_{1, 24} = 7.07$; p-value = 0.0137) (Figure 9). Temperature alone did not have a significant effect upon worm numbers, (ANOVA, $F_{1, 24} = 0.65$; p-value = 0.4290); however, the interaction between variant and temperature was significant (ANOVA, $F_{2, 24} = 8.84$; p-value = 0.0013). All other interactions were not significant (p-value > 0.05). Student t-tests revealed that temperature had a significant effect upon reproductive success for the GR variant ($t_{10} = 2.26$; p-value = 0.0472), which reproduced better at 8° C, and the MR variant ($t_{10} = -3.37$;

p-value = 0.0071), which had greater success at 15° C. Temperature did not significantly affect worm reproductive success for the CA variant (t_{10} = -0.69; p-value = 0.5071).

Total degree-days required for TAM release was calculated using 0¡ C as the developmental threshold. The 89 days required for TAM production at 15¡ C resulted in a degree-day total of 1335 (15 * 89), while the 8¡ C treatment required 1360 (8 * 170) degree-days.

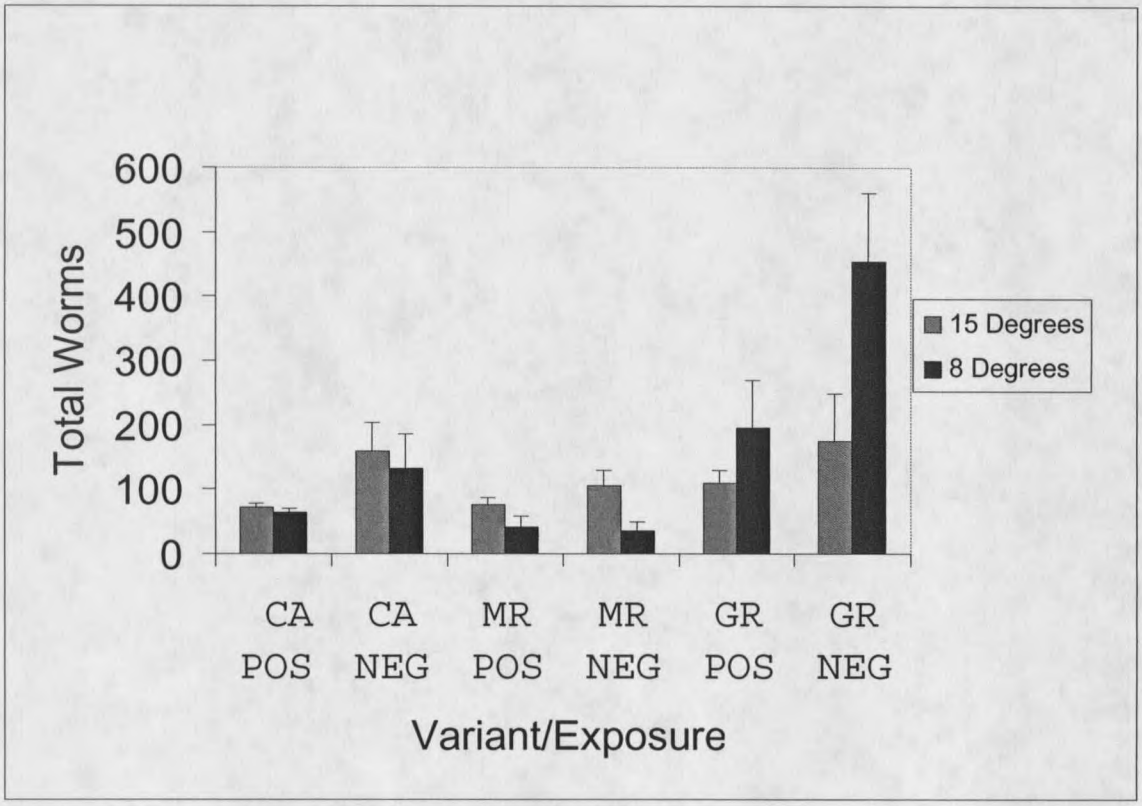

Figure 9. Mean number of worms by variant and *M. cerebralis* exposure, plus one standard error.

DISCUSSION

Variant Experiment I

The failure of this experiment to produce the TAM life stage of the parasite has not been positively explained. However, several possible factors may have contributed to the lack of results. Genetic analysis of the three variants of *T. tubifex* conducted at the US Geological Survey Laboratory at the University of Washington, Seattle, revealed that the Great Lakes and Spanish variants were closely related (C. Rasmussen, personal communication). Thus, if the Great Lakes variant was resistant to the parasite, it seems reasonable that the Spanish variant was as well. The homogeneity of these two populations is not surprising, given the history of the Great Lakes region as a receptacle for exotic species arriving by water (Leach 1995). The possibility also exists that the Great Lakes and Spanish variants may have been cross-contaminated prior to this experiment, and the test worms may actually represent hybrids of the two variants. In addition, the wrong worms may have been sent from the laboratory in Canada (T. Reynoldson, personal communication).

The failure of the Willow Creek variant to produce TAMS is more problematic however. These worms were collected from a stream in which fish held in live cages have consistently developed severe infection levels based upon clinical signs of whirling disease, population data, and the McConnell-Baldwin scale, a ranking system used by researchers to quantify the severity of *M. cerebralis* infection (Baldwin et al. 2000). It is possible that there were several distinct genetic variants of *T. tubifex* in the stream, and

the culture from which the worms were selected contained only a strain that was resistant to the parasite.

A more likely possibility involved the myxospores that were used in the experiment. Although the myxospores tested viable using the methylene blue method (Hoffman and Markiw 1977), it is unknown if viability equates with virulence. It is possible the myxospores required additional maturation in order to be capable of propagating the parasite.

A final potential explanation for the failure of this experiment to produce TAMS involves environmental variability in the laboratory during the experiment, specifically, fluctuations in the temperature. An earlier study found that temperatures of 25¡ C and higher caused *T. tubifex* to purge *M. cerebralis* regardless of the stage of development attained by the parasite (El-Matbouli et al.1999). While the temperature of the laboratory was typically 15¡ C – 2¡, problems with the heating/cooling system occasionally resulted in spikes of warmer temperature. Given the small volume of the experimental containers, the treatment groups would quickly equilibrate with the room temperature. The possibility that such a temperature spike caused the worms to rid themselves of the parasite was perhaps supported by the PCR analysis, which indicated a decreasing incidence of infection over time. However, the PCR data was too inconsistent to be considered very reliable.

Finally, it should be noted that other laboratories working with *T. tubifex* and *M. cerebralis* have also experienced difficulties propagating the TAM phase of the parasite. The host-parasite relationship is a complex one, and the failure of routine laboratory

experiments points out the need for continued research at scales ranging from the ecological to the bio-molecular.

Variant Experiment II

As noted in experiment one, distinct genetic races of *T. tubifex* have been identified and found to have varying responses to environmental conditions in a laboratory study (Anlauf 1994). My results suggest that intraspecific differences may also impact TAM production.

The results of the genetic analysis conducted by C. Rasmussen suggest that individual *T. tubifex* races can be distinguished based on the length of the ITS-1 amplification product. The ITS-1 region is located immediately downstream of the highly conserved 18S ribosomal RNA (18S rRNA) gene. This region of the genome is unlikely to undergo mutation, and may therefore serve as a marker for different *T. tubifex* subspecies, with differing levels of parasite resistance (C. Rasmussen, personal communication).

The analysis of the RAPD banding patterns also appears to correlate with the TAM data the most distantly related variants (C alifornia and Gallatin River) also displayed the greatest disparity in TAM output. The Madison River variant was intermediate on both counts. It would be premature, however, to conclude that the variation in TAM production is determined by the genetic makeup of the variant.

The results of this experiment add some evidence to the hypothesis that *T. tubifex* is the sole oligochaete host. Nonetheless, it would be premature to conclude that *L. hoffmeisteri* and *I. templetoni* do not propagate the parasite based upon the results of one

laboratory experiment. It is possible that these species will do so under different environmental conditions.

The parasitology literature contains numerous examples of host reproductive impairment occurring as a result of parasite infection (Forbes 1993). My data strongly suggest that *T. tubifex* reproductive success was significantly lower when *M. cerebralis* infection was present. As with the TAM production data, genetic analysis mirrored the patterns of reproductive success found in *M. cerebralis* exposed variants. The variant with the lowest net reproductive rate (California) was most distantly related to the variant with the highest rate (Gallatin River). The Madison River variant was again intermediate, in both reproductive rate and genetic similarity. One of the Madison River negative replicates suffered a net loss of population during the experiment; however, this particular replicate was heavily contaminated with a species of oligochaete from the family Naididae. It is unknown if the lack of reproductive success was the result of this contamination.

It is unclear what effects this reproductive impairment will have in wild communities of oligochaetes, where infection rates in sampled *T. tubifex* have generally been low (Rognlie and Knapp 1998, Zendt and Bergersen 2000). These findings may be of significant import for laboratory studies, however, which require long-term production of TAMS, and where infection rates among *T. tubifex* cultures may approach 100%.

Variance in TAM production may be a factor in determining the level of disease severity within the fish population of a specific watershed, and the possibility warrants further study. Of particular importance is the question of whether lower TAM production

in some *T. tubifex* variants is the result of resistance to the parasite, and whether that resistance can be maintained in the face of chronic exposure to *M. cerebralis.* The literature on host-parasite relationships suggests that host resistance is often met with parasite adaptation (Anderson 1994). The ability of all *T. tubifex* variants to produce at least some TAMS argues that the host-parasite relationship in each case is still a viable one, poised for further coevolution.

Dose Experiment

When the oligochaetes tested in this experiment began producing TAMS, the data appeared to indicate that the replicates of both variants which had received a dose of 500 *M. cerebralis* myxospores were producing significantly greater numbers of TAMS. Subsequent statistical analysis however, found no significant difference in TAM numbers between dose treatments. The lack of significant difference was true of both total TAM numbers, and the number of days each replicate required to produce at least 50% of the total TAMS released during the experiment.

M. cerebralis infection levels in wild populations of *T. tubifex* have typically been very low, less than 5% of the individuals in the population (Rognlie and Knapp, 1998, Zendt and Bergesen 2000). This suggests that myxospores are not abundant or well distributed in the wild. We do not yet know what range of myxospore dose individual oligochaetes encounter in the wild, but my results indicate that relatively low doses can still result in large numbers of TAMS being released. Recent laboratory experiments have documented the existence of a proliferative phase of the parasite life cycle within its

oligochaete host (El-Matbouli and Hoffmann 1998, Hedrick et al. 1998). This apparent ability of *M. cerebralis* to fully utilize the resources of the *T. tubifex* individual suggests that relatively few colonizing parasitic myxospores may result in high numbers of TAMS being released into the local salmonid population. The death of a single infected fish in an area previously free of the parasite may initiate a significant disease condition in relatively few generations of the parasite life cycle. Recent research also indicates that individual oligochaetes, once infected by the parasite, remain infected and capable of releasing TAMs for the remainder of their natural lifespan (Gilbert and Granath 2001).

The negative effect of *M. cerebralis* infection upon the worm s reproductive rate demonstrated here conforms to the results demonstrated in Variant Experiment II described above. This negative effect was more pronounced at 164 days post-exposure than at the conclusion of the experiment. I suspect this to be the result of a container effect , i.e., the length of the experiment exceeded the ability of the small replicate containers to maintain a healthy environment for the worms. The effect of overcrowding, the accumulation of toxic waste products, and perhaps disease might all contribute to the decline seen in most of the replicate populations, regardless of myxospore dose. As mentioned in the earlier discussion, the impact of any reproductive impairment upon wild oligochaete populations is likely to be slight, due to the low infection rates observed in wild populations (Rognlie and Knapp 1998, Zendt and Bergesen 2000). This reproductive impairment should, however, be of greater importance to laboratory research efforts.

Temperature Experiment

Variant Experiment II demonstrated that the Gallatin River variant of *T. tubifex* is capable of producing *M. cerebralis* TAMS in relatively low numbers. During this experiment, the Gallatin River variant failed to produce TAMS at either tested temperature. These results, taken together, suggest that the Gallatin River variant has very low susceptibility to infection from the parasite, and may require very specific environmental conditions, parasite exposure level, or parasite virulence to allow the maturation and release of TAMS. As in previous experiments, the California variant of worms was a more prolific TAM producer than the Madison River variant. The consistency of this result at least suggests that the specific genome of the *T. tubifex* variant may determine TAM abundance to a more significant degree than temperature. A number of other environmental factors are known to affect *T. tubifex* communities however, including water chemistry and the biotic communities endemic to the respective tubificid sites of origin (Brinkhurst 1970, Lestochova 1994). The variance was high in this experiment, particularly among the Madison River replicates, a result similar to that found in Variant Experiment II. The reason for this high variance within this variant of *T. tubifex* is unknown. Reproductive success is clearly affected as a result of exposure to the parasite, an effect that was true even for the Gallatin River variant which was never observed releasing TAMS in this experiment. The response of all three variants to *M. cerebralis* exposure included reduced reproductive success.

Temperature also had a significant effect upon reproduction for the two Montana variants, with the Gallatin River variant preferring the colder temperature while the

Madison River variant had greater reproductive success in the warm temperature. This phenomenon is almost certainly related to the temperature regimes found in their home water bodies. The Gallatin River variant originated in a spring pond where temperatures remain cool throughout the year, while the Madison River variant came from a river system influenced by several dams which allow water temperature to become quite warm during summer months (B. Kerans, personal communication). Interestingly, temperature had no significant effect upon reproduction for the California variant, which seems capable of adapting to either environment.

One final statistic that can be derived from this experiment is the number of degree-days required for TAM release at the two temperatures. Previous research has found that the time required for TAM development is related to temperature (El-Matbouli and Hoffmann 1998, Gilbert and Granath Jr. 1998). The small difference in total degree-days seen in this experiment suggests that temperature driven metabolic rates are a primary determinant of TAM development time.

This result may have considerable relevance to the disease condition in the wild, since the timing of TAM release may affect disease severity (Hedrick et al. 1998). In colder waters, mature TAMS may not be released until the cartilage of young of the year salmonids has sufficiently ossified to provide them some ability to resist the most debilitating symptoms of infection. If this is correct, it is possible the parasite may proliferate in cold water systems with little impact upon fish populations.

Closing Comments

Studying the ecology of aquatic invertebrates presents field researchers with numerous difficulties. Consequently, much of what we know about aquatic oligochaetes is the result of laboratory experiments such as the ones I have documented here. These creatures are extremely tolerant of laboratory environments, which makes them relatively easy to maintain and study, a fact for which this researcher is grateful. My hope is that these experiments were designed and carried out with sufficient rigor so as to allow the findings to be applied to our understanding of the etiology of whirling disease.

I believe our understanding of host-parasite interactions supports the results of my research. Clearly, *T. tubifex* pays a price for infection with *M. cerebralis*, irrespective of the outcome for the parasite. The other tubificid species tested did not appear affected by exposure to the parasite, a finding that adds to the growing body of evidence which supports host specificity. However, I have no conclusive evidence that the parasite causes direct mortality of *T. tubifex* individuals, and I believe this is a question worth pursuing.

Discovering the differential ability of geographically and genetically distinct populations of *T. tubifex* to propagate *M. cerebralis* is the most significant result of my research. This finding may offer at least a partial explanation for the spatial variation we see in disease severity, and may ultimately lead to management options aimed at mitigation of the disease among wild fish populations. The apparent resistance of some variants of the worm host to the parasite suggests the potential for implementation of biological control methods. It likewise reinforces the importance of efforts to prevent the

accidental spread of oligochaetes from one water body to another, since the introduction of a more susceptible variant of the worm may lead to increased disease severity in an area where the endemic *T. tubifex* population is more resistant.

Finally, establishing baseline data on patterns of genetics and parasite susceptibility for specific variants of *T. tubifex* will allow us to track the evolution of the host-parasite relationship over time. This will be of value from a purely ecological perspective, and may also contribute to a proactive response to whirling disease.

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