EFFECTS OF ENVIRONMENTAL STRESSORS AND ANTHROPOGENIC DISTURBANCES ON INFECTIONS WITH MYXOZOAN PARASITES

by

DEBORAH DIANN IWANOWICZ

(Under the Direction of Marsha C. Black)

ABSTRACT

Environmental influences on myxozoan parasites are poorly understood. The objective of this dissertation was to determine the effects of environmental stressors and anthropogenic disturbances on myxozoan parasites. The two myxozoan parasites that were studied included a previously undescribed species of myxozoan parasite, *Myxobolus stanlii* n. sp. that infects largescale stonerollers (*Campostoma oligolepis*) and *Myxobolus cerebralis*, a salmonid pathogen. *Myxobolus stanlii* n. sp. was found in the connective tissue of various organs, renal tubules, glomeruli, macrophage aggregates within the kidney, and in nerve tissue behind the eye. The intensity of infection and host response to *M. stanlii* n. sp. in stonerollers was also assessed along an urban land use gradient (ULUG). *Myxobolus stanlii* n. sp. densities were highest in largescale stonerollers inhabiting sites at lower and middle ULUGs and lowest at sites with the high ULUGs. This suggests an abundance of the host(s) and vectors, or a decrease in the natural resistance of the host to the parasite at sites at the lower and middle ULUGs. Conversely, at sites with higher ULUGs anthropogenic disturbances such as urbanization can affect the intermediate host populations such that the abundance of their parasite load was

reduced. With *M. cerebralis,* the effects of riparian zone and associated stream substratum on the oligochaete host, *Tubifex tubifex* was investigated. *Tubifex tubifex* inhabiting streams of New York and Pennsylvania from four different land cover categories (deciduous, deciduous/meadowland, meadowland and coniferous) primarily consisted of genetic lineages I and III, which are the most susceptible lineages to *M. cerebralis* infection. Field and laboratory studies indicated that substrate type associated with land cover influenced the selection of lineages of *T. tubifex* that differ in their susceptibility to *M. cerebralis*. Lineage I and non-tubificid oligochaetes were greatest in deciduous sites; lineage III and *M. cerebralis* infection was greatest in deciduous/meadowland and coniferous sites. Laboratory studies further indicated that substrate from the various sites affected *T. tubifex* infection with *M. cerebralis*, triactinomyxon release, and duration of release. While site variability was evident, environmental factors associated with land cover clearly impact the host pathogen relationship between *T. tubifex* and *M. cerebralis*.

INDEX WORDS: Urban Land-use Gradient, *Campostoma oligolepis*, *Myxobolus stanlii*, *Myxobolus cerebralis*, Myxozoan, Environment, Stressors, Whirling Disease, *Tubifex tubifex,* Urbanization, Land Cover, Riparian Habitat

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DEDICATION

This dissertation is dedicated to my husband, Luke Iwanowicz, who is everything everyone should try to be.

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Chapter One

Introduction and Literature Review

Myxozoans are a diverse and economically important group of microscopic metazoan parasites. Historically, the classification of myxozoans was based exclusively on spore structure due to the difficulties of characterizing vegetative stages and life cycles of these complex organisms. Genetic classification has prompted the reassignment of the phylum Myxozoa from the protozoan to the metazoan subkingdom. Recent advances in molecular biology have also improved classification methods and in many cases have resulted in the renaming of these metazoans at the genus and species level. Development of molecular techniques have aided in the advancement of identification, life cycle, biochemical, and specific diagnostic tests. All myxozoans that infect fish are in the class Myxosporea. This class is further divided into two orders, the bivalvulida and the multivalvulida. Division of the two orders is based on the position of polar capsules in relation to spore shape and to the line of dehiscence of the shell valves. The number of polar capsules and shell valves and the shape and structure of these are also important characteristics.

Although myxozoans infect mainly fish, they have also been found in annelids, platyhelminths, reptiles, amphibians, and mammals (Friedrich et al. 2000), and tend to elicit minimal host immune response. Those that are recognized by the host cause only a low degree of humoral response. Common responses at the cellular and tissue level are generally not destructive to the parasite or host during early development. For example, early histozoic plasmodia only elicit formation of a host cell envelope. This envelope is

derived from the local population of connective tissue cells or from compressed cells of the neighboring tissue (Lom and Dykova´ 1995). Granulomatous inflammation is evident only after the plasmodium is replete with mature spores.

A small percentage of the ~1400+ described species of myxozoans are best known for the disease they cause in their commercial fish hosts (Kent et al. 2001). The transcontinental dissemination and economic importance of myxozoan parasites has paralleled the increased development in fin fish aquaculture since the 1990's (Halliday 1976, Kent et al. 2001). *Kudoa* species can cause large unsightly cysts or regions of lysis in the musculature of commercial fish (Moran et al. 1999). The most important association of *Kudoa* with commercially sold Atlantic salmon (*Salmo salar*) is referred to as 'soft flesh' (Tsuyuki et al. 1982). Soft flesh is a post-mortem myoliquefaction resulting from the release of proteolytic enzymes into the musculature by the parasites (Moran et al. 1999). *Sphaerospora dicentrarchi* and *Sphaerospora testicularis* are common parasites of European sea bass (*Dicentrarchus labrax*), a popular Mediterranean aquaculture species, that eventually cause the parasitic castration of valuable broodstock males (Sitjabobadilla and Alvarezpellitero, 1993). Other species such as *Myxidium leei* infect intestinal tracts of multiple species causing severe chronic enteritis that can cause emaciation and death (Athanassopoulou et al.1999, Branson et al. 1999, Padros et al. 2001). The most well known *Myxobolus* sp. in the United States is *Myxobolus cerebralis*, the causative agent of whirling disease. Whirling disease causes the destruction of cartilage and associated tissues in juvenile salmonids (Kent et al. 2001). The cartilage associated with the spinal column and cranium (skull, jaws and gills) of juvenile salmonids can be massively infected leading to death of the fish or life-long

skeletal deformities (Hedrick et al. 1999, Kent et al. 2001). These impairments, if not initially fatal, are thought to severely compromise survival and, in part, explain losses of up to 90% in certain year classes of wild rainbow trout (Nehring and Walker 1996, Vincent 1996, Kent et al. 2001).

Myxozoa are mostly intercellular parasites, a few of which have intracellular stages in their life cycles (El-Matbouli et al. 1992). *Myxosporea* are capable of parasitizing organ cavities or tissues, and morphologically vary in size and shape (ovoid to elongate) across species. Mature spores stain weakly with hemotoxylin and eosin, but are strongly acid-fast and stain intensely blue with giemsa. Externally myxozoa are lined by two or more valvogenic cells that form the shell valves and join along a sutural line. Spores are multicellular and contain two or more polar capsules that contain a polar filament capable of extruding for host attachment.

Life cycles of myxozoan parasites are relatively unknown, except for a few species (Markiw and Wolf 1984, Kent et al. 2001, MacKenzie et al. 2005, Golomazou et al. 2006, Morris and Adams 2006). In many species, an actinosporean (triactinomyxon) is a required alternate life cycle stage (Lom and Dykova′ 1995). Some general trends are apparent when correlating actinosporean morphotypes with myxozoans: *Myxobolus* species (suborder Platysporina) usually develop into triactinomyxons, while members of the suborder Variisporina (*Ceratomyxa, Myxidium, Sphaerospora*) develop into aurantiactinomyxon, neoactinomyxum, or tetraactinomyxon forms (Kent et al. 2001). With some exceptions, the life cycle and development of many myxozoans are similar to the life cycle of *Myxobolus cerebralis*. As explained by Markiw and Wolf (1983, 1984), the life cycle begins when the actinospore attaches to the surface of the fish and releases

sporoplasm into the fish. Cells within the sporoplasm divide by endogeny and begin the onset of presporogonic or extrasporogonic vegetative replication. Sporulation occurs with the formation of multi-cellular spores within plasmodia. Mature spores remain in the fish until the fish dies, or are released in feces. Once fully developed myxospores are liberated from their fish host, they may be ingested by oligochaetes, polychaetes, or other invertebrates depending on the species of parasite. However, not all species need an intermediate host. If an intermediate host is needed, schizogony occurs in the gut epithelium of the worm, resulting in binucleate cells that undergo gamogony. In gamogony, internal cells in pansporocyts undergo three mitotic and one meiotic division. The resulting gametes fuse to form a pansporocyst with four to eight zygotes. These zygotes are the beginning of a second sporogony for the parasite, where multicellular spores are formed with valves, polar capsules, and a sporoplasm. Finally, inflated spores are released with the worm's feces, float in the water, and contact the fish host to complete the life cycle.

The prevalence of myxozoan infections may vary considerably from site to site (Lom and Dykova′ 1995). These differences may be accounted for by changes in the hosts (final or intermediate) or the environment of the host. Appearance and distribution of myxozoan parasites in the environment may be favored by certain environmental conditions such as eutrophication, increased temperature or low dissolved oxygen. Environmental stress as a predisposing factor coupled with overcrowding, increases the susceptibility of fish to infection by myxozoans (El-Matbouli et al. 1992).

An environmental factor is an external physical, chemical, or biological component of the environment that may affect an organism's well-being or health. When

environmental factors negatively affect an organism's health, it is called an environmental stressor. Thus, environmental stressors are suboptimal conditions that can change an organism at the biological, physiological, or microscopic level. The presence of these changes, molecular, cellular and/ or genetic alterations, serves as biomarkers, signaling exposure to one or more environmental stressors. The ability of any animal to adapt to environmental changes increases its chance of survival, proliferation, and at times, resistance to disease.

Environmental stress may influence interactions between a parasite, its host, or the intermediate host. Although many factors can affect host-parasite interactions, drought, anoxia, heavy metals, and pesticide activation of the stress response mechanisms of all organisms is well documented (Landsberg et al. 1998, Lafferty and Kuris 1999, Schisler et al. 2000). Different species cope with variations in climate differently. These adaptive mechanisms reflect differences in their life cycles, physiology, biochemistry, and molecular biochemistry of all organisms in a specific habitat. Consequently, optimal conditions for a particular parasite may be tolerable for its host, yet unsuitable for the intermediate host. In this situation the life cycle of the parasite would be broken and the prevalence of infection within a host population would be expected to decrease. Little research has addressed the role of environmental factors on the intermediate host or post-infection for myxozoans.

The purpose of this dissertation was to look at both environmental and anthropogenic stressors on the infections of myxozoan parasites. Myxozoan parasites were chosen due to the variety of fish they infect. More specifically, *Myxobolus cerebralis* was chosen because of the detailed knowledge of this parasite's life cycle and

mode of action in both hosts (Kent et al. 2001) and importance for both cultured and wild fish health.

Environmental science is continually confronted with issues related to assessing and evaluating the effects of stressors on the health of aquatic ecosystems. Some of the more challenging issues are validating ecologically significant biomarkers, determining the importance of temporal and spatial variability (of physicochemical and biological factors in modifying responses to stress), and establishing cause and effect relationships between specific stressors as they relate to environmental damage (Adams 2001). Chapter two of this dissertation evaluated the health of the largescale stoneroller (*Campostoma oligolepis)* populations in streams affected by urban land use with selected biomarkers (glutathione, histology, and hepatic apoptosis). I further looked into the development of a new myxozoan parasite within the musculature of the largescale stoneroller from different urban land use gradients. Chapter three names and describes the new myxozoan parasite, *Myxobolus stanlii* n. sp. that was found in the musculature of the largescale stoneroller. In chapter four, I describe the results of a field study to determine if land cover affected *T. tubifex* density (the invertebrate host of *M. cerebralis*), and to assess the genetic populations of *T. tubifex* collected from different habitats on the east coast and compare them to each other as well as to populations described from other parts of the U.S.A. Chapter five describes a laboratory study that determined if land cover affected *T. tubifex* density and the ability of resident worms to become infected with *M. cerebralis* spores and to release infective spores.

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CHAPTER 2

EFFECTS OF URBAN LAND USE ON FISH HEALTH BIOMARKERS IN LARGESCALE STONEROLLERS IN THE MOBILE RIVER BASIN, BIRMINGHAM, AL[1]

[1]Iwanowicz D, Black M, Blazer VS, Zappia H, Bryant W. Submitted to Ecotoxicology

Abstract

During the spring and fall of 2001 and the spring of 2002 a study was conducted to evaluate the health of the largescale stoneroller (*Campostoma oligolepis)* populations in streams affected by urban land use using selected biomarkers (glutathione, histology, and hepatic apoptosis). Sites were selected from a pool of naturally similar subbasins (ecoregion, basin size, and geology) of the Mobile River basin, using an index of urban intensity derived from infrastructure, socioeconomic, and land use data. This urban land-use gradient (ULUG) is a multimetric indicator of urban intensity, ranging from 0 (background) to 100 (intense urbanization). Liver glutathione levels were positively associated with increasing urban land use ($R^2 = 0.98$). Histopathological examination determined that some abnormalities and lesions in largescale stonerollers were dependent on the ULUG and generally increased in prevalence or severity with increasing urbanization. Liver macrophage aggregates were positively correlated to the ULUG. The occurrence of nucleosomal ladders (indicating apoptotic cell death) did not correspond with urban intensity in a linear fashion. Apoptosis, as well as prevalence and severity of a myxosporidian parasite, appeared to have a hormetic dose-response relationship. The majority of the biomarkers suggested fish health was compromised in areas where the ULUG ≥ 36.00.

Keywords: Biomarkers · Largescale stoneroller · *Campostoma* · Hormesis · Liver Glutathione · Urban Land Use Gradient (ULUG)

Introduction

Urbanization impacts many freshwater systems, particularly small streams. These impacts include effects on stream geomorphology (Hammer, 1972; Paul and Meyer, 2001; Short et al., 2005; Tate et al., 2005), hydrology (Finkenbine et al., 2000; Wang et al., 2000; Paul and Meyer, 2001; Brown et al, 2005), stream temperature (Galli, 1991; Paul and Meyer, 2001) and water quality (Wilber and Hunter, 1977; Klein, 1979, Lenat and Crawford, 1994; Paul and Meyer, 2001). Concentrations of nutrients, pesticides, organic chemicals and heavy metals are often elevated in urban runoff and treated wastewater (Klein, 1979; Heany and Huber, 1984; Field and Pitt, 1990; Ahel et al., 2000; Hoffman et al., 2000; Shinya et al., 2000; Paul and Meyer, 2001; Brown et al., 2005). Alterations in physical habitat and water quality due to urbanization are also associated with changes in aquatic biota (Weaver and Garman, 1994; Wichert, 1994; Wang and Lyons, 2003; Brown et al., 2005). Understanding the effects of numerous stressors on aquatic assemblages is extremely important to preserve, rehabilitate, and manage ecosystems as urbanization continues (Nilsson et al., 2003; Cottingham et al., 2004; Brown et al., 2005).

Many urban studies have used a single measure; such as population density, percent urban land, or percent imperviousness (Arnold and Gibbons, 1996; Tate et al., 2005), to gauge ecosystem responses to urbanization. Interpretation of these results varies depending on the selected measure (Yoder and Rankin, 1995; Tate et al., 2005). In an attempt to address the complexity of urbanized areas (i.e., differing infrastructure, human populations and socioeconomic characteristics) and assign a single impact value, the urban land use gradient (ULUG) was developed (McMahon and Cuffney, 2000). The

ULUG is a multimetric indicator of urban intensity, ranging from 0 (background) to 100 (intense urbanization) that combines individual condition measures, and together provides distinct information on all of the dimensions (infrastructure, human populations, and socioeconomic characteristics) of these complex systems (McMahon and Cuffney, 2000; Short et al., 2005; Tate et al., 2005).

The National Water-Quality Assessment Program (NAWQA), part of the U.S. Geological Survey, conducted a five-year investigation of the Mobile River Basin (MRB). The MRB is the sixth largest basin in the Nation and encompasses 44,000 square miles in the states of Alabama, Georgia, Mississippi and Tennessee. Of this, 71% of the MRB lies within Alabama (Johnson et al., 2002). Chemical and physical data were collected from selected areas throughout this study unit to describe water quality (Johnson et al., 2002; McPherson et al., 2002; McPherson et al., 2003; Robinson, 2003; Atkins et al., 2004). In addition, this area was part of a study examining changes in aquatic biological communities, physical habitat, hydrology, temperature and water quality along gradients of urban intensity, in multiple environmental settings throughout the United States (Tate et al., 2005). As part of the urban gradient studies, 30 sites were sampled within the Ridge and Valley ecoregion of Alabama to assess responses of fishes, invertebrates, and algae to urbanization (Cuffney et al., 2005; Meador et al., 2005; Tate et al., 2005; Zappia et al., 2005). Sites were selected based on ecoregion, basin size and geology and expanded the range of ULUG parameters.

Atkins et al. (2004) reported that most urban streams and rivers in the MRB were high in insecticides (i.e.: chlorpyrifos, diazinon, malathion, DDT, dieldrin and carbaryl), PCBs, and volatile organic compounds (VOCs). The most frequently detected VOCs

included trichloromethane (chloroform - a byproduct of water chlorination), tetrachloroethylene, trichloroethylene (commercial and industrial solvents and degreasers), cis-1,2-dichlroethene (breakdown product of trichloroethylene), methyl tert-butyl ether (gasoline oxygenate), and the hydrocarbons benzene and toluene. Atkins et al. (2004) also determined concentrations of chlordane and hepatochlor epoxide in fish tissue increased with increasing ULUG. Zappia (2002) measured organochlorines and trace elements in fish tissue and streambed sediments in the Mobile River Basin. Four of the sites in the current study were included in this survey (Table 2.1: sites 1, 3, 6 and 9) and the following chemicals were found at these sites: cis-chlordane, p,p'-DDE, p,p'-DDT, endrin, hexachlorobenzene, PCB's, trans-nonachlor, dieldrin, heptachlor epoxide, oxychlordane, cis-nonachlor, and trans-chlordane. Their presence was attributed to urbanization and positively correlated with ULUG. Although sites with a ULUG $\geq$ 36.0 (sites 6 and 9) were highest in most organochlorines and trace elements sampled in the sediment and fish, Zappia (2002- Appendix 2-5) reported that sediment from selected sites with a ULUG <11.36 (sites 1 and 3) were highest in some organochlorines (o,p'-DDE, p,p'-DDE, o,p'-DDT, octochlorbiphenyl, 3-5-dichlorobiphenyl, and cis Nonachlor) from agricultural application. Fish from these same sites were highest in the trace elements mercury, arsenic, cadmium, lead, aluminum, barium, copper, and manganese and the organochlorine p,p'-DDE.

Although the effects of urbanization on fish community structure have been studied in a number of areas (Weaver and Garman, 1994; Wang et al., 2000; Wang et al., 2001; Siligato and Böhmer, 2002), there are few studies that evaluate the effects of urbanization on fish health. These studies tend to measure fish health in terms of fish

populations, population composition and tissue contaminant concentrations (Zandbergen, 1998). An effective, alternative method of assessing fish health in a stream is to use tolerant, short-lived species as sentinels. Largescale stonerollers (*Campostoma oligolepis*) are widespread and abundant throughout the Central and Mideastern United States, and are often a major component of stream fish communities (Trautman, 1981; Mettee et al., 1996). The characteristics of rapid growth, a short lifespan and widespread distribution make largescale stonerollers good candidates for sentinel species. Largescale stonerollers occupy flowing water habitats ranging from small streams to large rivers, and are tolerant species that dominate the urban fish assemblage in the Birmingham area (Meador et al., 2005).

The primary objective of this study was to evaluate the health of the largescale stoneroller using biomarkers including gross lesions, condition factor, histopathology, hepatic glutathione, and hepatic apoptosis. The second objective was to determine if there was a linear response of any biomarkers along a gradient of urban land-use in the MRB.

Materials and Methods

Site Selection

The ULUG was derived using a modification of the approach used by McMahon and Cuffney (2000). When appropriate, variables were normalized based on basin area (e.g., population density, percentage of basin in forest, number of toxic releases per 259 km^2).

Physical characteristics of each stream were an assessment of instream habitat and continuous measurements of stream stage and temperature. Instream habitat characteristics (velocity, channel depth and width, aspect of flow, bed substrate, habitat

cover, canopy closure and vegetation, and bank morphology) were assessed via standard NAWQA Program protocols (Fitzpatrick et al., 1998). Nine sites for this study were selected around Birmingham, AL (Figure 2.1). Sites similar in water depth, stream width, water flow and temperature within the Birmingham, AL metropolitan area were chosen with very low (2.97 to 3.11); low (11.36 to 13.99); moderate (36.0 to 39.0) and high (69.7 to 83.5) ULUG or urban intensity values (Table 1).

Fish Collection

Largescale stonerollers were collected using a Coeffelt Mark 10 backpack electrofisher™ in March 2001, November 2001, and March 2002 (Table 2.2). Water quality variable including water temperature, pH, dissolved oxygen and conductivity were measured with portable meters during each sampling. Global positioning system readings were recorded for each site. The target fish number for March 2001 was 20 fish per site. The target fish number for November 2001 and March 2002 was 30 fish per site.

Fish were euthanized with a lethal dose of MS-222, weighed, measured, and examined for external and internal lesions. During March 2002, pieces of livers were snap frozen in liquid nitrogen for glutathione and apoptosis analyses. During November 2001 and March 2002, otoliths were removed for aging. All fish were cut open and preserved whole in 10% phosphate buffered formalin and returned to the National Fish Health Research Laboratory for histopathological examination.

Condition Factor

Condition factor was calculated as $K = [\text{body weight in gms} / (\text{total length in mm})^3] \times 10$.

Glutathione Analysis

Liver reduced glutathione (GSH) concentrations (total glutathione including GSH and glutathione disulfide, i.e. reduced and oxidized forms combined) of individuals was determined by the 5,5'-Dithiobis(2-Nitrobenzoic Acid)-Oxidized GSH (GSSG) reductase recycling assay following the modified methods of Conners and Ringwood (2000). Briefly, frozen liver samples were thawed and weighed. Liver samples were homogenized in 10 volumes of 5 % sulfosalicyclic acid (SSA), and centrifuged (14000 rpm, 5 min, 4 °C). The supernatant was removed and diluted 1:1 with 5% SSA and mixed with the sodium phosphate buffer containing nicotinamide adenine dinucleotide phosphate (NADPH) and dithio-bis-nitrobenzoicacid (DTNB). GSSG reductase was quickly added and the rate of thio-nitrobenzic acid (TNB) formation was monitored at 412 nm over a 90s interval. GSH concentrations were estimated from a standard curve and reported as nM GSH/g wet weight.

Apoptosis - Ligation-mediated PCR

Genomic DNA was extracted from frozen liver tissue for apoptosis screening using the QIAmp Tissue Kit (Qiagen). Images were analyzed using an ApoAlert™ Ligand mediated PCR ladder assay kit designed by Clontech™. The ApoAlert™ LM-PCR ladder assay kit is a semi-quantitative assay that detects nucleosomal ladders generated during apoptosis. The ApoAlert™ assay made quantifying the relative extent of apoptosis in different samples from scanned gels possible. Genomic DNA (1 μg) was mixed with 1 nmol each of 24 - mer and 12 - mer unphosphorylated oligonucleotides in 60 μL of T4 DNA ligase buffer (Fast-link DNA ligation kit, Epicentre Technologies, Madison, WI).

Oligonucleotides were annealed by heating to 55 °C for 10 minutes and allowing the mixture to cool to 10 °C over 55 minutes. The mixture was then incubated at 10 °C for 10 minutes. One μL of ATP and 1.5 μL of ligase buffer were added to the mixture and incubated for 16 hours at 16 °C. The reactions were diluted to a final concentration of 5 ng/μL with TE buffer (10mM Tris-HCL, 1 mM EDTA, pH 7.5). Samples were stored at –20 °C prior to PCR.

Blunt-end linkers (24-mer: 5'-AGCACTCTCGAGCCTCTCACCGCA-3' and 12-mer: 5'- TGCGGTGAGAGG – 3') were used to amplify the nucleosomal ladders as described by Staley et al. (1997). The PCR mixture was made by adding 5 μL of 24-mer (20 pM), 7.5 μL DNA and 12.5 μL of water to puRe Taq™Ready-To-Go™PCR Beads (Amersham Biosciences, Piscataway, NJ). Samples were overlaid with 40 μL of sterile mineral oil. Tubes were heated to 72 °C for 3 minutes and 1.25 U of Taq polymerase (Gibco, Bethesda, MD) were added. The PCR amplification was conducted in a Hybaid Omnigene thermo-cycler using the following parameters (ApoAlert® LM-PCR Ladder Assay Kit User Manual PT3172-1): holding at 72 ˚C for 8 minutes, followed by 35 cycles with 1 minute at 94 °C, 3 minute at 72 °C, and 5 minute at 72 °C. The final extension was performed at 72 ˚C for 10 minutes. Calf-thymus DNA was used as an internal control. PCR products were resolved in a 0.75 % agarose and 1.5 % synergel matrix equilibrated in Tris-Acetate-EDTA buffer (TAE; 40 mM Tris base, 20 mM glacial acetic acid, and 10 mM EDTA, pH 8.5) for 4 hours at 90 V. Gels were stained with ethidium bromide and photographed on a UV transilluminator. Images of scanned gels were analyzed with TotalLab analytical software (Biosystematica, Wales, United

Kingdom). For apoptosis, nucleosomal ladders were measured at 200 bp intervals (data shown at 200, 400, and 600 base pairs lengths).

Histopathology

Fixed tissues (liver, kidney, spleen, gill, gonad, and grossly visible lesions) were prepared for histopathological analysis. Paraffin-embedded tissue sections (6 μm) mounted on glass slides were stained with hematoxlyin and eosin, Perl's prussian blue, or Giemsa stains (Luna, 1992). For histological examination, sagittal midline sections of the entire body were examined at 40-100X magnifications. All internal organs and muscle sections were examined for any abnormalities.

A previously undescribed myxozoan parasite was found and staged. Parasites were staged on a level of 0-3 where the value is related to the quantity of areas occupied by the myxozoan parasite (areas include: muscle and connective tissue, kidney, and nerve tissue).

Statistical analysis

One-way analysis of variance (ANOVA; Statistica v11.00.01, Statsoft Inc. Software) was used to determine significant differences in condition factor, age of fish and ligand-mediated apoptosis ($p \leq 0.05$) among sample sites. The unequal N HSD *post-hoc* test was used to determine statistical differences among sites, sex, or sampling date (Winer et al., 1991). All other data were assessed by forward stepwise multiple regression models, based on ordinary least squares for continuous dependent variables and ordered logit for categorical variables. Inspection of the bivariate plot of the dependent variable and lower

order independent variable indicated that this model was appropriate (Cohen and Cohen, 1983). To establish possible correlations between biomarkers and sites, the Pearson Correlation coefficient (r) and Bonferroni Probability (p) were calculated. For statistical significance, preliminary and final models were tested at stations where $n \geq 8$ for all endpoints. Data were significant if $P < 0.05$.

Results

Site Selection

Temperature was positively correlated with ULUG regardless of time sampled (March 2001, Pearson's r = 0.85, $p = 0.01$; November 2001, Pearson's r = 0.88, $p = 0.12$; March 2002, Pearson's r = 0.69, $p = 0.04$).

Organism-level indicators

External Abnormalities

External abnormalities primarily included black spots on the body surface and fins due to helminth metacercariae and the presence of leeches. A few fish exhibited reddened or hemorrhagic areas on the body surface. In March 2001, there was a positive correlation between ULUG and black spot on the body surface (Pearson's r = 0.84, $p = 0.01$) (Table 2.2). In November 2001 fish collected at all sites had a high prevalence of black spots that were not significant, and no other abnormalities were noted. In March 2002, a similar spatial trend to that seen the previous year was observed in terms of black spots and leeches but they were not significant.

Condition Factor and Age

Condition factor was significantly different among sites (F = 2.75, $p = 0.01$) only in the March 2002 collection and only between site 5 and site 8. Condition factors were different among sampling dates, with the condition factor lower in the November sampling than either March sampling (F = 5.05, $p = 0.01$). Condition factor did not differ between male and female largescale stonerollers from any site at any sampling date (F = 1.36, and $p > 0.05$). Fish age was not significantly different among the sampling sites (F = 2.82, $p = 0.26$).

Cellular-Level Indicators

Histopathology

Lesions were most frequently observed in the liver and muscle tissue. In the liver, necrosis, fibrosis, vacuolated foci, macrophage aggregates, regenerative foci, and inflammation were the most common histological observations. In the muscle, digenetic trematode metacercariae were the most common histological observation.

In March 2001 histopathological samples from largescale stonerollers from all nine sites were examined (Table 2.3). In the liver, necrosis (F = 14.18, p = 0.01), macrophage aggregates (F = 8.40, p = 0.03) and inflammation (F = 12.83, p = 0.02) were significantly affected by ULUG. Necrosis (Pearson's r = 0.89, p = 0.003), macrophage aggregates (Pearson's r = 0.81, p = 0.01) and inflammation (Pearson's r = 0.83, p = 0.01) were positively correlated with ULUG, as were digenetic trematode metacercariae in the muscle (Pearson's r = 0.77, p = 0.03).

In November 2001, histopathological samples from largescale stonerollers from four stations were examined (Table 2.3). In the liver, presence of macrophage aggregates ($F = 10.42$, $p = 0.04$) and fibrosis ($F = 72.44$, $p = 0.01$) were significantly different among ULUG values. In the muscle, metacercariae were not significantly different between sites ($F = 6.69$, $p = 0.08$) in this season.

In March 2002, histopathological samples from largescale stonerollers from all nine stations were examined (Table 2.3). In the liver, macrophage aggregates were positively correlated with ULUG (Pearlson's $r = 0.71$, $P = 0.04$). Digenetic trematode metacercariae were significantly different between sites ($F = 24.65$, $P = 0.002$).

A newly described myxosporidian parasite, *Myxobolus stanlii* n.sp., was found in 100 % of the largescale stonerollers collected (Iwanowicz et al., In Press). Urban land use gradient was not a good predictor of parasite spores per gram fish tissue ($F = 0.78$, $p = 0.41$). Parasite load (spores per gram fish tissue) was highest in sites 3 and 5 and lowest at site 9. Tissue distribution of this parasite included connective tissue of various organs, renal tubules, glomeruli, macrophage aggregates within the kidney, and in nerve tissue behind the eye (Iwanowicz et al., In Press). Myxozoan parasites were only found in the latter location during heavy infections. The locations of the parasite lead us to believe that the parasite matures in the connective tissue but may travel through the renal tubules. Of all fish collected, 95% were either stage 1 or 2. Less than 5% of all fish collected were at stage 3 parasitism (Table 2.4). There was no significant difference between parasite stage and the ULUG ($F = 0.86$, $p = 0.38$).

Sub-cellular Level Indicators

Glutathione

Urban land use gradient was positively correlated with hepatic glutathione levels (Pearlson's r = 0.97, p = 0.00) (Figure 2.3). Glutathione was highest at Sites 8 and 9 and lowest at Sites 1 and 3.

Ligand-mediated PCR

Apoptotic nucleosomal ladders (measured as percent frequency) at base pair lengths of 200, 400 and 600 were measured. Liver apoptosis was significant at 200 bp (F = 8.85, p = 0.02), 400 bp (F = 6.03, p = 0.04) and 600 bp (F = 5.98, p = 0.04) lengths when compared to ULUG. Data for the 200 base pair length is illustrated (Figure 2.4). In this case a U-shaped or biphasic dose-response relationship was noted (Figure 2.4a), particularly in fish from sites with a ULUG value between 36.00 and 83.54 (Figure 4b).

Discussion

Previous research in the MRB has demonstrated the presence of insecticides, PCBs, and volatile organic compounds (VOCs). Additionally, chemicals including chlordane and heptachlor epoxide have been identified in fish tissues. With respect to ULUG, these chemicals are found at increasing concentrations as ULUG increases (Atkins et al., 2004). Studies of other fish species have found that hepatic glutathione levels increase as these chemicals increase (Martinez et al., 1996; De Luca-Abbott et al., 2005; Rosety et al., 2005; Schmidt et al., 2005; Panahi et al., 2006; Parvez et al., 2006; Rocher et al., 2006). Glutathione is a non-enzymatic antioxidant that scavenges free radicals, facilitates

intracellular transport, detoxifies electrophiles and maintains thiol-disulfides (DeLeve and Kaplowitz, 1991). Elevated levels of glutathione are induced by toxic substances and hence have been used as a biomarker of exposure in a variety of fish species (Hasspieler et al., 1994, Otto and Moon, 1995, Sayeed et al., 2003). In the present study, we found that liver glutathione concentrations in largescale stonerollers increased proportionally to the ULUG value. Our results suggest that largescale stonerollers were exposed to increasing levels of chemicals that initiate oxidative damage (Lemaire et al., 1994, Rana et al., 1995).

Another biomarker that was investigated, liver apoptosis, appeared to have more U-shaped or biphasic dose-response over the ULUG values studied. Apoptosis or programmed cell death is important in development and homeostasis for the removal of infected, transformed or damaged cells. It involves a series of morphological and nuclear changes that subsequently allow the cells to be ingested by phagocytic cells. This type of cell death protects tissue from the release of degradative enzymes that accompanies necrosis (Lockshin and Zakeri, 2001). The apoptotic process includes an endonuclease-mediated fragmentation of the chromatin. Cellular endonucleases cleave genomic DNA between nucleosomes, producing fragments whose lengths vary by multiples of 180 – 200 base pair (Arends et al., 1990; Staley et al., 1997). The resulting oligonucleosome-sized DNA fragments when visualized by gel electrophoresis, are described as a 'nucleosomal ladder', and have become a widely accepted biochemical indicator of apoptotic cell death (Staley et al., 1997).

Credible evidence has shown that apoptosis displays a biphasic dose-response relationship in a wide range of animal models and cell types (Calabrese, 2001). Our data

suggest the biphasic dose-response relationship is occurring with regard to hepatic apoptosis and degree of urbanization as well, particularly in sites with ULUG values between 36.00 and 89.00. At these sites a J-shaped dose response (Figure 4b) was noted, very similar to that produced by x-ray irradiation of mice thymocytes with inhibited low-dose response and stimulated high-dose response (Liu et al., 1996; Calabrese, 2001). This general relationship of biphasic dose-response relationships is termed hormesis (Jonas, 2001; Calabrese and Baldwin, 2001a). The definition of hormesis is an adaptive response to low levels of stress or damage resulting in improved fitness for some physiological systems for finite periods (Calabrese and Baldwin, 2001b). The key conceptual features of hormesis are disruption of homeostasis, modest overcompensation, reestablishment of homeostasis, and the adaptive nature of the process (Calabrese and Baldwin, 2001b). At the present time, offering firm conclusions about such findings except to indicate that biphasic apoptotic responses reliably occur is premature, and more research is needed to understand these responses.

We found that fish had increasing densities of parasite load (spores per gram fish tissue) through the lower and middle land-use gradients and a decreased density at sites with the highest urban land-use values. At the sites with ULUG $\leq$ 36.00 water quality, eutrophication and thermal effluent can raise rates of parasitism because the associated increased productivity can increase the abundance of intermediate hosts and vectors (Lafferty and Kuris, 1999), while stress on the host can decrease natural resistance to the parasites. At sites with ULUG $\geq$ 36.00 there could be a decrease in parasitism if infected hosts suffer differentially higher mortality or the parasites were more susceptible to pollution than their hosts (Lafferty and Kuris, 1999). Also habitat alterations such as

urbanization can affect the intermediate host populations such that the abundance of their parasite load is reduced (Lafferty and Kuris, 1999).

Histopathological changes in a variety of tissues have been used as biomarkers of contaminant exposure (Myers and Fournie, 2002; Stentiford et al., 2003; Au, 2004). The presence of macrophage aggregates, indicative of a hepatotoxic response, were dramatically increased in site 9 (ULUG = 83.5). Macrophage aggregates have long been used as a biomarker of prior exposure to toxicants (Blazer et al., 1987; Murchelano and Wolke, 1991; Chang et al., 1998). In the present study, positive relationships were seen between urbanization and prevalence of some lesions and abnormalities including liver necrosis, liver fibrosis and liver inflammation in largescale stonerollers. Interestingly, the occurrence of regenerative foci was highest in sites with the lowest ULUG value. Many factors could account for such data, including: dietary protein level, genetic variation within species, route of exposure, chemicals present, temperature, and dose (Bailey et al., 1989; Chang et al., 1998).

Urban land-use gradient information may be an important factor when determining impacts on freshwater ecosystems. In this study we found strongly positive correlations between ULUG index values and hepatic glutathione levels, where hepatic glutathione levels were highest in ULUG sites $\geq$ 36.00. There were also positive correlations between ULUG index values and macrophage aggregates at both spring samplings. We can also predict that at ULUG sites $\geq$ 36.00, within the stonerollers, parasite spores per gram fish tissue decrease significantly. Overall the findings suggest that at ULUG values greater than 36.00, largescale stoneroller health may be affected.

The deleterious effects of urbanization on water quality are evident across the United States. Urbanization is reported to adversely affect the physical, chemical, and biological characteristics of the aquatic environment. Concern about the effects of urbanization has galvanized efforts to understand and manage urban development on the part of governmental organizations at all levels (McMahon and Cuffney 2000). Environmental science is continually confronted with issues related to assessing and evaluating the effects of stressors on the health of aquatic ecosystems. Some of the more challenging issues are validating ecologically significant biomarkers, determining the importance of temporal and spatial variability (of physicochemical and biological factors in modifying responses to stress), and establishing cause and effect relationships between specific stressors as they relate to environmental damage (Adams 2001).

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Table 2.1 Site name, locations and urban land use gradient (ULUG) values of nine sites surrounding Birmingham, AL.

Site	Site Name	Latitude	Longitude	ULUG Value
1	Little Cahaba River at Trussville, AL	33.38	-86.36	3.0
2	Unnamed trib to Big Canoe Creek, Springville, AL	33.79	-86.49	3.1
3	Cahaba Valley Creek at Pehlam, AL	33.18	-86.48	11.4
4	Five Mile Creek at McCalla, AL	33.36	-87.02	12.0
5	Little Cahaba River at Braggsville, AL	33.57	-86.12	14.0
6	Shades Creek at Homewood, AL	33.27	-86.47	36.0
7	Shades Creek at Mountain Brook, AL	33.44	-86.84	39.0
8	Village Creek at East Lake, AL	33.57	-86.73	69.7
9	Valley Creek at Powderly, AL	33.47	-86.89	83.5

Table 2.2 External lesion incidence for largescale stonerollers (*Campostoma oligolepis*) collected along an urban land use gradient (ULUG) in different years and seasons.

						Condition	Individual Lesions (%)		
	Site	ULUG	Temp ˚ C	Sample Size	Mean Age ± SE	Factor Mean ± SE	Black Spots	Red Areas	Leeches Copepods
March 1, 2001									
	1	3.0	8.5	20	NA[1]	1.1 ± 0.16	20.0	0.0	5.0
	2	3.1	11.5	17	NA	1.16 ± 0.29	5.8	0.0	76.4
	4	12.3	9.9	22	NA	1.19 ± 0.16	32.0	0.0	0.0
	5	14.0	13.4	20	NA	1.20 ± 0.13	25.0	0.0	5.0
	6	36.0	12.2	20	NA	1.25 ± 0.17	20.0	0.0	0.0
	7	39.0	11.6	20	NA	1.19 ± 0.15	20.0	0.0	0.0
	8	69.7	15.5	20	NA	1.17 ± 0.16	70.0	5.0	0.0
	9	83.5	15.9	20	NA	1.08 ± 0.12	95.0	0.0	0.0
November 1, 2001									
	1	3.0	8.2	30	1.0 ± 0.5	0.94 ± 0.10	86.7	0.0	0.0
	6	36.0	9.8	30	1.0 ± 0.0	0.92 ± 0.07	100.0	0.0	0.0
	8	69.7	13.4	30	1.0 ± 0.3	0.85 ± 0.07	83.3	0.0	0.0
	9	83.5	11.6	30	1.0 ± 0.0	0.95 ± 0.09	90.0	0.0	0.0
March 1, 2002									
	1	3.0	15.1	25	1.0 ± 0.3	1.12 ± 0.13	60.0	0.0	12.0
	2	3.1	17.9	17	1.0 ± 0.4	1.25 ± 0.27	47.1	0.0	47.1
	3	11.4	19.1	10	1.0 ± 0.3	1.10 ± 0.12	20.0	0.0	0.0
	5	14.0	17.8	30	1.0 ± 0.4	1.40 ± 0.25	3.3	3.3	0.0
	6	36.0	18.6	21	1.0 ± 0.2	1.01 ± 0.11	100.0	0.0	0.0
	7	39.0	17.2	30	1.0 ± 0.3	1.07 ± 0.13	100.0	5.0	0.0
	8	69.7	18.9	30	1.0 ± 0.5	0.97 ± 0.12	66.7	0.0	0.0
	9	83.5	21.0	30	1.0 ± 0.2	1.07 ± 0.16	90.0	0.0	0.0

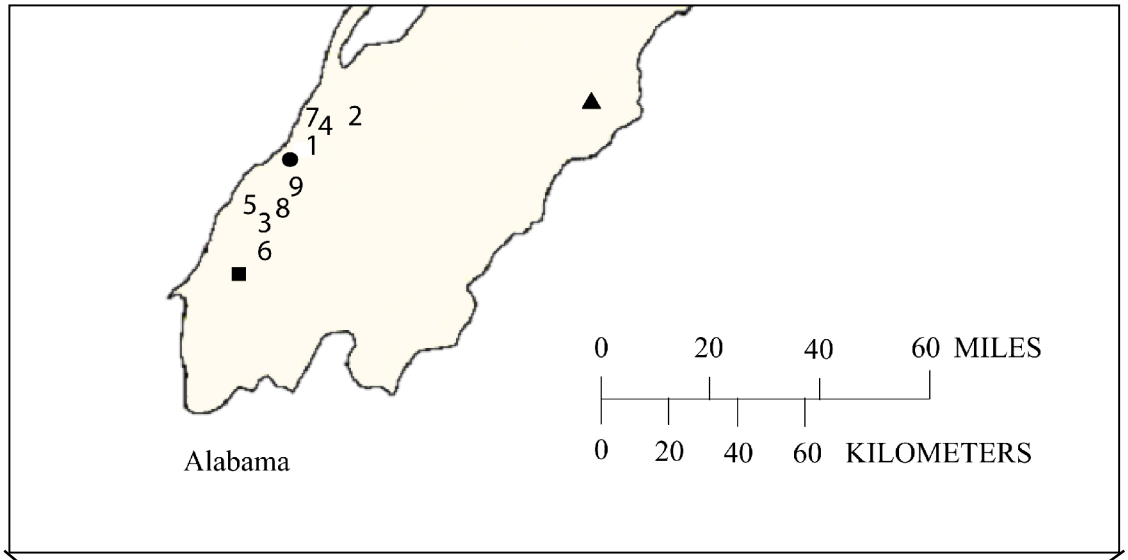
[1]NA = Otoliths were not collected for aging fish in the March 2001 Sampling

Table 2.3 Liver lesion incidence for largescale stonerollers (*Campostoma oligolepis*) collected along an urban land use gradient (ULUG).

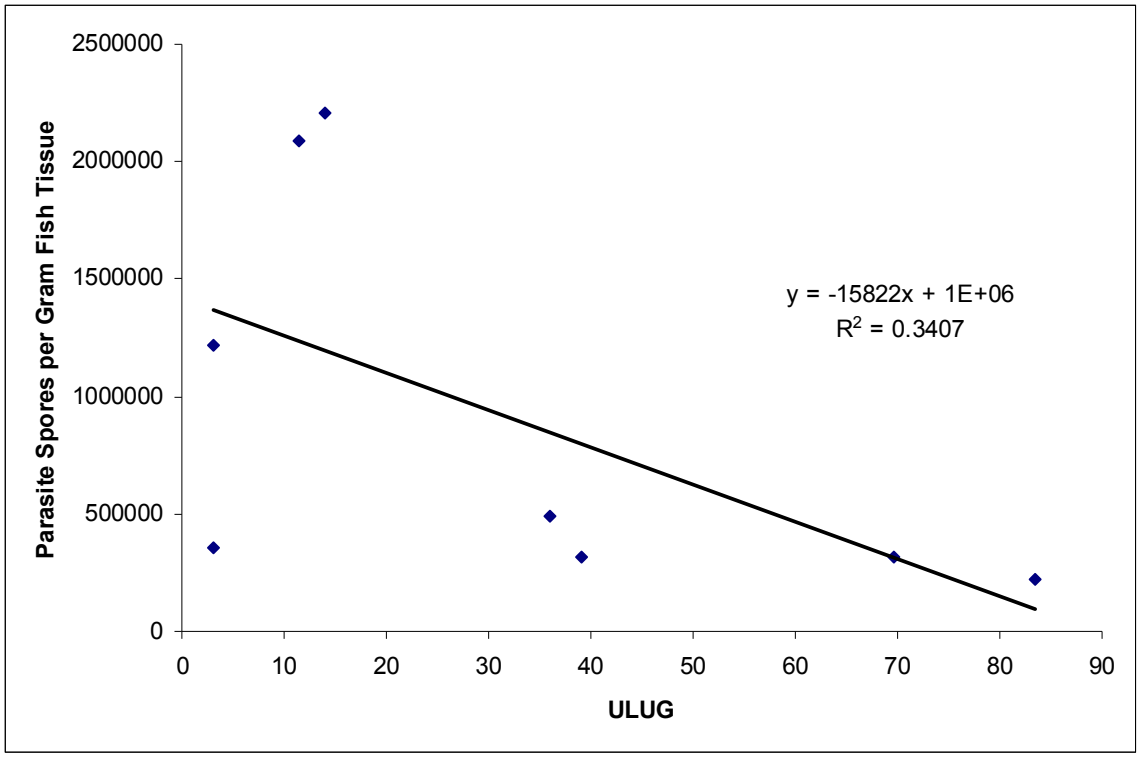
Site	ULUG	Sample Size	Necrosis %	Fibrosis %	Vacuolated Foci %	Macrophage Aggregates %	Regenerative Foci %	Inflammation %	Digenetic Trematodes %
March 2001									
1	3	20	5	25	0	5	55	0	0
2	3.1	17	0	29	65	24	0	6	0
4	12.3	22	5	14	27	5	27	9	0
5	14	20	0	25	20	5	20	0	0
6	36	20	0	35	20	20	0	5	0
7	39	20	5	30	0	0	0	0	5
8	69.7	20	15	50	30	45	10	10	0
9	83.5	20	15	50	40	75	0	25	15
November 2001									
1	3	30	0	10	7	20	50	0	7
6	36	30	0	23	27	0	0	0	0
8	69.7	30	3	43	17	13	0	0	37
9	83.5	30	0	10	0	27	0	13	7
March 2002									
1	3	25	28	16	0	4	12	0	0
2	3.1	17	6	41	35	0	6	0	0
3	11.4	10	20	10	0	10	10	0	0
5	14	30	7	20	10	3	20	0	0
6	36	21	0	24	24	5	0	0	0
7	39	30	0	27	0	3	0	0	3
8	69.7	30	7	43	27	7	0	0	20
9	83.5	30	3	27	0	33	0	7	3

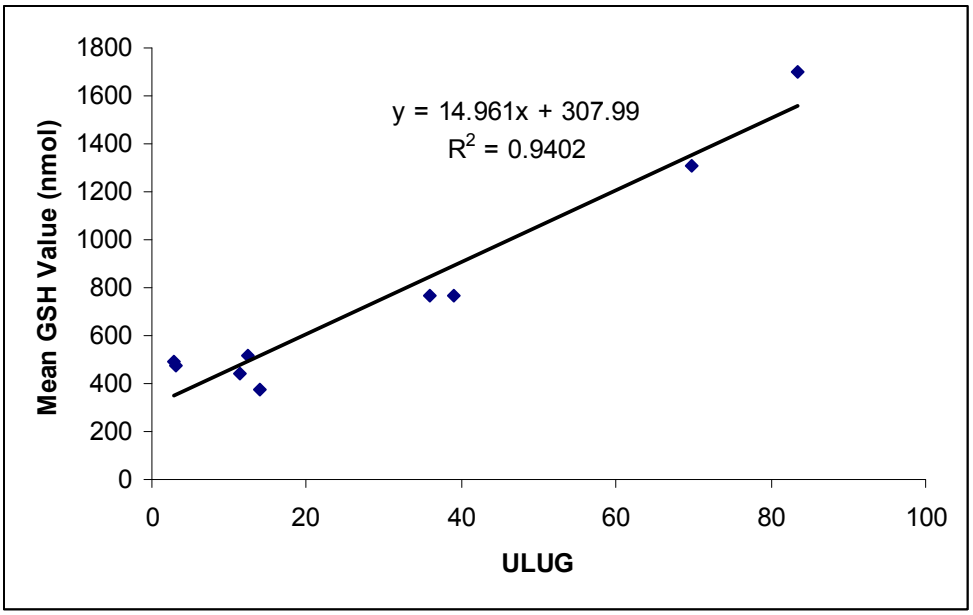
Table 2.4 Parasite staging on a scale of 1-3, where the number is related to the quantity of areas occupied by the myxozoan parasite (areas include: Muscle and connective tissue, kidney, and nerve tissue). Also shown is the percent number of fish in each stage.

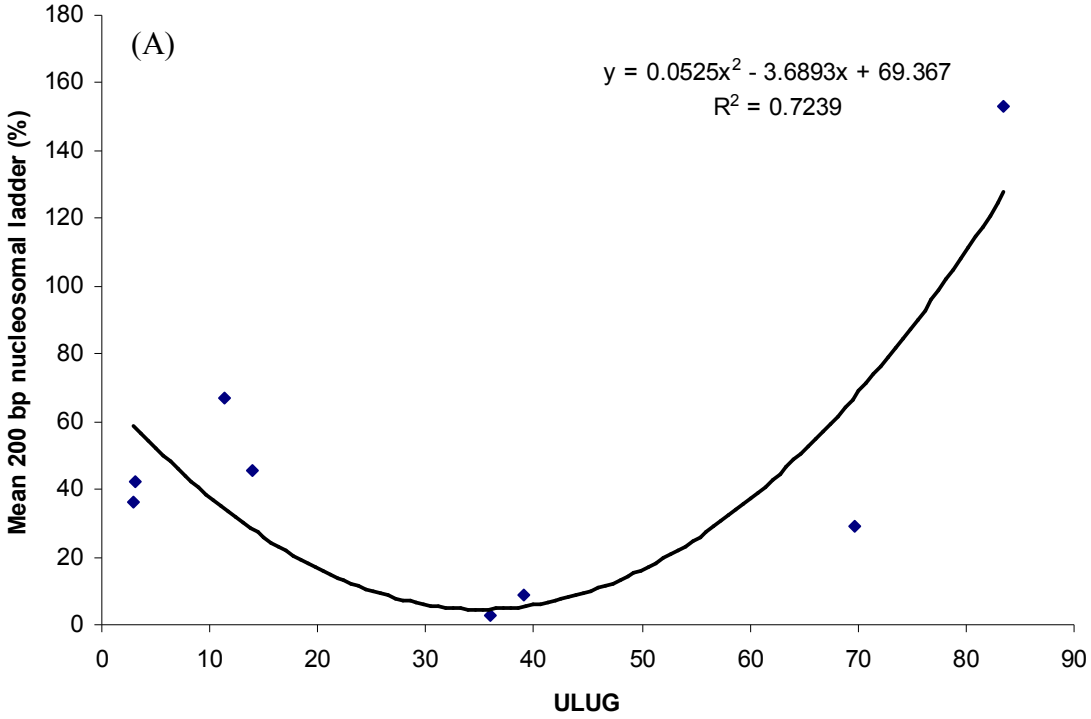
			Stage			Percentage		
Site	ULUG	*n*	1	2	3	1	2	3
1	3.0	75	60	13	2	80.0	17.3	2.7
2	3.1	34	27	4	3	79.4	11.8	8.8
3	11.4	10	6	4	0	60.0	40.0	0.0
4	12.3	22	8	14	0	36.4	63.6	0.0
5	14.0	50	43	7	0	86.0	14.0	0.0
6	36.0	71	59	10	2	83.1	14.1	2.8
7	39.0	50	46	4	0	92.0	8.0	0.0
8	69.7	80	23	51	6	28.8	63.8	7.5
9	83.5	80	35	35	10	43.8	43.8	12.4

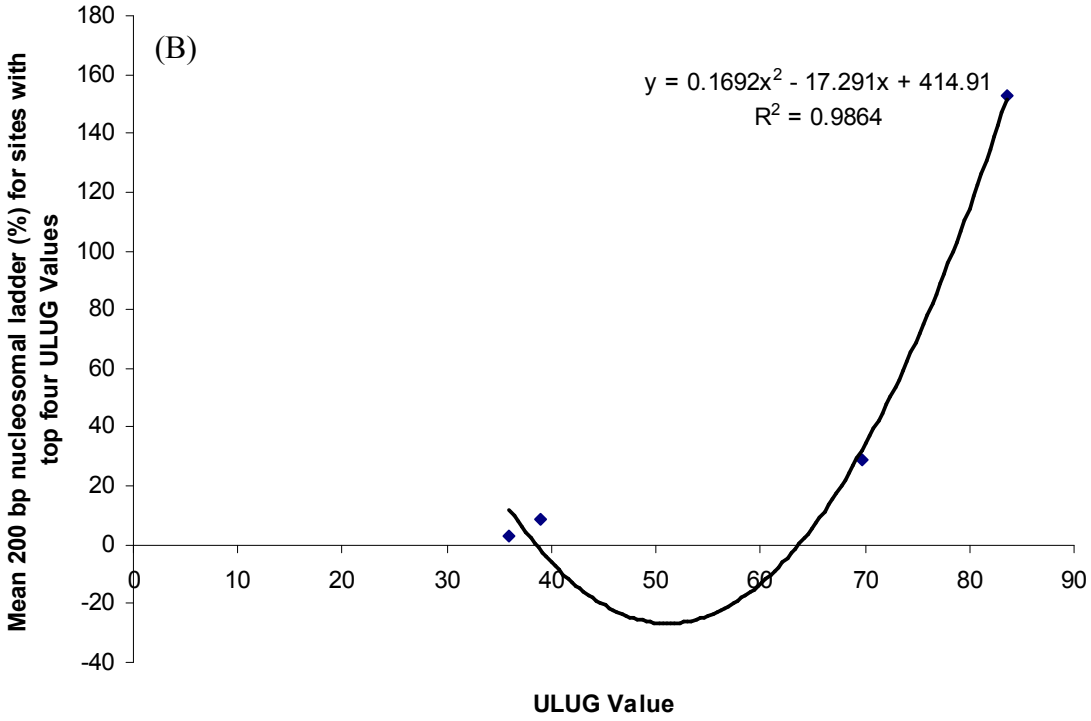

7
4
2
1
9
5
3
8
6
0 20 40 60 MILES
0 20 40 60 KILOMETERS
Alabama


Florence
Huntsville
Decatur
Gadsden
Anniston
Birmingham
Bessemer
Tuscaloosa
Auburn
Phenix City
Selma
Montgomery
Dothan
Mobile
Alabama


2500000
2000000
1500000
1000000
500000
0
Parasite Spores per Gram Fish Tissue
0
10
20
30
40
50
60
70
80
90
ULUG
y = -15822x + 1E+06
R2 = 0.3407


y = 14.961x + 307.99
R2 = 0.9402
Mean GSH Value (nmol)
ULUG
1800
1600
1400
1200
1000
800
600
400
200
0
0
20
40
60
80
100


(A)
y = 0.0525x2 - 3.6893x + 69.367
R2 = 0.7239
Mean 200 bp nucleosomal ladder (%)
ULUG
0
20
40
60
80
100
120
140
160
180
0
10
20
30
40
50
60
70
80
90


(B)
y = 0.1692x2 - 17.291x + 414.91
R2 = 0.9864
Mean 200 bp nucleosomal ladder (%) for sites with top four ULUG Values
ULUG Value
-40
-20
0
20
40
60
80
100
120
140
160
180
0
10
20
30
40
50
60
70
80
90

CHAPTER 3

CHARACTERIZATION OF *MYXOBOLUS STANLII* N. SP. (MYXOZOA: MYXOBOLIDAE: MYXOSPOREA) IN LARGESCALE STONEROLLERS (*CAMPOSTOMA OLIGOLEPIS*) FROM THE MOBILE RIVER BASIN[1]

[1]Iwanowicz D, Howerth EW, Schill WB, Blazer VS, Johnson R. Submitted to the Journal of Parasitology

ABSTRACT: A species of *Myxobolus* was isolated from largescale stonerollers (*Campostoma oligolepis*). The parasite was described using critical identifying features with the inclusion of 18S small subunit ribosomal RNA (SSU rRNA) gene sequence to facilitate future identification. The spore body was ovoid, 10.03 ± 0.7 (7.5 to 11.0) µm in length and 8.8 ± 1.5 (6.3 to 11.3) µm in width in frontal view. Spore thickness was 6.3 ± 2.7 (6.2 – 8.6) µm in sutural view. Polar capsules are pyriform, of equal size, oriented in plane with the sutural ridge. Polar capsules were 2.45 ± 1.5 (range 2.1 – 4.3) µm in width and 4.6 ± 2.7 (range 4.5 – 6.9) µm in length. Based on the SSU rRNA gene sequence of this *Myxobolus* sp. and others available, it is most closely related to *M. bartai*.

INTRODUCTION

Myxozoans are a diverse and economically important group of microscopic metazoan parasites. The genus *Myxobolus* is the largest genus within the Myxosporea with over 744 species currently described (Eiras et al., 2005). Parasites within this genus have received much attention due to the economic effects of specific diseases in their commercial or recreational fish hosts (Kent et al., 2001). Although only a fraction of the currently described *Myxobolus* species are responsible for diseases of economic impact, this impact has increased the research and knowledge of these parasites particularly in the area of myxozoan development and host interactions (Kent et al., 2001).

As with most myxozoan parasites, descriptions of *Myxobolus* species are primarily based on spore morphology, ultrastructure or host/tissue specificity (Lom and Arthur, 1989; Eiras et al., 2005). However, there are studies suggesting variable spore morphology within and between species and a varying degree of host and tissue specificity among myxozoans. Sequence data of 18S has been used extensively to

evaluate phylogenetic relationships and assess the reliability of spore morphology, host and tissue specificity for identification. Kent et al. (2001) reported that myxozoan taxa cluster more by development and tissue location than by spore morphology. Andree et al. (1999) reported that *Myxobolus* species also tend to cluster more according to their tissue location than spore morphology or host specificity, as did Eszterbauer (2004) for gill-infecting *Myxobolus* species. For intramuscular *Myxobolus* species of cyprinids, sequence analysis demonstrated clustering according to fish species but not spore morphology (Molnár et al., 2002). Conversely, Salim and Desser (2000) using the 18S rDNA sequences of *Myxobolus* species from cyprinids, demonstrated spore morphology, but not host specificity or tissue tropism, was useful in the systematics.

Largescale stonerollers (*Campostoma oligolepis*) are abundant cyprinids found throughout the Central and Mideastern United States, and are often a major component of stream fish communities (Trautman, 1981; Mettee et al., 1996). While approximately 100 species of myxozoans have been described from cyprinid fishes worldwide and include members of the genera *Myxobolus, Myxidium, Myxobilatus, Dicauda, Unicauda, Hoferellus* and *Sphaerospora* (Hoffman and Walker, 1978; Minchew, 1981; Mitchell et al., 1983; Lom et al., 1992; Yokoyama et al., 1996; Székely et al., 2001; Koprivnikar et al., 2002; Molnár et al., 2002; Kent et al., 2004; Eszterbauer, 2004; Longshaw et al., 2005), no myxozoan parasite has been described in the largescale stoneroller. In this paper we present the description of a new species of *Myxobolus*, the microscopic appearance of infected fish, ultrastructural data using transmission electron microscopy, sequence information from 18S SSU rDNA, and phylogenetic analyses.

MATERIALS AND METHODS

Sample collection

Approximately five hundred largescale stonerollers were collected from nine streams surrounding Birmingham, AL via electroshocking. Fish were sampled during March 2001, November 2001, and March 2002 as part of a fish health assessment study (regarding the effects of urban land use) (Iwanowicz et al., Submitted). Ten largescale stonerollers were also collected via electroshocking from the South Branch of the Potomac, near Romney, WV.

Fish were euthanized with a lethal dose of tricaine methanesulfonate (MS-222, Argent Finquel®, Redmond, WA), weighed, measured, and examined for external and internal lesions. Most fish were preserved in 10% phosphate buffered formalin and prepared for histopathological examination. An additional 10 fish from each site were frozen (-20 ºC) for spore enumeration and wet mount preparation during the November 2001 and March 2002 collections.

Spore Identification

Spores found within the musculature of the largescale stonerollers were examined in fresh wet mounts, measured and photographed. For the transmission electron microscopy, pieces of tissue were removed from paraffin blocks and paraffin extracted with three washes of xylene. The tissue was then placed in 100% ethanol, processed and embedded in Epon-Araldite (EMS, Hatfield, Penn). Ultrathin sections were placed on 200-mesh copper grids and post-stained with 5% methanolic uranyl acetate and Reynold's lead citrate and examined with a JEOL JSM-1210 transmission electron microscope.

Spore Enumeration

The skin and internal organs were removed from the fish and each fish was individually weighed for spore enumeration. Individual fish bodies were macerated, blended with spring water, and then sequentially filtered through a series of sieves (500 μm, 250 μm and 45 μm). The filtrate was then pelletted (12000 rpm x 12 min) and weighed. Pellets were suspended in five mL of spring water and the spores were quantified via hemacytometer. Spores per gram of fish tissue were determined.

Histopathology

Fish tissues were processed, embedded in paraffin, sectioned at 6 μm and stained using hematoxylin and eosin, Perl's prussian blue, or Giemsa procedures (Luna, 1992). Saggital midline sections of the entire body were processed in smaller fish. In the larger fish organs were removed, and the head and body were cut in half saggitally. All tissues and organs were processed and examined for abnormalities.

DNA Sequencing and Analysis

Genetic screening of the spores was based on the 18S PCR. Briefly, a sample of stoneroller muscle was removed and frozen at -80 C prior to DNA extraction. Genomic DNA was extracted from frozen muscle using the Qiagen DNeasy Tissue Kit (Valencia, California). Genomic DNA was stored at 4 C prior to amplification. For PCR amplification, a PCR cocktail containing 1 μM of each primer: MyxFor (5'- ACC GTG GGA AAT CTA GAG CTA A – 3') and MyxRev (5' – GTT CCA TGC TAT YAA CAT TCA A – 3') was added to PuReTaq™ Ready-To-Go™ PCR Beads (Amersham Biosciences, Buckinghamshire, England). All primers were designed as universal

myxozoan primers and purchased from Integrated DNA Technologies (www.idtdna.com).

The primers were used with a PCR cycling profile consisting of 5 min denaturation step at 95 °C; 35 cycles of 1 min at 95 °C, 1 min at 56 °C, 1 min at 72 °C, and a final 10 min extension at 72 °C. After amplification, 10 µL of the DNA sample was identified by electrophoresis at 90 V for four hours on a gel containing 1.5% synergel (Diversied Biotech, Boston, Massachusetts), and 0.75% I.D.NA® agarose (FMC Bioproducts, Rockland, Maine).

PCR products were cleaned by adding 1.4 µL Exonuclease I (New England Biolabs, Ipswich, Massachusetts) and 4 µL Shrimp Alkaline Phosphatase (Promega, Madison, Wisconsin). Sequencing reactions were performed using Applied Biosystems (Foster City, California) Big Dye Cycle Sequencing Kit and the reactions were cleaned with ethanol and sodium acetate. Briefly, 2 µL of Big Dye, 1 µL of one primer (either MyxFor or MyxRev), and 2 µL of the template DNA were added to 5 µL of water. This was done for both the forward and reverse primer. The samples were then run on the following PCR cycling profile: 25 cycles of 30 sec at 96 ºC, 15 sec at 50 ºC, and 4 min final extension at 60 ºC. The cleaned samples were sequenced in both using an Applied Biosystems 3100 Genetic Analyzer. Samples were sequenced in both directions. Resulting sequences were aligned, and the 614-base pair sequence for *Myxobolus stanlii* n. sp. was deposited in GenBank (accession numbers: DQ779995 and DQ779996).

Myxozoan SSU rDNA sequences were acquired from the NCBI database, aligned with ClustalX v1.83(Thompson et al., 1997) and cropped to yield sequences of equal length (614 bp including gaps). Sequences (n = 21) used for analysis were as follows: *M.*

bibullatus (AF378336), *M. bartai* (AF186835), *M. pseudodispar* (AF380145), *M. musculi* (AF380141), *M. cyprini* (AF380140), *M. elegans* (AF448445), *Sphaerospora molnari* (AF378345), *M. dispar* (AF507972), *M. muelleri* (AY325284), *M. siddalli* (AF186840), *M. algonquinensis* (AF378335), *M. intimus* (AY325285), *M. obesus* (AY325286), *M. hungaricus* (AF448444), *M. pseudokoi* (AF186839), *M. bilobus* (DQ008579), *M. longisporus* (AY364637), *M. pendula* (AF378340), *M. cultus* (AB121146), *Tetracapsuloides bryosalmonae* (U70623) and the case isolate *M. stanlii* n. sp. (DQ779995). *Tetracapsuloides bryosalmonae* was set as an outgroup for phylogenetic analysis. Maximum parsimony and maximum likelihood phylogenetic analyses were conducted in PAUP*4.01b1 and Treefinder v. May 2006, respectfully (Swofford, 1998). Bayesian analysis was conducted using Mr. Bayes 3.1.1 software (Huelsenbeck et al., 2001; Ronquist and Huelsenbeck, 2003). Gaps were treated as missing data in all analyses. Maximum parsimony analysis utilized the Goloboff fit criterion (kappa = 2) using the heuristic search algorithm with 1000 random additions of sequences and tree bisection-reconnection (TBR) branch swapping. Bootstrap values were calculated using 1000 replicates using the same heuristic search. Maximum likelihood analysis used a heuristic search algorithm with 100 random sequence additions and TBR branch swapping. Bootstrap values were calculated with 1000 replicates using the same heuristic search. Maximum likelihood analysis used the default analysis settings in Treefinder with the appropriate substitution model, and bootstrap values calculated from 1000 replicates. Maximum likelihood analysis and Bayesian inference of phylogeny were conducted using the general time reversible (GTR+I+G) model based on the most appropriate DNA substitution model as calculated by Mr. Modeltest v.2.2

(Nylander, 2004). Parameters for the Bayesian analysis included 1,000,000 generations of Markov chain Monte Carlo sampling sampled once every 100 generations. A consensus phylogram was constructed in Treefinder (Jobb et al., 2004) based on the trees sampled in the asymptotic phase of the Bayesian analysis (burnin = 101, trees used for consensus = 9000).

DESCRIPTION

Myxobolus stanlii n. sp.

The spores are typical of the genus *Myxobolus* Bütschli, 1882. The spore body is ovoid, 10.03 ± 0.7 (n=41; range 7.5 to 11.0) µm in length and 8.8 ± 1.5 (n=41; range 6.3 to 11.3) µm in width in frontal view. The spores, pleomorphic in wet mounts and histologic sections, consist of two equal valves adhering together along the sutural line (Figure 1; Figure 2). Spore thickness is 6.3 ± 2.7 (n=41; range 6.2 – 8.6) µm in sutural view. Polar capsules, pyriform, of equal size, oriented in plane with the sutural ridge, 2.45 ± 1.5 (n=31; range 2.1 – 4.3) µm in width and 4.6 ± 2.7 (n=31; range 4.5 – 6.9) µm in length. Polar filament with 5 – 7 turns in a single layer surrounded by a dense matrix (Figure 3A). Sporoplasm contains one nucleus surrounded by dense, irregular matter. The nucleus is surrounded by a thick, less dense wall. Shell valves smooth, without projections (Figure 3B).

Taxonomic Summary

Type host: Largescale stonerollers *Campostoma oligolepis* (Cypriniformes: Cyprinidae).

Other hosts: Unknown.

Type locality: Birmingham, AL, USA, and South Branch of the Potomac River, Romney, WV, USA.

Site of infection: Connective tissue, muscle, nerve, kidney macrophage aggregates.

Type material: Histological sections of infected tissue stained with H&E and Geimsa deposited in the United States National Parasite Collection (USNPC 98811), Beltsville, MD. Genbank accession nos.: DQ779995 (Alabama isolates) and DQ77996 (West Virginia isolates).

Prevalence of infection: 100% of the 510 largescale stonerollers sampled from Birmingham, AL and from Romney, WV were positive for spores.

Etymology: The specific name is in honor of a colleague and friend John Stanley.

Remarks

Spores were most often found in loosely formed cysts or free within loose connective tissue between muscle bundles (Figure 4A) and surrounding cartilage (Figure 4B). Only occasionally were spores actually observed within muscle bundles (Figure 4C). Most often there was little to no inflammatory response; however, the presence of melanin often accompanied the spores (Figure 4D). Spores and melanin were observed around and within peripheral nerve tissue (Figure 4E) and sometimes associated with the central nervous system (Figure 4F). In heavy infections and in association with free spores, inflammation was observed (Figure 5A). Spores were also commonly found around blood vessels (Figure 5B) and within macrophage aggregates in the kidney (Figure 5C). Occasionally structures that may have been trophozoite stages were observed within the lumen of the kidney tubules (Figure 5D) and within the glomeruli.

Neighbor-joining, maximum likelihood, maximum parsimony (length = 1100, CI = 0.5818, HI = 0.4182, RI = 0.5958; 699 parsimony informative characters), and Bayesian Inference place *M. stanlii* most closely related to *M. bartai* and within a clade including *M. bartai, M. pseudodispar, M. musculi,* and *M. cyprini* (Figure 6). Morphologically and within its host, *Myxobolus stanlii* n. sp. is not similar to any other *Myxobolus* spp. previously described (Table I). Although stonerollers were only sampled around Birmingham, AL and near Romney, WV, it appears that the range of the parasite may extend through the range of the host however, more collections are necessary.

DISCUSSION

The genus *Myxobolus* Bűtschlii, 1882 is one of the most intensely studied genera in the phylum Myxozoa. These parasites can be found in every organ of a fish and have been known to cause serious disease to both wild and cultured fishes. Increased knowledge in molecular genetics and life cycles of myxozoans have shown that certain of the traditional descriptive characters (morphology, size, host tissue specificity) may be misleading (Bahri et al., 2003). In fact, spore morphology or tissue location of a given myxozoan may vary with fish species and due to environmental influences (Lom, 1987; Mitchell, 1989; Hedrick et al., 1999; Baldwin and Myklebust, 2002).

Our phylogenetic analyses of *M. stanlii* n. sp. support its description as a distinct species. Following methods of Bahri et al. (2003), we also assessed the reliability of tissue specificity as a reliable indicator of species and as an indicator of evolutionary relationships. Our data suggested that spores grouped according to tissue specificity more than spore morphology. For example, *M. stanlii* n. sp. belonged to the clade of muscle associated myxozoan parasites including *M. bartai, M. pseudodispar, M. musculi*,

M. cyprini, and *M. bibullatus* (Salim and Desser, 2000; Longshaw et al., 2005). Likewise, *M. intimus, M. obesus, M. hungaricus, M. pseudokoi, M. bilobus, M. longisporus*, and *M. pendula* all infect gills of fish and grouped together (Salim and Desser, 2000; Eiras et al., 2005). These parasites are not morphologically similar but they do have similarities in host and tissue specificity. Only one group formed that infected multiple tissues (gills, skin, kidney, and ovary) and included: *M. elegans, Sphaerospora molnari, M. dispar, M. muelleri, M. siddalli* and *M. algonquinensis* (Salim and Dresser, 2000; Eiras et al., 2005; Longshaw et al., 2005).

Although spores of *M. stanlii* n. sp. appear to develop most commonly within the connective tissue and nerves of the skeletal muscle and head region, this parasite shares many characteristics with the other muscle-infecting species. Similar to that observed in histological sections of *M. pseudodispar* and *M. musculi* spores, *M. stanlii* spores are pleomorphic (Longshaw et al., 2005). Spores of *M. cyprini* develop in the skeletal muscle and are disseminated via the circulatory system. Spores can be found in numerous organs often within macrophage aggregates as a result of rupture of the intramuscular plasmodia (Molnár and Kovács-Gayer, 1985). Spores of *M. pseudodispar* are also found within macrophage aggregates of the kidney and similar to *M. cyprini.* It has been suggested that spores released from the muscle are carried to the kidney via the bloodstream (Baska, 1987). The close proximity of plasmodia to the circulatory system and the presence of spores within macrophage aggregates of kidney suggest a similar dispersion of *M. stanlii* n. sp. spores in the largescale stoneroller.

The presence of *M. stanlii* within the largescale stoneroller does not appear to affect the health of the fish under normal conditions. Environmental stressors such as an

increase in temperature, poor water quality and anthropogenic disturbances such as those associated with increasing urban land use may increase severity of infections (Iwanowicz et al., *In prep*). However, fish mortality has not been recognized and the pathogenicity of this parasite species needs to be studied. Myxozoan parasites do not always kill fish, in fact, most do not.

Complete life cycles have been described for a few species of *Myxobolus* (Markiw and Wolf, 1983; Ruidisch et al., 1991, Molnar et al., 1999; Eszterbauer et al., 2000; Koprivnikov and Desser, 2002; Koie, 2005) and it has been found that *Myxobolus spp*. utilize multi-host (fish and aquatic oligochaete) for infection. To date, neither the aquatic oligochaete nor the actinomyxon stage of *M. stanlii* n. sp. have been identified.

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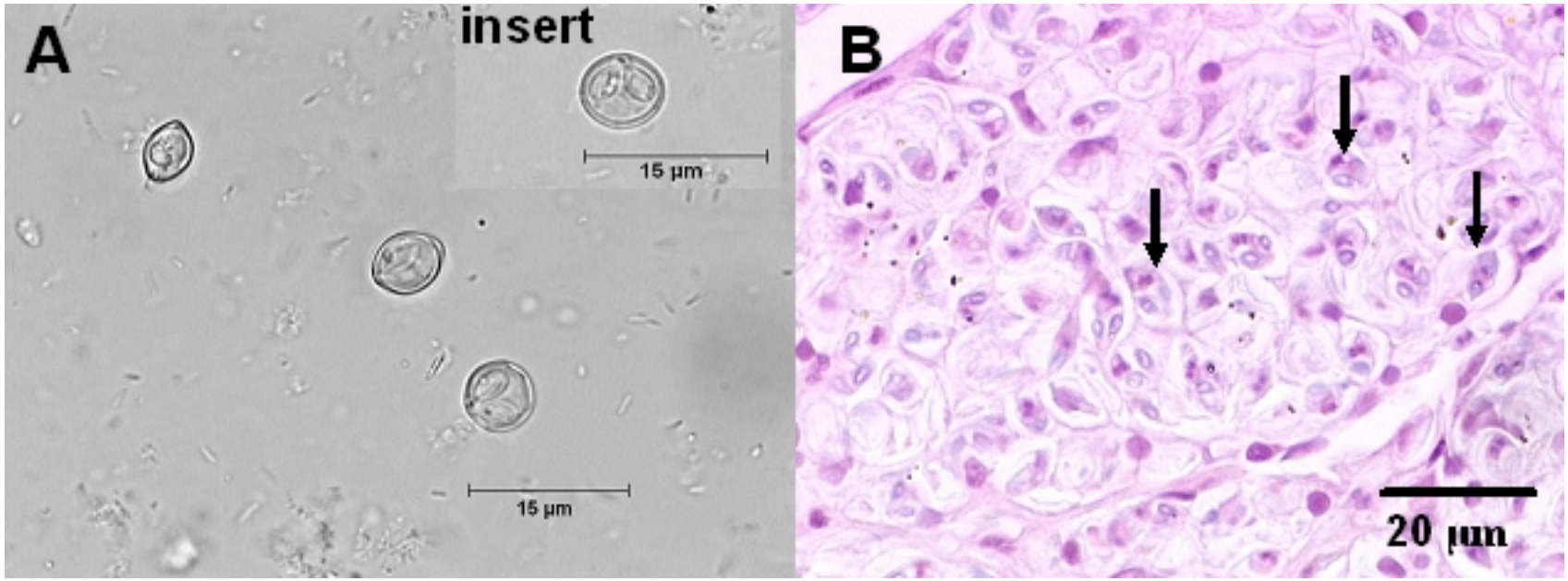
within a macrophage aggregate (a) of the kidney. D. Trophozoites (arrows) within tubules lumens of the kidney (a). H&E stain.

FIGURE 3.6. Phylogram derived from Bayesian Inference analysis of myxozoan 18S SSU rDNA (641 bp including gaps). Bootstrap values are noted beside the branches. Phylogram was similar to those derived from maximum parsimony and maximum likelihood).

Table 3.I. Morphological features of *Myxobolus* species most closely related to *Myxobolus stanlii* n sp. within a clade. Includes *Myxobolus bartai, Myxobolus pseudodispar, Myxobolus musculi*, and *Myxobolus stanlii* n sp.*

Species	LS	WS	TS	LPC	WPC	PC	Type-host
Myxobolus bibullatus Kudo, 1934	14-15	11.5-12.5	6-7.5	7	3.5	=	*Catostomus commersonii*
Myxobolus bartai Salim and Desser, 2000	11.0 (10.3-11.4)	10.8 (10.0-11.3)	7.1(6.1-7.5)	6.3 (5.9-7.0)	3.8 (3.2-4.2)	≠	*Notropis cornutus*
Myxobolus pseudodispar Gorbunova, 1936	12-Oct	7-9.5	5.3-6	4.5-6.2	3-3.7	≠	*Rutilus rutilus*
Myxobolus musculi Longshaw et al., 2005	8.67-11.86	6.82-9.16		3.32-11.86	1.83-3.68	≠	*Gobio gobio* *Phoxinus phoxinus*
Myxobolus cyprini Doflein, 1898	16-Oct	12-Aug		5.2-7		=	*Cyprinus carpio*
***Myxobolus stanlii* n sp.**	10.03 (7.5-11)	8.8 (6.3-11.3)	6.3 (6.2-8.6)	4.6 (4.5-6.9)	2.45 (2.1-4.3)	=	*Campostoma oligolepis*

Note— Abbreviation descriptions include: LS, length of the spore; WS, width of the spore; TS, thickness of the spore; LPC, length of the polar capsules; WPC, width of the polar capsules; PC, relative size of the polar capsules (equal, not equal). All measurements are in micrometers. Extended table found in Eiras et al. (2005).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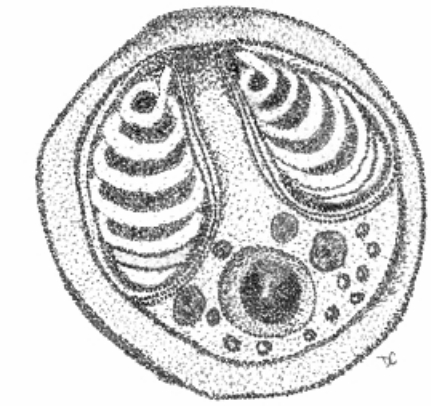
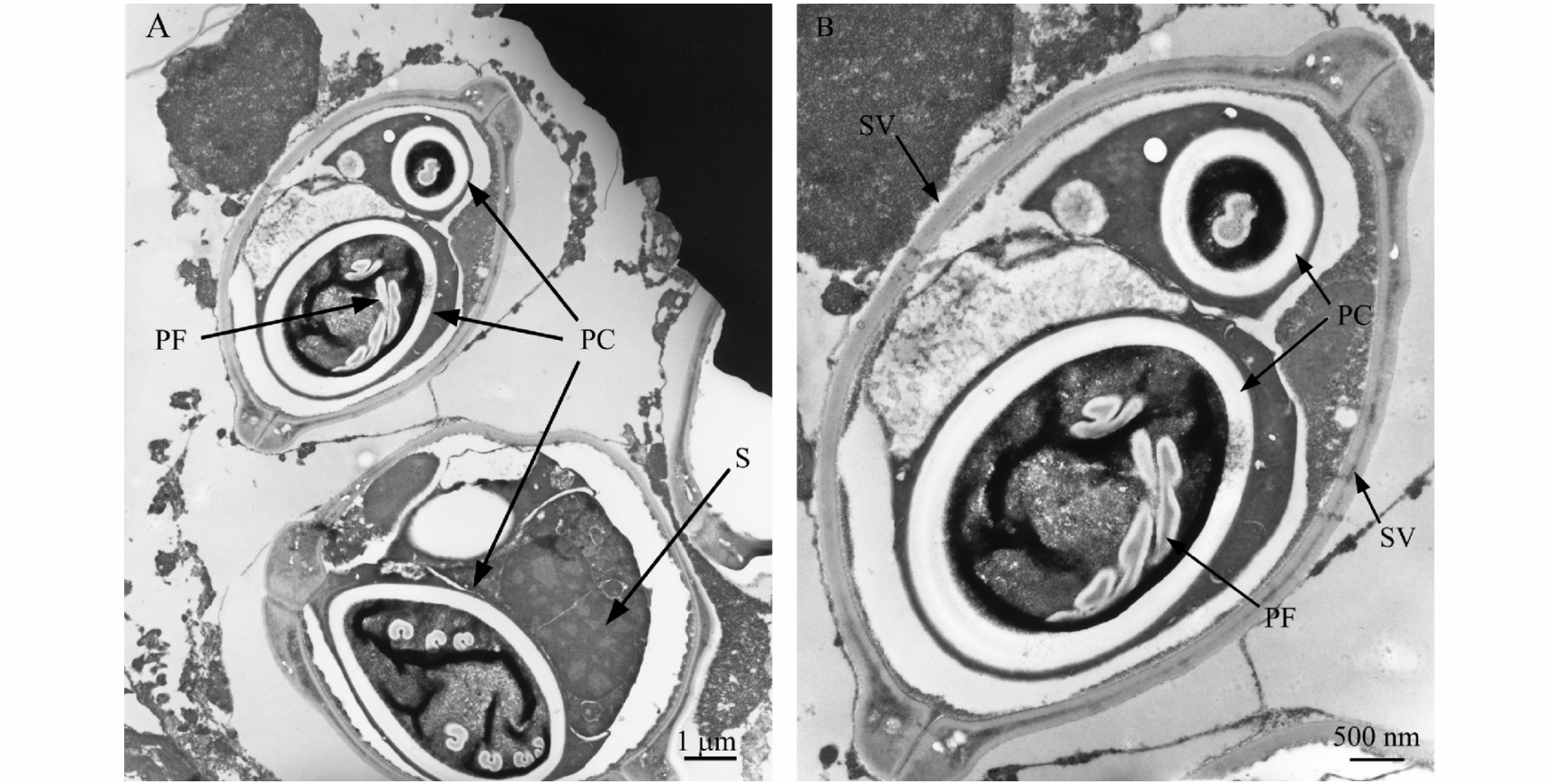
insert
15 µm
15 µm
B
20 µm


10 μm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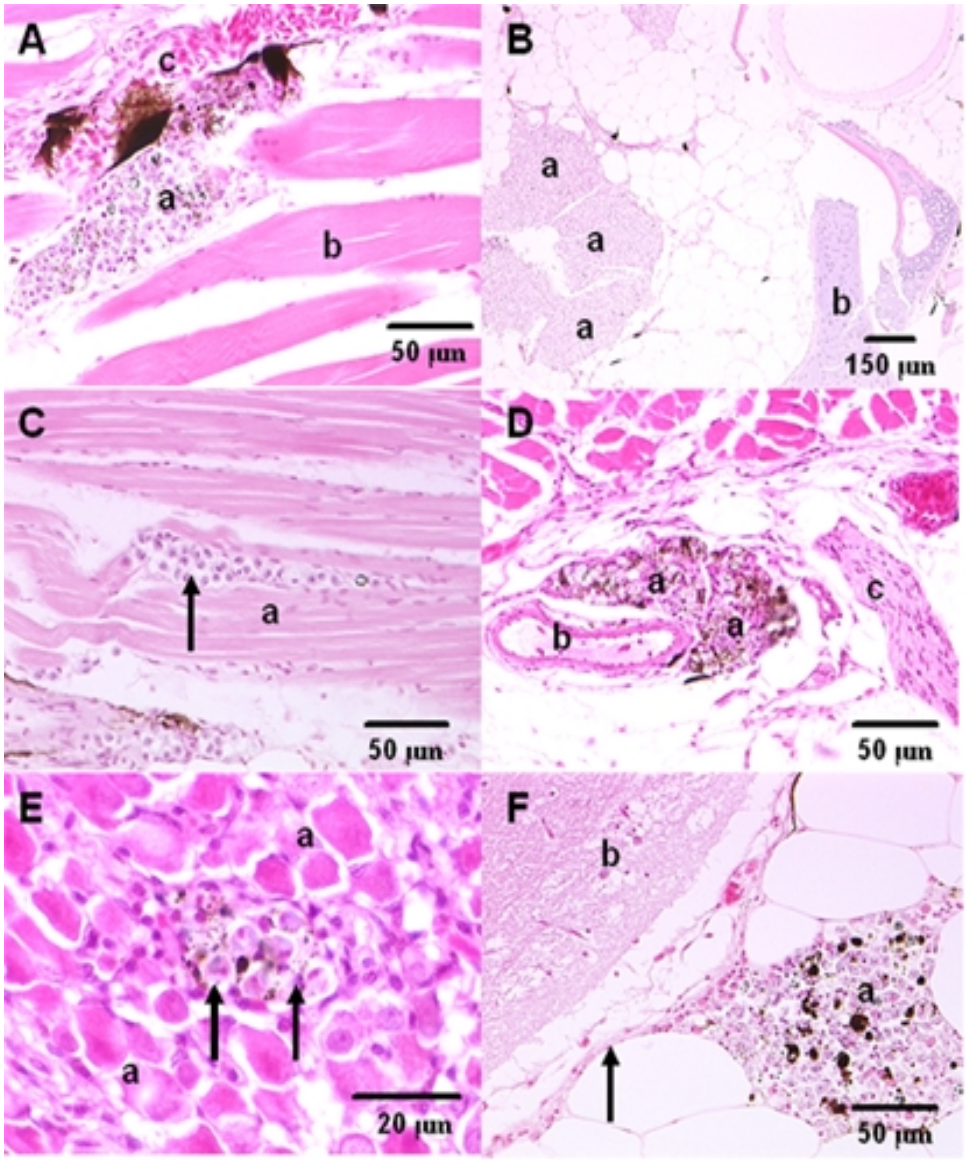
S
1 µm
B
SV
PC
SV
PF
500 nm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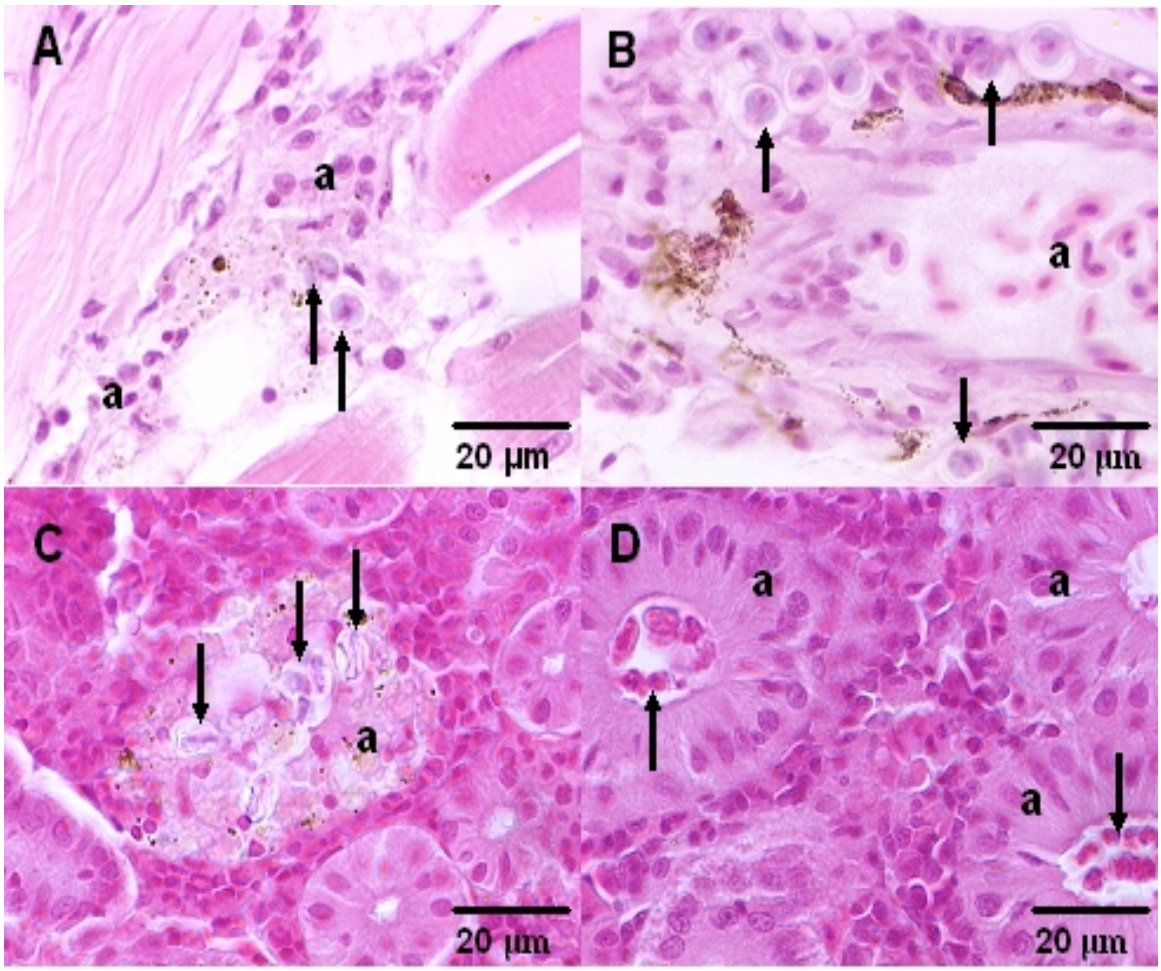
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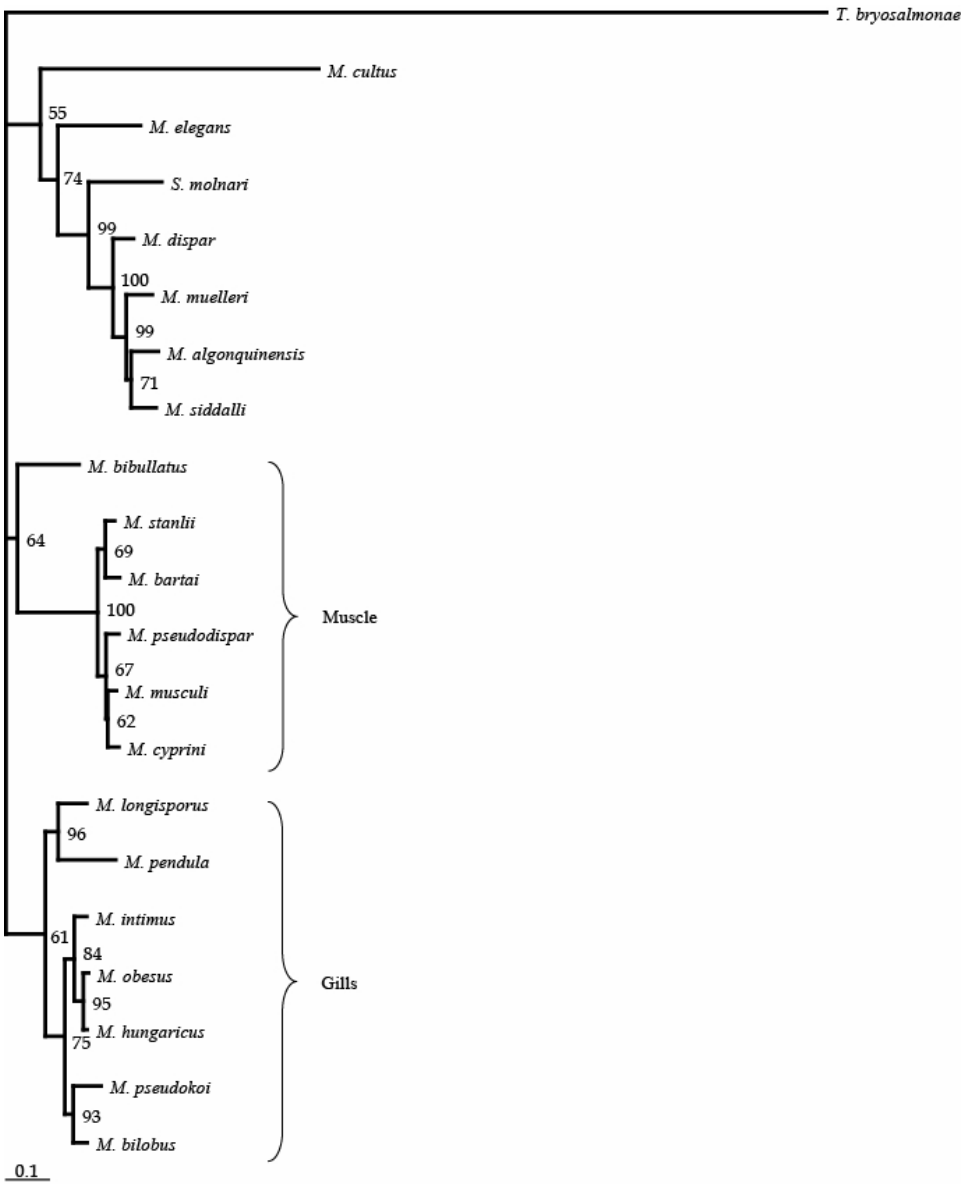
T. bryosalmonae
M. cultus
55
M. elegans
74
S. molnari
99
M. dispar
100
M. muelleri
99
M. algonquinensis
71
M. siddalli
M. bibullatus
64
M. stanlii
69
M. bartai
100
M. pseudodispar
67
M. musculi
62
M. cyprini
Muscle
M. longisporus
96
M. pendula
M. intimus
61
84
M. obesus
95
M. hungaricus
75
M. pseudokoi
93
M. bilobus
Gills
0.1

CHAPTER 4

EFFECT OF RIPARIAN ZONE AND ASSOCIATED STREAM SUBSTRATA ON *TUBIFEX TUBIFEX* DENSITY AND INFECTIONS WITH MYXOZOAN PARASITES

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[1]Iwanowicz DD, Blazer VS, Schill WB, Griffin AR. To be submitted to the Journal of Aquatic Animal Health

Abstract

Whirling disease is caused by *Myxobolus cerebralis*, a metazoan parasite found throughout the United States. This parasite has a two-host life cycle with both an invertebrate host (*Tubifex tubifex*) and a vertebrate host (salmonid fishes). Although the parasite was first identified on the East Coast in 1956, population effects on salmonid populations have only been documented in the West/Intermountain West. Environmental influences and anthropogenic disturbances may play a significant role in whirling disease by affecting the salmonid or worm host. Few studies have addressed how environmental factors affect *T. tubifex* populations and their ability to become infected with *M. cerebralis* and produce infective triactinomyxon (TAM) spores, particularly in Eastern populations. Hence the primary objective of this study was to determine if substrate and associated riparian zone would affect *T. tubifex* density. The secondary objective was to assess the genetic populations of *T. tubifex* collected from different habitats and compare them to each other as well as to populations described from other parts of the U.S. Specific lineages of *T. tubifex* and presence of myxozoan parasites were determined via PCR. Genetic screening of the New York and Pennsylvania oligochaete populations revealed that most *T. tubifex* belonged to lineage I and III. Deciduous sites had the largest percentages of lineage I (76 and 86%) populations and were significantly higher than those from deciduous/meadowland sites ($P < 0.001$), coniferous sites ($P < 0.001$), and meadowland sites ($P < 0.001$). Deciduous sites also had the lowest percent of *T. tubifex* within the oligochaete population and the lowest percentage of lineage III. The highest percentages of lineage III *T. tubifex* were found at a deciduous/meadowland site (90%). This site had significantly larger populations of lineage III *T. tubifex* than those

populations found in meadowland ($P < 0.001$), and coniferous sites ($P < 0.001$). *M. cerebralis* was the most common myxozoan parasite found at any site. However, *Myxidium truttae* was positively identified in worms from a meadowland site, while positive bands for *Myxobolus pseudodisbar* were identified in worms from a deciduous/meadowland site. Myxozoan parasites were only found in lineage III worms. Apparently, certain environments select for certain lineages of *T. tubifex* that differ in their susceptibility to *M. cerebralis* infection, and perhaps other myxozoan parasites as well.

KEYWORDS – *Tubifex tubifex*, *Myxobolus cerebralis*, riparian habitat, Myxozoan

Introduction

Whirling disease is caused by the metazoan parasite *Myxobolus cerebralis* and affects salmonids across the United States (Hoffman 1990; Bartholomew and Reno 2002). This parasite has a complex two-host life cycle involving separate stages of sporogony in salmonid fishes and the aquatic oligochaete *Tubifex tubifex* (Markiw and Wolf 1983; Wolf et al. 1986). *Tubifex tubifex* produces the triactinomyxon (TAM) spore that infects salmonid hosts. Population effects on wild salmonid stocks have been reported in some geographic areas (Walker and Nehring 1995; Vincent 1996) in the Western United States. Although the parasite is endemic in other areas of the United States, similar population declines have not been observed (Hnath 1996; Modin 1998; Kaeser et al. 2006). The specific reasons for this regional dichotomy are unknown. Understanding the impacts and potential for management of this disease in wild populations requires an understanding of the environmental factors, both climatic and anthropogenic that affect the ability of the parasite to infect and proliferate within both worm and fish hosts.

First diagnosed on the East Coast in Pennsylvania in 1956 (Hoffman 1962), whirling disease spread across the contiguous US (Bartholomew and Reno 2002). In 1994, *M. cerebralis* was diagnosed in hatchery and wild fish in New York. Between 1994 and 1996, the New York Department of Environmental Conservation (NYDEC) conducted field exposure studies while monitoring hatchery and wild fish populations. *Myxobolus cerebralis* was detected in wild and cultured brook (*Salvelinus fontinalis*), brown (*Salmo trutta*) and rainbow trout (*Onchorhynchus mykiss*) throughout the state. Further research has shown that the disease does not spread rapidly from infected waters

to uninfected waters, or within watersheds. Additionally both resistant species (brown trout) and highly susceptible species (rainbow trout) were not affected at the population level in the areas examined (Hulbert 1996). None of the East Coast state with *M. cerebralis* in wild trout populations has documented population declines due to whirling disease, it has caused the decimation of wild trout populations in the West and Intermountain West. In fact, up to 90% of yearling rainbow trout are infected and die of whirling disease in infected reaches of the Madison, Gunnison, and Colorado Rivers in Colorado and Montana (Vincent 1996; Nehring and Walker 1996; Wagner 2002), but the affects of *M. cerebralis* on wild fish populations is highly variable within and between river systems (MacConnell and Vincent 2002).

Considerable research has focused on differential susceptibility of salmonid species, subspecies, and strains to *M. cerebralis* (Hedrick et al. 1999a,b; Densmore et al. 2001; Vincent 2002, Blazer et al. 2004). The effects of water temperature and optimal parasite dose required for development of the parasite in both hosts has also been investigated (Schisler et al. 2000; Kerans et al. 2005; Dubey et al. 2005; Krueger et al. 2006). Others have assessed the geographic distribution and spread of *M. cerebralis* (Uspenskaya 1955; Baldwin et al. 1998; Bergerson and Anderson 1997; Zendt and Bergerson, 2000).

Recently studies have focused on *T. tubifex* and its potential role in geographically based differences in whirling disease (Beauchamp et al. 2005; Beauchamp et al. 2006; Kaeser et al. 2006; Kaeser and Sharpe 2006). *Tubifex tubifex* is a cosmopolitan freshwater oligochaete that is morphologically similar to closely related species (Chapman and Brinkhurst 1987; Kathman and Brinkhurst 1999). Phylogenetic

analysis of mitochondrial 16S ribosomal DNA from geographically distinct populations of *T. tubifex* provides evidence of genetic differences between morphologically identical strains (Beauchamp et al. 2001; Sturmbauer et al. 1999; DuBey and Caldwell 2004). Six genetically distinct lineages are present in Europe (I - VI), while only four of these six lineages have been identified in the US (Sturmbauer et al. 1999; Beauchamp et al. 2002). Interestingly, all four lineages differ in susceptibility to infection with *M. cerebralis.* In general, lineages I and III are the most susceptible to *M. cerebralis*, and lineages V and VI are comparatively more resistant to *M. cerebralis*. There are also differences in susceptibility of individuals within lineages (Beauchamp et al. 2002).

The abundance and genotype of *T. tubifex* certainly influence the severity of this disease on fish populations; however, environmental factors may be important in selecting for certain lineages and influencing the infection of susceptible worms. Beauchamp et al. (2005) suggested more susceptible worm populations (lineages I and III) were more prevalent in man-made habitats (e.g., reservoirs, dams, drainage channels) and near urban development and other anthropogenic disturbances in the Colorado River. Kaeser and Sharpe (2006) also found this to be true in Pennsylvania, where sites with highly enriched organic substrate were a requisite for detectable *T. tubifex* populations. Previous work in our laboratory has demonstrated effects of substrate type (mud, sand, leaf litter) on spore production and release in *T. tubifex* worms infected with *M. cerebralis* (Blazer et al. 2003). The primary objective of this study was to determine if stream substrate (leaf litter) and hence, riparian zone affected *T. tubifex* density. A secondary objective was to assess the genetic populations of *T. tubifex* collected from

different habitats and compare them to each other as well as to populations described from other parts of the U.S.

Materials and Methods

Site Selection—Site selection was based on watersheds where whirling disease is enzootic. Sites were chosen based on information provided by the New York Department of Environmental Conservation and the Pennsylvania Fish and Boat Commission regarding the presence of brook, brown, and rainbow trout. Elton Creek was selected as the negative control because it was negative for whirling disease in 2001. Sites were selected to include each of four land cover categories (deciduous, coniferous, meadowland and deciduous/meadowland mixture). Land cover was defined by the most prevalent type of land cover at the sampling site. Sites in NY with a deciduous land cover included: Elton Creek and Trout Brook. Sites in NY with a coniferous land cover included: Lime Lake Outlet and Mongaup Creek. Sites in NY with a deciduous/meadowland mixture included North Branch of Wiscoy Creek and Wiscoy Creek. Cheney Brook (cow pasture with a fence that was constructed in 2002) and Upper Clear Creek have a meadowland cover. Big Fishing Creek was the only site within Pennsylvania that had a coniferous land cover. Stream transects were 100 m in length and included a 50 m buffer on each side. Land cover for each site's drainage basin was described using geographic information (GIS) technology and analyzed with ArcView v3.3/ArcMap v8.02 software.

Sample Collection—During July and August of 2003 and June of 2004, benthic samples were collected with a Petite Ponar sampler from areas within each of the eight

sites. Invertebrates were collected by targeting specific pools that contained oligochaetes. All invertebrate samples were washed on a 500 μm mesh screen and preserved in 70% ethanol for sorting. All oligochaetes were identified morphologically (Kathman and Brinkhurst 1998) and counted. A subsample for those identified as *T. tubifex* were subjected to quantitative PCR analyses to determine the prevalence of *M. cerebralis* infection. Fifty individuals from each site were preserved for genetic screening (see below).

Water Quality—Water quality measurements were taken with portable meters on each sampling trip (July and August of 2003 and June and August of 2004). Variables included water temperature, pH, dissolved oxygen, and velocity (Table 4.1). Riparian habitat was evaluated as described by Platts et al. (1987). Briefly, a spherical densitometer (Forestry Products, Model-A) was used to measure forest over story density. The spherical densitometer works by the investigator counting dots in a grid that are equivalent to quarter-square canopy openings. Once the grid was counted, the number obtained was multiplied by 1.04 to obtain percent of overhead area not occupied by canopy. The difference between this number and 100 was then used as to estimate over story density. Four readings were taken per location facing North, East, South and West. These numbers were recorded and averaged (Table 4.2). GPS readings were also recorded for each site (Table 4.2).

Substrate Nutrient and Metal Analyses— In June 2003, substrate samples from both New York and Pennsylvania were collected and sent to the USGS Water Resources Division Denver Federal Center for chemical analyses of nutrients and metals (Table 4.3). Samples were dried, refrigerated, and metals were analyzed with 48h ion

chromatography (IC) (Dionex Model DX-120) (Theodorakos et al. 2002). Sample sites were grouped by primary stream canopy type for analysis.

Genetic Screening—Benthic oligochaetes collected from the field were morphologically sorted. *Tubifex tubifex* were identified by the presence of chaete. Genetic screening of the *T. tubifex* was based on the mt16S lineage-specific PCR as described by Sturmbauer et al. (1999) and Beauchamp et al. (2002). Briefly, oligochaetes were separated based on morphological characteristics and frozen at -80 ° C until DNA was extracted. Whole genomic DNA was extracted from frozen worms by using the Qiagen DNeasy Tissue Kit (Catalog No 69506, pages 18-20). DNA was stored at 4 °C until PCR was completed. PCR amplification was performed as described by Beauchamp (2002) for PCR amplification. A PCR cocktail was prepared containing 1 μM of each of the four lineage-specific forward primers: I (5'-AAA TTG CAA TAT CGG CAA ATA AG -3'); III (5'- TTA TCA CCC CCA AAC TAA AAG ATA -3'); V (5'- AAG TGA AGA AGC TTA AAT AAA CG -3'); VI (5'-CAA ACA CGT CAT TAA AAA GAT CCT A -3') and the reverse primer Tub 16SR (5'- TAA RCC AAC ATY GAG GTG CCA -3'). General myxozoan primers (Mc) were used on all *T. tubifex* that were genetically screened. Both forward and reverse primers were added to the *T. tubifex* PCR cocktail. The two primers added for myxozoan parasites, consisted of MyxF (5'-ACC GTG GGA AAT CTA GAG CTA A – 3') and MyxR (5' – GTT CCA TGC TAT YAA CAT TCA A – 3'). All primers were purchased from Integrated DNA Technologies (Coralville, IA, www.idtdna.com).

A PCR product of a characteristic size (V, 320 bp; I, 196 bp; III, 147 bp; VI, 125 bp; Mc, 740 bp) was generated for each major mitochondrial lineage. The primers were

used with a PCR cycling profile consisting of 5 min denaturation step at 95 °C; 35 cycles of 1 min at 95 °C, 1 min at 56 °C, 1 min at 72 °C, and 10 min extension at 72 °C. After amplification, PCR products were resolved by electrophoresis at 90 V for four hours on a gel containing 1.5% synergel (Diversied Biotech, Boston, MA, Catalog No Syn-100), and 0.75% I.D.NA® agarose (FMC Bioproducts, Rockland ME, Catalog No 50172) (Figure 4.1).

For sequencing of *M. truttae* and *M. pseudodisbar*, PCR products were cleaned by adding 1.4 µL Exonuclease I (New England Biolabs, Ipswich, Massachusetts) and 4 µL Shrimp Alkaline Phosphatase (Promega, Madison, Wisconsin). Sequencing reactions were performed using Applied Biosystems (Foster City, California) Big Dye Cycle Sequencing Kit and the reactions were cleaned with ethanol and sodium acetate. Briefly, 2 µL of Big Dye, 1 µL of one primer (either MyxFor or MyxRev), and 2 µL of the template DNA were added to 5 µL of water. This was done for both the forward and reverse primer. The samples were then run on the following PCR cycling profile: 25 cycles of 30 sec at 96 ºC, 15 sec at 50 ºC, and 4 min final extension at 60 ºC. The cleaned samples were sequenced in both directions using an Applied Biosystems 3100 Genetic Analyzer.

Statistical Analysis—One-way analysis of variance (ANOVA; Statistica v11.00.01, Statsoft Inc. Software) was used to determine significant differences ($p \leq 0.05$) among sample sites (Sokal and Rohlf 1981). The ad hoc Tukey test was performed for a pairwise comparison of the means. Data were tested for normality and homogeneity of variance using Shapiro-Wilk's W Test and the Brown & Forsythe Test, respectfully.

Genetic screening data were set up with lineages V, I , III, VI, *Myxobolus cerebralis*, and other myxozoan parasites labeled as either 1 (positive) or 0 (negative).

Results

Water Quality Analysis—Water quality parameters were not significantly different between sites (Table 4.1). The mean water quality parameters (temperature, 13.46 ºC, SE ± 1.59; dissolved oxygen, 11.09 mg/L, SE ± 0.66; pH, 4.46, SE ± 0.39) reflected relatively homogeneous habitat conditions within the study area.

Sediment Analysis—All stream sediments were analyzed for nutrients and metals (Table 4.4). Barium (P=0.01) was the only sediment metal significantly lower in both deciduous and deciduous/meadowland sites than coniferous sites. Concentrations of Ce (P = 0.02), Cu (P = 0.01), La (P = 0.007), Nd (P = 0.006), and Y (P = 0.02) were significantly higher in deciduous sites than in coniferous sites. Concentrations of Ce (P = 0.04), Eu (P = 0.04), La (P = 0.04), Nd (P = 0.02), Y (P = 0.008), and Yb (P = 0.03) were significantly higher at deciduous/meadowland sites when compared to coniferous sites. Concentrations of Ce (P = 0.01), Cu (P = 0.006), Eu (P = 0.01), La (P = 0.007), Nd (P = 0.007), Y (P = 0.01), and Yb (P = 0.02) were significantly higher in meadowland sites than in coniferous sites.

Genetic Screening of Oligochaete Population— Percentage of *T. tubifex* in the total oligochaete population was determined (Table 4.4). *Limnodrilus hoffmeisteri* and *Ilyodrilus templetoni* were the dominant non-tubificid oligochaetes present at all sites. *Tubifex tubifex* was the dominant oligochaete found in the deciduous/meadowland sites Wiscoy Creek (94%, n = 229) and the North Branch of Wiscoy Creek (99%, n = 744).

They were the least abundant oligochaete at the deciduous sites Elton Creek (14%, n = 33) and Trout Brook (20%, n = 483). Intermediate levels of *T. tubifex* were found in the coniferous sites Lime Lake Outlet (42%, n = 209), Big Fishing Creek (47%, n = 272) and Mongaup Creek (57%, n = 504) and in the meadowland sites Upper Clear Creek (53%, n = 110) and Cheney Brook (52%, n = 66). These percentages are based on the morphological identification followed by PCR confirmation. Populations of non-tubificid oligochaetes at deciduous sites were significantly higher than those at deciduous/meadowland ($P < 0.001$), coniferous ($P < 0.001$), and meadowland ($P < 0.001$) sites. Populations of non-tubificid oligochaetes were significantly higher at meadowland sites than at deciduous/meadowland ($P < 0.001$), and coniferous ($P < 0.001$) sites.

Specific lineages of *T. tubifex* were determined via PCR (Table 4.5). Genetic screening of the New York and Pennsylvania oligochaete populations revealed that most *T. tubifex* belonged to lineage I and III. The highest levels of lineage I were found at Trout Brook (deciduous, 76%), Elton Creek (deciduous, 86%), Big Fishing Creek (coniferous, 59%), and Mongaup creek (coniferous, 63%). Wiscoy Creek (deciduous/meadowland, 32%), Cheney Brook (meadowland, 23%), Upper Clear Creek (meadowland, 25%) and Lime Lake Outlet (coniferous, 20%) had intermediate levels of lineage I. North Branch Wiscoy Creek (deciduous/meadowland, 10%) had the lowest percentages of lineage I. Populations of lineage I were significantly higher in the deciduous sites than from deciduous/meadowland sites ($P < 0.001$), coniferous sites ($P < 0.001$), and meadowland sites ($P < 0.001$).

The highest populations of lineage III *T. tubifex* were found at the North Branch of Wiscoy Creek (deciduous/meadowland, 90%), Lime Lake Outlet (coniferous, 80%),

and Upper Clear Creek (meadowland, 75%). Intermediate levels of lineage III were found at Cheney Brook (meadowland, 54%), Wiscoy Creek (deciduous/meadowland, 68%), Big Fishing Creek (coniferous, 41%), and Mongaup Creek (coniferous, 37%). Elton Creek (deciduous, 14%) and Trout Brook (deciduous, 13%) had the lowest levels of lineage III. Deciduous/meadowland sites had significantly larger populations of lineage III *T. tubifex* than populations found in meadowland sites ($P < 0.001$), and coniferous sites ($P < 0.001$). Lineage V *T. tubifex* were only found in Cheney Brook (Meadowland, 23%) and Trout Brook (deciduous, 11%).

Genetic Screening for Myxozoan Parasites—Myxozoan parasites were positively identified in worms from five sites (Lime Lake Outlet, Trout Brook, Cheney Brook, North Branch of Wiscoy Creek, and Wiscoy Creek) (Figure 4.2) and were not found in worms from Upper Clear Creek, Mongaup Creek or Big Fishing Creek. Myxozoan parasites were not found in *T. tubifex* at the control site, Elton Creek. *Myxobolus cerebralis* was the most common myxozoan parasite found at the positive sites. *Myxidium truttae* was present in worms from Cheney Brook (meadowland) and *Myxobolus pseudodisbar* was present in worms from the North Branch of Wiscoy Creek (deciduous/meadowland) (Figure 4.2). Of particular note, myxozoan parasites were only found in lineage III *T. tubifex*. PCR products were sequenced for positive identification.

At sites infected with *M. cerebralis*, the highest percentage infected *T. tubifex* were from Lime Lake Outlet (coniferous, 20%). The lowest percentage of *M. cerebralis* infected *T. tubifex* were from Trout Brook (deciduous, 2%) and Wiscoy Creek (deciduous/meadowland, 4%). Intermediate levels of *M. cerebralis* infection were from North Branch Wiscoy Creek (deciduous/meadowland, 15%) and Cheney Brook

(meadowland, 14%) (Figure 4.2). The amount of *T. tubifex* positive for *M. cerebralis* were significantly higher in coniferous sites than deciduous ($P < 0.001$), deciduous/meadowland ($P < 0.001$), and meadowland ($P < 0.001$) sites.

Discussion

Genetic screening of the New York and Pennsylvania oligochaete populations revealed that most *T. tubifex* were lineage I and III. This agrees with the findings of Kaeser et al. (2006) during their Pennsylvania survey, and DuBey and Caldwell (2004) for worms from New Mexico. Only Trout Brook (deciduous) and Cheney Brook (meadowland) had lineage V *T. tubifex*. Deciduous sites were highest in lineage I *T. tubifex* populations, which are moderately susceptible to whirling disease. In turn, deciduous sites were lowest in their populations of lineage III *T. tubifex* which are generally the most susceptible *T. tubifex* to *M. cerebralis*. Deciduous sites also had higher non-tubificid oligochaete populations than deciduous/meadowland, meadowland, and coniferous sites. These non-tubificid oligochaetes comprise the majority of the oligochaete populations found at deciduous sites. Given that the predominant lineages present in the East coast are the most susceptible to *M. cerebralis*, that whirling disease is not a more significant issue is surprising.

Some metal sediment concentrations quantified in this study were significantly different among sampling sites. Sediment serves as a repository for toxic chemicals that if bioavailable may pose a risk to aquatic life (Ingersoll et al. 2002; Chapman et al. 1999). This is a useful but difficult factor to research because for the relationship between sediment concentrations of contaminants and toxicity to freshwater invertebrates are not

clear and varies between species and sediment type (Ankley et al. 1994; Ingersoll et al. 2002; Chapman et al. 1999). Copper was highest in sediment from the deciduous (30.0 ppm) and meadowland sites (23.5 ppm), and lowest in sediment from the coniferous sites (17.0 ppm). *Tubifex tubifex* are extremely sensitive to copper (Phipps et al. 1995) at levels ranging from 0.1 - 10.0 ppm, increased copper levels significantly decreases *T. tubifex* egg production (Chapman et al. 1999; Pasteris et al. 2003). Copper levels higher than 50 ppm can cause death in *T. tubifex* (Chapman et al. 1999). Therefore, copper levels may be affecting total *T. tubifex* assemblages in deciduous and meadowland sites. Interestingly, non-*Tubifex* oligochaete populations were highest in the deciduous sites; hence, they may be less sensitive to copper and have a competitive advantage at these sites. What is not known is how different *T. tubifex* lineages are affected by copper toxicity. Large amounts of deciduous leaves covering the substrate is another factor that could affect *T. tubifex* infection at deciduous sites. Deciduous leaves retain protective substances such as tannins, polyphenols, and pigments that repel invertebrates from the surface (Sridhar and Bärlocher 1993; Naamane et al. 1999). If *T. tubifex* are deep in the substrate in deciduous sites they may not be available to ingest *M. cerebralis* spores for continuation of the life cycle.

Tubifex tubifex abundance has often been positively correlated with sedimentation and organic matter (Bergerson and Anderson 1997; Robbins et al. 1999; DuBey and Caldwell 2004). In this study *T. tubifex* were always a component of oligochaete populations. This was true whether the particulate consisted of organically rich fine sediment (eutrophic, lentic) habitat or coarse pebbles/sand habitat. However, *Tubifex tubifex* preferred fine silty sediment and were much more prevalent in these areas. This is

similar to the findings of studies in the Intermountain West (Sandell et al. 2001; Zendt and Bergerson 2000; Beauchamp et al. 2005). Kaeser and Sharpe (2006) also noted that in sites from Pennsylvania *T. tubifex* were found in typical areas of eutrophic, lentic habitats. However, they also found a restricted distribution of *T. tubifex*, and a greater dominance of *I. templetoni* in Pennsylvania streams. They reported that organic pollution sources appeared to be a requirement for the occurrence of detectable *T. tubifex* populations in Pennsylvania (Kaeser and Sharpe 2006). In the current study, organic pollution sources apparently were not necessary to support *T. tubifex* populations, but such sources did increase the abundance of *T. tubifex*. *Tubifex tubifex* were the primary oligochaetes found at all sites in New York and Pennsylvania except the two deciduous sites in New York. However, except for one site, there were few organically rich habitats available for *T. tubifex*. One site in New York, North Branch of Wiscoy Creek, had *T. tubifex* widely distributed throughout the 100 m transect. The sampling site at North Branch of Wiscoy Creek is also downstream of a trout hatchery. At the other sites, however, conditions such as high water velocity, shallow depth, and increased temperatures of these eastern streams, appeared to limit habitat availability for *T. tubifex* survival. Apparently, environmental factors such as sediment, water velocity, and land cover play an important role in *T. tubifex* distribution.

The establishment of *M. cerebralis* in geographic watersheds is largely determined by the spatial distribution and abundance of *T. tubifex* within a system in respect to myxospore deposition (Kaeser and Sharpe 2006). Moreover, the presence of all susceptible hosts is required to establish the life cycle of *M. cerebralis*. Kaeser and Sharpe (2006) suggest that *T. tubifex* may not be as common or widely distributed within

Pennsylvania streams compared to Intermountain West streams affected by whirling disease. In the current study, oligochaete assemblages were separated by wide spaces of unsuitable stream habitat. The idea that the isolation of suitable habitat for *T. tubifex* and consequent isolation of habitat available for *M. cerebralis* limits the growth, spread, and long-term persistence of the parasite has been studied (Kaeser and Sharpe 2006). Kaeser and Sharpe (2006) hypothesized that a spatially widespread *T. tubifex* population is an essential community-level requirement for whirling disease outbreak after introduction in a stream community. Our work supports this hypothesis.

Land cover appears to influence the presence of *M. cerebralis*. In the current study, the highest percentages of *M. cerebralis* infections were found in *T. tubifex* from a coniferous site and a deciduous/meadowland site. Lowest percentages of *M. cerebralis* infections were found in *T. tubifex* from a deciduous site. There was a big difference in infectivity between the two deciduous/meadowland sites North Branch of Wiscoy Creek and Wiscoy Creek. Wiscoy Creek is a small creek that intersects upstream of our sampling site into North Branch of Wiscoy Creek. Fewer trout were seen in this section of Wiscoy creek. With fewer trout, fewer parasite spores would be present for ingestion by *T. tubifex*. With coniferous sites the differences in lineage composition (Lime Lake Outlet had very high lineage III *T. tubifex* compared to the other two coniferous sites) may have influenced the high percentage of *M. cerebralis* infection. In addition to *M. cerebralis* infection, mixed myxozoan populations were only found at meadowland and deciduous/meadowland sites.

Studies investigating the proportions of *M. cerebralis* infected *T. tubifex* range between <5% to 7.3% where infected worms are found (Wolf et al. 1986; Zendt and

Bergersen 2000). Beauchamp et al. (2002) found that 0.4 – 1.5% of *T. tubifex* are infected with *M. cerebralis* at sites in California and Colorado. DuBey and Caldwell (2004) found *T. tubifex* infection between 0.51 – 3.01% in worms from New Mexico, Rognlie and Knapp (1998) found *T. tubifex* infected around 2.6%, and Zendt and Bergerson (2000) found *T. tubifex* infected between 1.2 – 6.8% in the Upper Colorado Basin. *T. tubifex* infections in NY and PA worms positive for *M. cerebralis* were between 3.5 - 20%. The highest *T. tubifex* infectivity was found at the coniferous site Lime Lake Outlet (20%). The North Branch of Wiscoy Creek (deciduous/meadowland) and Cheney Brook (meadowland site) both had *T. tubifex* susceptibility > 15%. *Tubifex tubifex* susceptibility at Wiscoy Creek (deciduous/meadowland) and Trout Brook (deciduous) were < 5%. Given these results, *T. tubifex* susceptibility was influenced by more than riparian habitat.

On the West/Intermountain West coast highly impacted sites are defined by the severity of infections in wild and sentinel trout and high numbers of TAMs of *Myxobolus cerebralis* found in the water (Nehring 1999; Thompson and Nehring 2000; Beauchamp et al. 2002). Furthermore Beauchamp et al. (2002) suggested that the abundance and genotype of *T. tubifex* are important in defining high- and low-impact areas. In the present study, the highly susceptible lineages I and lineage III *T. tubifex* were present, but distantly dispersed among the streams, and effects on salmonid population were not found. Further tests of environmental factors such as water velocity and contaminants should be completed.

Population declines of rainbow trout have long been observed to be related to *M. cerebralis*, this varies regionally, within a watershed, or within the same stream (Nehring

and Walker 1996; Modin 1998; Hiner and Moffitt 2001; de la Franco and Budy 2004). Environmental factors may explain these differences (Schisler et al. 2000; Blazer et al. 2003; de la Franco and Budy 2004). Parasites, their fish host, and alternative host(s) can be evaluated for changes in diversity, prevalence, richness, and abundance in response to changes in environmental factors (Landsberg et al. 1998). Factors such as declines in water quality, eutrophication, hypoxia, and thermal effluent often lead to increased rates of parasitism, as intermediate hosts tend to flourish in these environmental conditions (Lafferty and Kuris 1999).

In summary, the current study suggests that canopy/land cover influences the selection of lineages of *T. tubifex* that differ in their susceptibility to *M. cerebralis* infection and perhaps other myxozoan parasites as well. This study reiterates the importance of site variability and the influence of land cover (i.e., deciduous, coniferous, meadowland, and deciduous/meadowland mixed sites). Parasite load was highest at coniferous sites. Lineage I and non-tubificid oligochaetes were greatest in the deciduous sites. Lineage III *T. tubifex* were highest in the deciduous/meadowland sites and coniferous site. Interestingly deciduous sites also had the highest copper levels. Land canopy cover, and thus sediment, does in fact affect the type of benthic oligochaetes, *T. tubifex* density, *T. tubifex* lineages present, and the physiology of the worms present. Subsequent laboratory experiments were conducted to determine effects of land cover on *T. tubifex* infections and TAM production with *M. cerebralis*.

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Figures

Figure 4.1. Gels depicting average genetic lineages of *T. tubifex* from A) West Virginia, B) New York, C) New York with additional *Myxobolus cerebralis* primer. Abbreviations include: NC, negative control; S, standard; V, *T. tubifex* lineage V positive control (PC); I, *T. tubifex* lineage I PC; III, *T. tubifex* lineage III PC; VI, *T. tubifex* lineage VI PC; Mc+, Mc+ PC; 1-11, Samples from West Virginia or New York *T. tubifex*. C) Mc positive results can be seen in samples 7 and 8. The Mc primer was a general myxozoan primer that found *Myxobolus cerebralis* (sample 7) and *Myxobolus pseudodisbar* (sample 8).

Figure 4.2. *T. tubifex* susceptibility (% infections) with *Myxobolus cerebralis* and other myxozoan parasites. Sites include Elton Creek (Deciduous - EC), Trout Brook (Deciduous - TB), Wiscoy Creek (D/M - WC), North Branch of Wiscoy Creek (D/M - NBW), Cheney Brook (Meadowland - CB), Upper Clear Creek (Meadowland - UCC), Lime Lake Outlet (Coniferous - LL), Big Fishing Creek (Coniferous - BFC), and Mongaup Creek (Coniferous - MC). *Myxidium truttae* was also present in worms from Cheney Brook (Meadowland) and *Myxobolus pseudodisbar* was present in worms from the North Branch of Wiscoy Creek (deciduous/meadowland). In the figure, ND means none detected and D/M stands for deciduous/meadowland.

Table 4.1. Mean water quality values from July, August and September 2003, and June and August 2004 at sites sampled from New York and Pennsylvania. Values are given for temperature (°C), pH, D.O. (ppm) and stream water velocity.

	Water Averages			
Site Name	Temperature (°C)	pH	D.O. (ppm)	Velocity
Elton Creek	11.90	7.75	10.83	high
Trout Brook	13.80	7.25	11.10	mild
Wiscoy Creek	15.50	8.00	11.02	moderate
North Branch Wiscoy Creek	15.10	7.25	11.80	mild to moderate
Cheney Brook	11.80	7.25	11.70	mild to moderate
Upper Clear Creek	12.10	7.15	9.85	mild
Lime Lake Outlet	15.20	7.00	10.95	moderate
Mongaup Creek	12.30	8.00	11.50	mild
Big Fishing Creek	10.65	7.88	11.55	moderate to high

Table 4.2. Location, canopy type and cover and surrounding vegetation from sites sampled in New York and Pennsylvania.

Site Name	Latitude	Longitude	Major Canopy	Canopy Cover (%)	Surrounding Vegetation
Elton Creek	N42°27.981'	W78°26.589'	Deciduous	99.2	Willows
Trout Brook	N42°35.886'	W78°11.154'	Deciduous	99.1	Willows
Wiscoy Creek	N42°34.975'	W78°14.259'	Deciduous/Meadowland	10.0	Willows, Meadow
North Branch Wiscoy Cr.	N42°35.899'	W78°15.346'	Deciduous/Meadowland	79.9	Willows, Meadow
Cheney Brook	N42°29.937'	W78°22.476'	Meadowland	99.0	Willows, Elm
Upper Clear Creek	N42°29.141	W78°21.535	Meadowland	60.0	Willows, Alder
Lime Lake Outlet	N42°27.036'	W78°28.896'	Coniferous	98.0	Coniferous
Mongaup Creek	N41°56.931'	W74°71382’	Coniferous	100.0	Coniferous
Big Fishing Creek	N40°59.160'	W77°29.256'	Coniferous	100.0	Coniferous

Table 4.3. Metals and nutrients analyzed in sediments collected at all sites.

Aluminum	Manganese
Calcium	Molybdenum
Iron	Niobium
Potassium	Neodymium
Magnesium	Nickel
Sodium	Lead
Phosphorus	Antimony
Titanium	Scandium
Silver	Tin
Arsenic	Strontium
Gold	Tantalum
Barium	Thorium
Beryllium	Thallium
Bismuth	Uranium
Cadmium	Vanadium
Cerium	Yttrium
Cobalt	Ytterbium
Chromium	Zinc
Copper	Inorganic Carbon
Europium	Organic Carbon
Gallium	Total Carbon
Holmium	Mercury
Lanthanum	Selenium
Lithium	Sulphur

Table 4.4. Metal analyses of sediment samples. Data are presented in ppm for those metals significantly different between land cover types.

Site	Land Cover	ICPMS Ba ppm	ICPMS Ce ppm	ICPMS Cu ppm	ICPMS Eu ppm	ICPMS La ppm	ICPMS Nd ppm	ICPMS Y ppm	ICPMS Yb ppm
Elton Creek	Deciduous	370.0	49.0	33.0	1.3	23.0	26.0	22.0	2.5
Trout Brook	Deciduous	310.0	58.0	27.0	1.5	28.0	31.0	23.0	2.7
Wiscoy Creek	D/M*	320.0	46.0	16.0	1.1	22.0	24.0	19.0	2.3
N.B. Wiscoy Creek	D/M	340.0	42.0	21.0	1.2	20.0	23.0	19.0	2.1
Cheney Brook	Meadowland	380.0	58.0	24.0	1.7	27.0	31.0	27.0	2.9
Upper Clear Creek	Meadowland	300.0	53.0	23.0	1.4	25.0	29.0	24.0	2.7
Lime Lake Outlet	Coniferous	470.0	42.0	18.0	1.1	20.0	22.0	18.0	2.2
Mongaup Creek	Coniferous	ND	ND	ND	ND	ND	ND	ND	ND
Big Fishing Creek	Coniferous	360.0	35.0	16.0	1.0	16.0	20.0	16.0	2.1

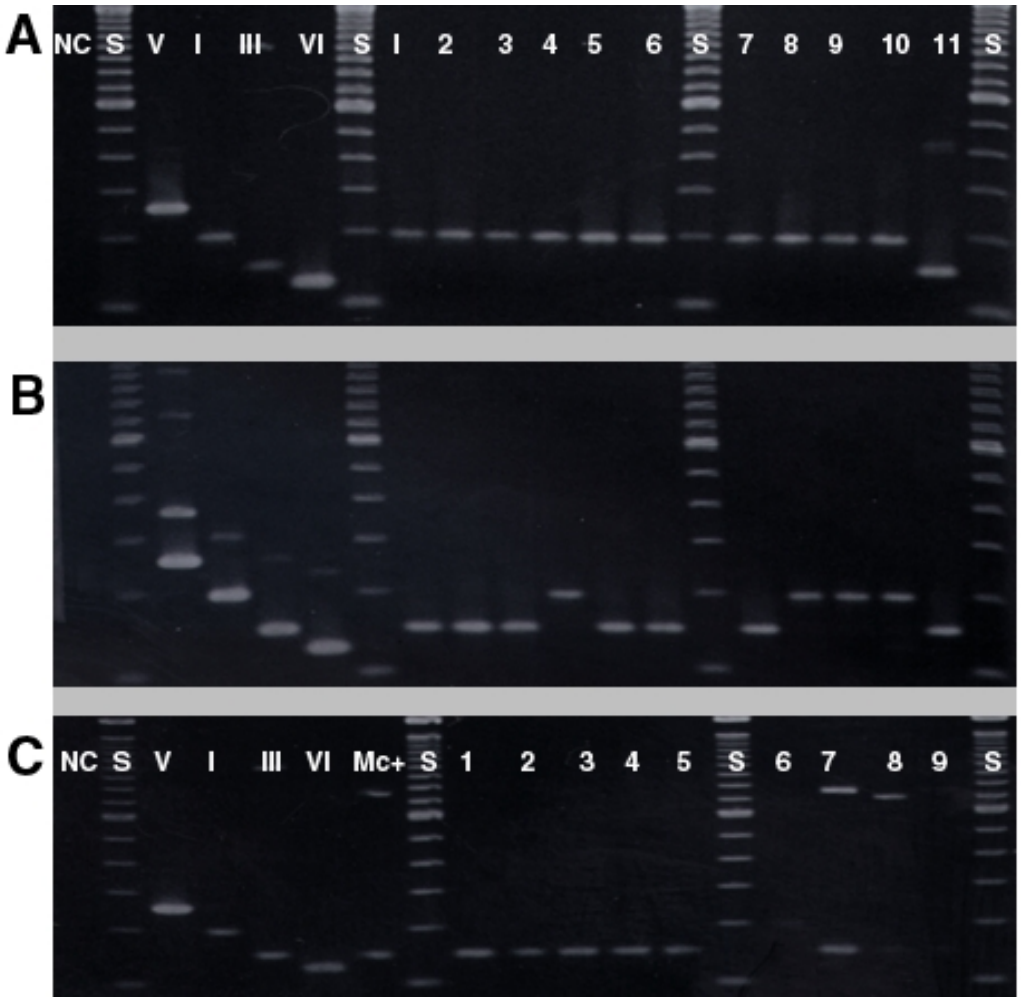
D/M* = Deciduous/Meadowland

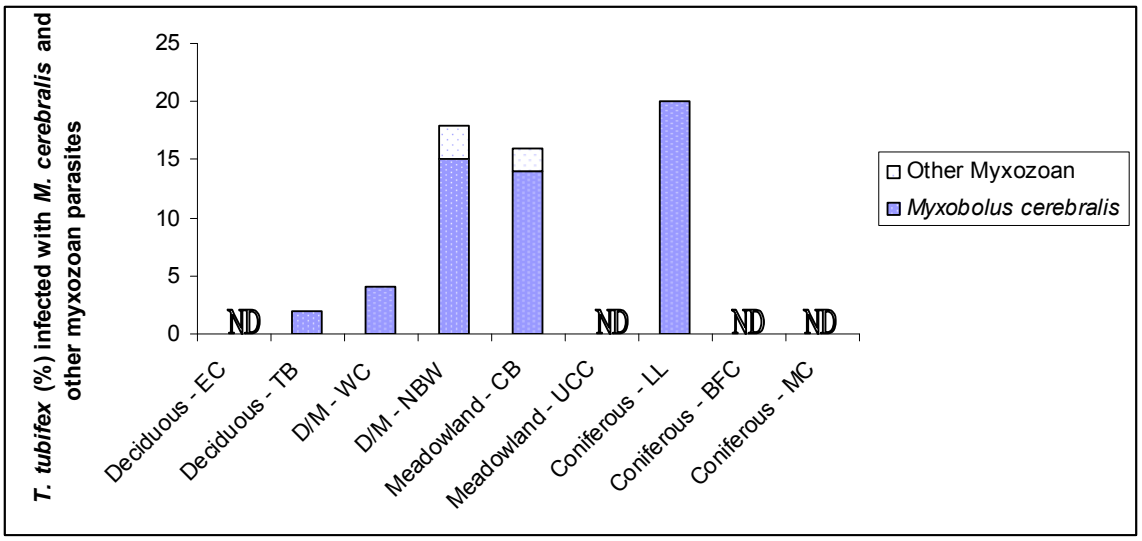
ND = Site was not part of field collection during field sediment analysis

Table 4.5. Summary of the total benthic oligochaete population assemblage collected from all sites.

Sites	Land Cover	% Oligochaete not *T. tubifex*	% Oligochaete *T. tubifex*	% *T. tubifex* Lineage I	% *T. tubifex* Lineage III	% *T. tubifex* Lineage V	% *T. tubifex* Infected with Myxozoan parasites	Other Myxos
Elton Creek	Deciduous	86	14	86	14	0	0	N
Trout Brook	Deciduous	80	20	76	13	11	2	N
Wiscoy Creek	D/M*	6	94	32	68	0	4	N
N.B. Wiscoy Creek	D/M	1	99	10	90	0	16	Y
Cheney Brook	Meadowland	48	52	23	54	23	14	Y
Upper Clear Creek	Meadowland	47	53	25	75	0	0	N
Lime Lake Outlet	Coniferous	58	42	20	80	0	20	N
Mongaup Creek	Coniferous	43	57	63	37	0	0	N
Big Fishing Creek	Coniferous	53	47	59	41	0	0	N

D/M* = Deciduous/Meadowland


A
NC S V I III VI S I 2 3 4 5 6 S 7 8 9 10 11 S
B
C
NC S V I III VI Mc+ S 1 2 3 4 5 S 6 7 8 9 S


T. tubifex (%) infected with M. cerebralis and other myxozoan parasites
25
20
15
10
5
0
ND
ND
ND
ND
Deciduous - EC
Deciduous - TB
D/M - WC
D/M - NBW
Meadowland - CB
Meadowland - UCC
Coniferous - LL
Coniferous - BFC
Coniferous - MC
Other Myxozoan
Myxobolus cerebralis

CHAPTER 5

EFFECTS OF RIPARIAN ZONES AND ASSOCIATED STREAM SUBSTRATA ON *TUBIFEX TUBIFEX* DENSITY AND INFECTIONS WITH *MYXOBOLUS CEREBRALIS* IN THE LABORATORY

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[1]Iwanowicz DD, Blazer VS, Schill WB, Griffin AR. To be submitted to the Journal of Aquatic Animal Health

Abstract

Few studies have investigated oligochaete assemblages or *T. tubifex* populations on the East coast. Our previous research examined *T. tubifex* and substrata from New York and Pennsylvania trout streams with coniferous, deciduous, meadowland and mixed deciduous/meadowland riparian zones. We found that the majority of *T. tubifex* in the Northeast are from lineages I and III, the most susceptible lineages to *M. cerebralis* infection, yet whirling disease is not an issue for wild salmonids in this region. The primary objective of this study was to determine if substrate/leaf litter and hence, riparian zone affected *T. tubifex* infectivity and triactinomyxon (TAM) production from *M. cerebralis*. Two laboratory studies were completed to meet the above objective. Stream substrata from five sample sites in New York or Pennsylvania were selected based on factors such as stream canopy cover, whirling disease prevalence, substrate differences, *T. tubifex* prevalence, and accessibility. Two laboratory studies were completed to meet the objective. In Study I, we used reference worms from West Virginia in site-specific substrates. All stream substrates used in laboratory experiments were analyzed for nutrients and metals. As with the field substrates, significant differences in metal copper concentrations were found between substrate from the deciduous and coniferous sites. There were no significant correlations when comparing the substrate chemical analyses to *T. tubifex* infection rate, duration of release of TAMs, average number of TAMS released per worm per day, or the average number of TAMS released per site ($P > 0.05$). In Study II we used site specific worms in their individual substrate. In both laboratory studies the percent *T. tubifex* infected, duration of TAM release, and the mean number of TAMs released per infected worm, were all consistently higher in worms maintained in substrate

from coniferous sites and deciduous/meadowland sites ($P < 0.01$). Both laboratory experiments supported field observations, but also suggested factors other than substrate may affect infectivity and viable TAM production at some sites.

KEYWORDS – *Tubifex tubifex, Myxobolus cerebralis*, riparian habitat, Myxozoan

Introduction

Whirling disease is caused by the myxozoan parasite *Myxobolus cerebralis*. As with other myxozoan parasites, this parasite has a complex two-host life cycle involving separate stages of sporogony in salmonid fishes and the aquatic oligochaete *Tubifex tubifex* (Markiw and Wolf 1983; Wolf et al. 1986). The oligochaete worm *T. tubifex* produces the triactinomyxon (TAM) spore that infects salmonid hosts. For decades, whirling disease in wild salmonid populations in some geographic areas on the West Coast has caused population declines (Walker and Nehring 1995; Vincent 1996). Although the parasite is endemic in other areas of the US, population declines have not been observed (Hnath 1996; Modin 1998; Kaeser et al. 2006).

Environmental stress is the result of many factors including climate change (temperature, rainfall), pollutants (urban and agricultural), habitat alterations, commercial fisheries and introduced species (Lafferty and Kuris 1999). These stressors influence the interactions between a parasite and its host(s) (Lafferty and Kuris 1999). Numerous investigators have reported the effects of environmental stressors on aquatic parasites (Khan and Thulin 1991; Overstreet 1993; Mackenzie et al. 1995; Williams and MacKenzie 2003; Marcogliese 2004; Sures 2004). Although many factors can affect host-parasite interactions, documented that drought, anoxia, heavy metals, acidification, and pesticides are well documented as activators of a stress response in some organisms (Landsberg et al. 1998; Lafferty and Kuris 1999; Schisler et al. 2000; Marcogliese 2005). Organisms acclimate to environmental stressors depending on their species. Species acclimation with environmental stressors reflects differences in their life cycles, physiology, biochemistry, and molecular biochemistry of all organisms in a specific

habitat. For instance, decreases in individual parasite species occurs when the parasite is negatively affected by direct exposure to the stress, or if the stressor has a negative impact on the parasites host(s) with or without infection of the parasite (Marcogliese 2005). Increases in individual parasite species have been observed when the host(s) resistance is compromised under stressful conditions, or if environmental stressors result in a proliferation of the intermediate host (Marcogliese 2005). On the other hand, as with any species of parasite, environmental stressors may alter the host(s) behavior, leading to unpredictable changes in encounter rates with free-living infective stages of the parasite (Poulin 1992). Consequently, optimal conditions for a particular parasite may be tolerable for one host, yet unsuitable for another. These changes alter the life cycle of a parasite and infection within its host population. Little research has addressed the role of environmental factors on the oligochaete host of myxozoan parasites.

Understanding populations of *T. tubifex* in their native aquatic environment is important. Few studies have investigated oligochaete assemblages or *T. tubifex* populations on the East coast. Kaeser et al. (2006) determined that *T. tubifex* were only found near point sources of organic pollution in Pennsylvania. Kaeser and Sharpe (2006) further postulated that the spatial distribution of *T. tubifex* in water systems in Pennsylvania made propagation for *M. cerebralis* difficult, they also determined that *T. tubifex* from Pennsylvania streams are predominately lineages I and III. Previous work from Iwanowicz et al. (In prep) found this to also be true for *T. tubifex* assemblages analyzed in New York and Pennsylvania. Interestingly, these are the most susceptible lineages to *M. cerebralis* infection, yet whirling disease is not an issue for wild salmonids

along the East Coast. The influences of substrate, water quality and other environmental factors on *M. cerebralis* infections are not clear.

The primary objective of this study was to determine if substrate/leaf litter and hence, riparian zone affected *T. tubifex* infectivity and TAM production from *M. cerebralis*. Two laboratory studies were completed to meet the above objective. Our experimental approach was different than previous studies; the first laboratory study was completed using reference worms in site-specific substrate from New York and Pennsylvania. The second laboratory study used site-specific worms in their individual substrate from New York and Pennsylvania.

Materials and Methods

Site Selection— Sites were chosen based on information from the New York Department of Environmental Contamination and Pennsylvania Fish and Boat Commission regarding the presence of salmonids, and based on watersheds where whirling disease was enzootic (Table 5.1). Sites chosen were also based on similarity of physical characteristics and land cover. Actual sites were selected to include each of three land cover categories (deciduous, coniferous, and deciduous/meadowland mixture). Deciduous sites from New York included Elton Creek and Trout Brook. Elton Creek was the negative control, last tested in 2001 and found to be negative for whirling disease. Coniferous sites from New York included Lime Lake Outlet and Mongaup Creek. Deciduous/meadowland sites from New York included the North Branch of Wiscoy Creek. Big Fishing Creek was the only site within Pennsylvania and had a coniferous land cover. In Study I sites chosen included Trout Brook, Elton Creek, Lime Lake

Outlet, North Branch of Wiscoy Creek, and Big Fishing Creek. Sites chosen for study II were originally the same as study I but worms at Elton Creek were not sufficiently abundant to provide an adequate sample size. Therefore, Elton Creek was exchanged for Mongaup Creek. Stream transect lengths were 100 m in length and included a 50 m buffer on each side. Land cover was determined using geographic information (GIS) technology and analyzed with Arc View v3.3/ArcMap v8.02 software.

Riparian Leaf-Litter Experiments:

The first experiment used substrate from only New York and reference worms from West Virginia maintained at the National Fish Health Research Laboratory (NFHRL), Kearneysville, WV. The second study used substrate and worms from New York and Pennsylvania sites. In both studies, reference populations of WV worms in sand substrate were used as a control.

Study I— Exposure of Reference Worms Maintained in Substrata from Individual Sites

During April of 2004, *T. tubifex* collected at a State Hatchery (Berkeley Springs, WV) were negative for *M. cerebralis*. This is the same population of worms used in the original work of Markiw and Wolf (1983) and Blazer et al. (2003). These reference worm populations were maintained in 37.8 L flow-through troughs at approximately 13 °C at the NFHRL. Worms were fed a mixture of spirulina disks and trout chow every two weeks throughout the study. During July (2004), substrate samples were collected from New York with a petite ponar and placed into individual 5 gallon plastic transports. Substrate was covered with stream water and aerated for transport. Once at the NFHRL,

substrate was placed over a bright table light and slowly sorted for all benthic invertebrates. All benthic invertebrates were removed and saved in 90% ethanol for later identification. Once cleaned, substrate was ready for laboratory experiments.

Stream substrate or sand (1 cup or ~350.0 grams dry weight) was added to 1 L glass jars. Following methods of Blazer et al. (2003), *T. tubifex* worms (200/jar in quadruplicate) were exposed to *M. cerebralis* spores (350 spores per worm). As a measure of quality control, 10% of the worms used for the study were identified via PCR, and 100% were confirmed as *T. tubifex*. Water was aerated and temperature was maintained at 13 ± 1 °C. Duplicate jars from each treatment were used to monitor progression of the infection via PCR and histopathology. Based on the methods of El-Matbouli and Hoffman (1998), 20 worms per replicate jar were removed at 5, 10, 25, and 60 days post-exposure for these analyses. Worms were cut in half so histopathological and PCR data could be collected for each individual. The anterior portion was fixed in 10% neutral buffered formalin and mounted for histopathological identification; the posterior section was used for PCR.

Starting at 60 days post-exposure, 500 mL of water from each of the other two treatment jars was filtered daily and monitored for the presence of waterborne TAMs. Upon discovery of a single TAM from an experimental jar, 72 *T. tubifex* were placed individually into the wells of three 24-well plates (one worm/well) filled with 1 mL of filtered spring water. These plates were floated on a water bath maintained at 13 ± 1 °C. A mixture of 10 µL spirulina water mixture (1/8th teaspoon spirulina: 10 mL water) was placed in the wells bimonthly as a food source for the worms. Spores released from individual infected *T. tubifex* on two of the plates were quantified to determine infection

with *M. cerebralis*, total number of TAMs released per worm, and duration of release. Worms on the third plate were monitored and separated into positive and negative for TAM production. These worms were compared genetically as described by Beauchamp et al. (2002).

TAM Enumeration—TAMs were enumerated by concentrating TAMs from 200 mL of water on a 25 μm mesh sieve. The sieve was then rinsed, and TAMs were suspended in 5 mL of spring water. Ten microlitres of the TAM concentrate were then transferred into each of five wells (10 mm) on a slide and mixed with pinacynol chloride dye (bis-(N-ethyl-2-quinolyl)-trimethinium chloride; 5 μL). Slides were dried in a 65 ˚C oven (Thermolyne Type 19200 oven/incubator) and read under a light microscope. Slides prepared for TAM enumeration were read daily to quantify TAM release.

Infection with M. cerebralis—An individual worm was considered to be infected if it released TAMs at any time during the experimental period. Infectivity was based on the 72 worms per replicate (picked randomly from the 200 originally exposed) in each substratum group. Data were presented as percent infected.

Duration of Release—Duration of release was defined as the total numbers of days TAMs were released from infected worms from each site. Data were presented as an average.

Mean TAM released per infected worm per day at each site—Mean TAM released per worm per day at each site was defined as the total number of TAMs released by an individual infected worm per day. These data were measured by the quantity of TAMs found per mL of water.

Histopathology—Worm tissues were processed, embedded in paraffin, sectioned at 6 µm and stained using Hematoxylin and Eosin and Giemsa procedures (Luna, 1992). TAM development within *T. tubifex* from the six experimental groups were compared with Image Pro Plus v3.0 (Symbol Universal Software).

Genetic Screening—T. tubifex were morphologically identified by the presence of chaete. Genetic screening of the *T. tubifex* was based on the mt16S lineage-specific PCR as described by Iwanowicz et al. (In Prep).

Substrate Nutrient and Metal Analyses— At the completion of the study, substrate samples from all sites were collected and sent to the USGS Water Resources Division Denver Federal Center for chemical analyses of nutrients and metals (Table 5.2). Samples were prepared and analyzed as described by Iwanowicz et al. (In Prep).

Study 2—Susceptibility of Worms and Substrate from New York and Pennsylvania

During July of 2005, oligochaete worms and substrate were collected from New York and Pennsylvania and transported to the NFHRL. Worms were maintained in the plastic 5 gallon transport until sorted. Worms were maintained in these containers for a maximum of 2 months. All worms were sorted based on morphology. Worms were fed trout chow every two weeks until they were plated and all transports and jars were aerated constantly.

Stream sediment from each site was used as substrate for the treatments, and sand was used as a control substrate. Methods followed those described in study I. However, in this study, 20 worms from each of two jars were removed at 10, 25, 60 and 90 days post-exposure for histopathology and PCR analyses. Also at approximately 120 days post-exposure, spirulina feeding to the worms maintained in plates was stopped and feeding them 3 grains of 2 mm trout chow per well, every two weeks was begun. The filtered water was changed daily, even during feeding, and the trout chow was removed after four days.

Statistical analysis—Statistical analysis was performed using STATISTICA v6.0 (Statsoft, Inc., Tulsa, OK). All data were compared using the analysis of variance (ANOVA). The post hoc Tukey test was performed for a pairwise comparison of the means (Sokal and Rohlf 1981). Data were tested for normality and homogeneity of variance using Shapiro-Wilk's W Test and the Brown & Forsythe Test, respectfully. Data for number of TAMs per worm, total number of TAMs and duration of release were log transformed prior to analysis to meet the assumptions of parametric statistics and considered significant if $P \leq 0.05$.

Results

Study I— Exposure of Reference Worms Maintained in Substrata from Individual Sites

Sediment Analyses—Stream sediments were analyzed for nutrients and metals (Table 5.3). Sediment concentrations of Ce (ppm, $P = 0.02$), Cu (ppm, $P = 0.01$), La (ppm, $P < 0.01$), and Nd (ppm, $P < 0.01$) were significantly higher in the deciduous sites than in the coniferous sites. Sediment concentrations of Ba (ppm, $P = 0.01$) were

significantly lower in the deciduous sites than in the coniferous sites. Differences in sediment did not statistically affect *T. tubifex* infection with *M. cerebralis*, duration of release of TAMs, average number of TAMS released per worm per day, or the average number of TAMS released per site ($P > 0.05$).

Total TAM Production—The number of TAMs released by *T. tubifex* maintained in deciduous substrate (Elton Creek) was statistically higher ($P < 0.01$) than those maintained in all other substrates (Figure 5.1). In general 50-100% more TAMs were released. Conversely, the number of TAMs released by *T. tubifex* maintained in deciduous substrate (Trout Brook) was statistically lower ($P < 0.01$) than those maintained in all other substrates. The number of TAMs produced by *T. tubifex* maintained in coniferous substrate, Lime Lake Outlet, was statistically higher when compared to TAMs collected from worms from other substrate, excluding Elton Creek ($P < 0.01$). TAMs collected from *T. tubifex* maintained in deciduous/meadowland substrate (North Branch Wiscoy Creek) peaked 96-102 days post-exposure, whereas other sites peaked approximate 96-110 days post-exposure.

Infection with M. cerebralis—Infectivity of *T. tubifex* was calculated for all experimental sites (Table 5.4). Only lineage III *T. tubifex* were positive for *M. cerebralis* infection. The highest infectivity was found in *T. tubifex* maintained in deciduous/meadowland substrate (North Branch of Wiscoy Creek, 52%), which was significantly higher than every other site ($P < 0.01$). Statistically more ($P < 0.01$) *T. tubifex* maintained in substrate from the deciduous site Elton Creek (33%) were infected with *M. cerebralis* than those maintained in deciduous substrate (Trout Brook, 9%). Additionally, statistically more *T. tubifex* maintained in coniferous substrate Big Fishing

Creek (23%, P = 0.04) and Lime Lake Outlet (26%, P = 0.03) were infected than those maintained in sand (control, 17%).

Mean TAMs released per infected worm per day—Significant differences were found between the mean numbers of TAMs produced per worm per day (Table 5.4). Worms maintained in the deciduous substrate Elton Creek produced significantly more TAMS (6.4×10^3) than worms from any other site ($P < 0.01$).

Duration of TAM Release—Duration of TAM release was affected by substrate (Table 5.4). *Tubifex tubifex* maintained in sand (control) released TAMs for more days than *T. tubifex* maintained in substrate from the deciduous site (Trout Brook, $P = 0.04$) or the deciduous/meadowland site (North Branch of Wiscoy Creek, $P = 0.03$). *Tubifex tubifex* maintained in substrate from the deciduous site (Elton Creek) also had a longer duration of TAM release than those maintained in substrate from the deciduous site (Trout Brook, $P < 0.01$), the coniferous sites (Big Fishing Creek, $P < 0.01$ and Lime Lake Outlet, $P = 0.03$), and the deciduous/meadowland site (North Branch of Wiscoy Creek, $P < 0.01$).

Histopathology—Worms maintained in different substrates were examined for development of *M. cerebralis* (Figure 5.2). In *T. tubifex* maintained in all substrates at 60 days post-exposure, pansporocyst with sporoblasts and dividing zygotes were visible. Approximately 90 days post-exposure, the apical part of a maturing TAM spore inside a pansporocyst was observed and 110 days post exposure mature TAMs and polar capsules were visible. Lesions were not observed in the infected worms.

Genetic Screening of T. tubifex— All worms frozen for QA/QC were identified as *T. tubifex* (100%) and were comprised of lineage I (83%) and lineage III (17%). *Tubifex*

tubifex maintained in substrate from all sites represented both lineage I and lineage III worms (Table 5.4). Of particular interest, by 94 days post-exposure, there was a increase in the dominant lineage I *T. tubifex* in populations maintained in deciduous (Trout Brook) or sand (control) substrate ($P < 0.01$) compared to worms maintained in coniferous or deciduous/meadowland substrate. The number of lineage III worms was lower when maintained in deciduous (Trout Brook) and sand (control) substrate than *T. tubifex* maintained in coniferous or deciduous/meadowland substrate ($P < 0.02$).

Study 2—Susceptibility of Worms and Substrate from New York and Pennsylvania

Total TAM Production—The number of TAMs released by *T. tubifex* from the deciduous/meadowland site (North Branch Wiscoy Creek) was statistically higher ($P < 0.001$) than those from all other sites (Figure 5.3). The numbers of TAMs produced by *T. tubifex* from the coniferous substrate, Lime Lake Outlet, were also much higher when compared to TAMs collected from worms from other sites ($P< 0.01$). Production of TAMs peaked for all sites between 107-114 days post-exposure, except for worms from the deciduous site (North Branch Wiscoy Creek) which peaked at 119 days post-exposure.

Infection with M. cerebralis— Infectivity of *T. tubifex* was calculated for all experimental sites (Table 5.5). More *T. tubifex* from the deciduous/meadowland site (North Branch of Wiscoy Creek, 81%) were infected than from any other site ($P < 0.01$). Worms from the deciduous site (Trout Brook, 13%) had significantly lower infectivity than worms from deciduous/meadowland sites or coniferous sites ($P < 0.01$). Worms

from coniferous sites had significantly higher infectivity than worms from the control sand or deciduous sites ($P < 0.01$).

Mean TAM released per infected worm per day at each site —Significant differences were found between the numbers of TAMs produced per worm per day (Table 5.4). *T. tubifex* from coniferous sites (9.5×10^3) had a significantly higher release of TAMS per infected worm at each site ($P < 0.01$).

Duration of TAM Release—The duration of TAM release was clearly affected by substrate (Table 5.5). TAMs were released for the most days from *T. tubifex* maintained in coniferous sediment than ($P < 0.01$). *Tubifex tubifex* maintained in sand (control) released more TAMs than those maintained in deciduous (Trout Brook, $P < 0.01$) or deciduous/meadowland (North Branch of Wiscoy Creek, $P = 0.03$) site sediment, but had a shorter duration of TAM release than worms from the coniferous sites (Lime Lake Outlet, Mongaup Creek, and Big Fishing Creek, $P < 0.01$).

Histopathology—As in study I, worms maintained in different substrates were examined for the development of *M. cerebralis* (Figure 5.2). Development of the TAM within *T. tubifex* occurred in the same time frame as study I.

Genetic Screening of T. tubifex—The oligochaetes for study II from West Virginia, New York and Pennsylvania morphologically identified as *T. tubifex* were confirmed via PCR. Similar to study I, *T. tubifex* from all sites were a mixture of lineage I and lineage III worms (Table 5.5). Worms from one coniferous site (Lime Lake Outlet, 18%) and the deciduous/meadowland site (North Branch of Wiscoy Creek, 17%) had significantly fewer lineage I worms than worms from the other coniferous sites Big

Fishing Creek (80%; P = 0.01), Mongaup Creek (75%; P = 0.02), or the sand control (81%; P < 0.01).

Discussion

In this study we examined whether substrate type affected the susceptibility of *T. tubifex* to *M. cerebralis* infection in a controlled laboratory setting. The first experiment of this two part laboratory study utilized reference worms as the test subjects for the site exposure study. The second experiment employed site specific worms in their individual substrate from New York and Pennsylvania. *Tubifex tubifex* assemblages, infection, duration, and quantity of TAMs released per worm were affected by land cover.

In the laboratory study, copper levels were highest in substrate from the deciduous sites and lowest in the coniferous sites. Although significant differences were not found on the total TAMs produced, infection of *T. tubifex* with *M. cerebralis,* mean number of TAMs released per infected worm and duration of TAM release with copper level, there may be some mixed effects on *T. tubifex*. The EC_{50} for *T. tubifex* is approximately 8.4-8.9 ppm for copper (Chapman et al. 1999). Copper levels in both field (Iwnowicz et al. in prep) and laboratory studies ranged from 5.20 - 33.00 ppm which is well above the EC_{50} for *T. tubifex* with copper. However, experimentally examining the combined effects of chemical mixtures on *T. tubifex* proliferation or their infection with *M. cerebralis* is difficult. This was similar to the field substrate analyses (Iwanowicz et al. In Prep). Contaminated sediments serve as a repository for toxic chemicals that may, if bioavailable, pose a risk to aquatic life (Ingersoll et al. 2002; Chapman et al. 1999). However, it is difficult to determine the relationship between substrate concentrations of

contaminants in this study and toxicity to freshwater invertebrates (Ankley et al. 1994; Ingersoll et al. 2002; Chapman et al. 1999).

Our findings suggest that stream substrata does influence oligochaete population dynamics and lineage selection of *T. tubifex*. Specifically, in study I, the proportion of lineage I:lineage III *T. tubifex* shifted from 92:8 at the completion of the study for worms maintained in the control sand and the deciduous site substrate (Trout Brook) compared to 75:25 lineage I:lineage III from all other sites. This shift suggests lineage III *T. tubifex* populations favor those sites with a deciduous/meadowland or coniferous land cover. Results of study II exhibited a similar trend in lineage specificity with the highest percentage of lineage III *T. tubifex* from the coniferous (Lime Lake Outlet) and deciduous/meadowland sites (North Branch of Wiscoy Creek). These data support our results from a similar study conducted in the field (Iwanowicz et al. In Prep). At the field sites, the highest proportion of lineage I *T. tubifex* were found at deciduous sites, while the highest proportion of lineage III *T. tubifex* were found at sites with a deciduous/meadowland or coniferous land cover. High copper levels in deciduous and meadowland sediment in the field data affected *T. tubifex* assemblages (Iwanowicz et al. In Prep). Data from the laboratory studies support this hypothesis. In both field and laboratory trials, Lime Lake Outlet's lineage structure was different from the other coniferous sites and was more similar to the deciduous/meadowland sites. This may be due to the lake and surrounding anthropogenic influences upstream of our site.

Only lineage III *T. tubifex* were found to be positive for infection with *M. cerebralis,* and infectivity was highest at the coniferous and deciduous/meadowland sites. Interestingly, this result coincides with the shifts in proportion of lineage I: lineage III *T.*

tubifex observed in study I. Furthermore, these shifts in lineage assemblages would impact the total production of TAMs within a stream. Interestingly, in the field experiment, *M. cerebralis* infection was only prevalent in lineage III *T. tubifex* and highest at the coniferous and deciduous/meadowland sites (Iwanowicz et al. In Prep). In addition, lineage III *T. tubifex* in the field were highest in the deciduous/meadowland sites and coniferous site (Lime Lake Outlet). For substrates such as the deciduous/meadowland site (North Branch Wiscoy Creek) and coniferous site (Lime Lake Outlet), shifts in lineage composition could greatly impact the quantity of TAMs produced. This is important, because the total production of TAMs is an environmentally relevant measure, as exposure of young-of-the-year salmonids to high numbers of TAMs is considered one of the single most important factors that determine the severity of whirling disease among wild trout populations (MacConnell and Vincent 2002; Beauchamp et al. 2006).

Production of infectious *M. cerebralis* TAMs by *T. tubifex* worms from NY, PA, and WV were significantly affected by land cover. Results from both laboratory studies showed similar trends in infectivity and TAM production within the worm host. In both studies, total TAMs produced, infections with *M. cerebralis*, mean number of TAMs released per infected worm per day at each site, and duration of release was highest in worms maintained in coniferous and deciduous/meadowland substrate, and lowest in worms maintained in deciduous substrate. In study II, the significant affect of land cover was not surprising as *T. tubifex* populations from the deciduous/meadowland site (North Branch Wiscoy Creek) and the coniferous site (Lime Lake Outlet) were dominated by lineage III *T. tubifex* (>80%). Surprisingly, this was also seen in *T. tubifex* from the other

coniferous sites (Big Fishing Creek, 20%; Mongaup Creek, 25%), which had fewer lineage III *T. tubifex*. These data support the theory from a previous field study (Iwanowicz et al. in prep) that there may be a competitive advantage for lineage III *T. tubifex* in the coniferous (Lime Lake Outlet) and deciduous/meadowland site (North Branch of Wiscoy Creek), and is supported by the field data (Iwanowicz et al. In Prep). In the field research *T. tubifex* from the coniferous site (Lime Lake Outlet) and the deciduous/meadowland site (North Branch of Wiscoy Creek) had the highest infectivity with *Myxobolus cerebralis*, and *T. tubifex* from deciduous sites had the lowest *Myxobolus cerebralis* infectivity (Iwanowicz et al. In Prep). Interestingly, in the second laboratory study, *T. tubifex* from coniferous substrate had a longer duration of release than *T. tubifex* from deciduous/meadowland substrate. Increased duration of TAM release would possibly influence the infection of salmonid hosts.

TAM production for both studies began approximately 94-96 days post-exposure and was greatest during the following 10-30 days after the initial day of TAM release. This was similar to the data from Blazer et al. (2003) where initial production of TAMs was observed between 97-99 days post-exposure for *T. tubifex* maintained in mud or sand at 13 ºC. The calculated yield of TAMs for individual worms in Blazer et al. (2003) was between 300-19,500 TAMs per worm, which is also similar for worms maintained in site sediment from both of our studies. The yield of TAMs for individual worms is higher than in other experiments (Markiw 1986; Beauchamp et al. 2006). However, TAMs per worm in those studies was deduced by assuming that all worms produce an equal number of TAMs (Markiw 1986) or was deduced from individual worms monitored twice a week, rather than on daily counts for individual worms known to be producing TAMs

(Beauchamp et al. 2006). Counting individual TAMs daily gave precise TAM release per worm that can not be obtained with general or bi-weekly TAM counts. Therefore, these quantitative differences can be explained by differences in experimental design.

Susceptibility of *T. tubifex* to *M. cerebralis* from different land covers was influenced by lineage composition. However, laboratory data supports field results that factors other than land cover plays an integral role in *T. tubifex* infectivity. *T. tubifex* on the East coast were primarily the highly susceptible lineages (lineage I and lineage III), but the salmonid population effects were not found (Iwanowicz et al. In Prep). In the West coast, highly impacted sites are defined by the severity of infections in wild and sentinel trout and high numbers of TAMs of *Myxobolus cerebralis* found in the water (Nehring 1999; Thompson and Nehring 2000; Beauchamp et al. 2002). Beauchamp et al. (2002) hypothesized that the abundance and genotype of *T. tubifex* are important in defining these high- and low-impact areas. Stevens et al. (2001) suggested that the host-parasite relationship and the ability to produce TAMs may vary among *T. tubifex* populations, and this variability may explain the geographic variation in the effects of the disease. The results of the present study do not support these hypotheses. Genetic differences do influence the infectivity of worms, but land cover and substrate are also important in regulating TAM production (Blazer et al. 2003). Further tests of environmental factors such as temperature, water velocity, and water quality (including contamination with Cu and other toxic substances) must be completed to understand their impact on TAM production.

The role of environmental factors on *T. tubifex* and their ability to produce TAMs is further affirmed by the differences in TAM production from worms maintained in the

two deciduous sites in study I. When comparing effects of substrata from individual sites on reference worms, we found fewer *T. tubifex* infected and significantly fewer TAMs produced at the one deciduous site (Trout Brook) when compared to the other deciduous site (Elton Creek). Reference worms maintained in substrate from the deciduous site, Elton Creek, had an intermediate number of worms infected and produced a high number of TAMs. However, in the field, Elton Creek *T. tubifex* were not abundant, were primarily lineage I and were not infected (Iwanowicz et al. In Prep). Hence, environmental factors in addition to land cover appear to be important in regulating infection at this site. In this instance, these environmental factors may include water velocity, lack of suitable habitat, and anthropogenic disturbances. Human alterations on land cover can cause changes in the community structures of phytoplankton, benthic invertebrates, and zooplankton (Rask et al. 1993; Burton and Ulrick 1994; Patoine et al. 2000), with more subtle alterations on fish communities (St.-Onge and Magnan 2000). Changes in invertebrate communities are expected to cause changes in parasite communities, as myxozoan parasites are dependent on multiple host to complete their lifecycle. For this reason, the laboratory data for Elton Creek appears to give incomplete information for what is actually occurring in the environment.

Cohabitation of susceptible and resistant *T. tubifex* also affects *M. cerebralis* infection (Beauchamp et al. 2006). In a recent study conducted by Beauchamp et al. (2006), the effects of cohabitation of susceptible (lineage I and III) and resistant (lineage V) *T. tubifex* populations and *M. cerebralis* infection were investigated. Interestingly, mixed populations of *T. tubifex* produce fewer TAMs than populations exclusively comprised of Lineage III *T. tubifex*. None of the worms used in our experimental trials

were lineage V *T. tubifex*; however, we did not notice a competition between lineage I and lineage III *T. tubifex* in regards to the production of TAMs. In both of our studies, only Lineage III *T. tubifex* produced TAMs after being infected with *M. cerebralis*. Beauchamp et al. (2006) also demonstrated that *T. tubifex* from Mt. Whitney, CA had a second peak in TAM production at approximately 140 days post-exposure. Within the time frame of 186 days post-exposure, worms from the East Coast (New York, Pennsylvania and West Virginia) never exhibited a similar second peak of TAM production after the initial release. These changes are not due to the development of the TAM within the worm host that has been described in detail (El-Matbouli and Hoffman 1998). The development of *M. cerebralis* within *T. tubifex* from both New York and Pennsylvania were the same as that previously described. Therefore, the development of the TAM within the oligochaete host, *T. tubifex*, is probably not the cause for the lack of salmonid population effects on the East Coast.

This study reiterates the presence of site variability and the influence of land cover (i.e.: deciduous, coniferous and deciduous/meadowland sites) on *T. tubifex* infection with *M. cerebralis*. Clearly, these studies demonstrate that infection of *T. tubifex* with *M. cerebralis* is affected by land cover and sediment. *Myxobolus cerebralis* infection rates, TAM release, duration of release, populations of lineage III *T. tubifex*, and parasite load were consistently higher in *T. tubifex* maintained in one substrate from one coniferous site and substrate from the deciduous/meadowland site. However, other biotic and abiotic factors obviously affect *T. tubifex* infectivity with *M. cerebralis* spores and their ability to produce and release TAMs. These laboratory studies using reference worms in site-specific substrate plus site-specific worms in their individual substrate also

supported the field observations, but suggested that factors other than substrate may affect infectivity at some sites.

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Figures

Figure 5.1. The effect of substrate from sites with different land cover on total triactinomyxon (TAM) release from reference *T. tubifex*. Sediment were collected from two deciduous sites (ELTON; Trout Brook, TB), a deciduous meadowland site (North Branch Wiscoy Creek, NBW), two coniferous sites (Big Fishing Creek , BFC; Lime Lake Outlet, LL), and a control sand substrate (CONTROL) post-infection with spores of *M. cerebralis*. (Study I)

Figure 5.2. Light microscope photographs representative of developing triactinomyxons (TAMs) in *T. tubifex* maintained in all substrates:

A) Representative photograph taken at 60 days post-exposure. Arrows are pointing to: a) pansporocyst, b) dividing zygote, c) still undivided zygote.

B) Representative photograph taken at 90 days post-exposure. Germ cells are beginning their advanced stage of development. Arrows are pointing to: a) the few still dividing zygotes that are still visible, b) along with less undivided zygotes, c) advanced stage of germ cell development.

C) Representative photograph taken at 110 days post exposure. Mature pansporocyst has developed. Fully developed TAMs with their polar capsule can be seen. Arrows are pointing to: a) polar capsule, b) sporoplasm and c) germ.

Figure 5.3. The effect of *T. tubifex* from and maintained in substrate from sites with different land cover on total triactinomyxon (TAM) production. Sediment and worms were collected from one deciduous site (Trout Brook, TB), a deciduous meadowland site (North Branch Wiscoy Creek, NBW), three coniferous sites (Big Fishing Creek , BFC; Lime Lake Outlet, LL; Mongaup Creek, MC), and the control sand substrate (CONTROL) following infection from spores of *M. cerebralis*. (Study 2)

Table 5.1. Mean water quality values from July, August and September 2003, and June and August 2004 at sites sampled from New York and Pennsylvania. Values are given for temperature (°C), pH, D.O. (ppm) and stream water velocity.

	Water Averages			
Site Name	**Temperature (°C)**	**pH**	**D.O. (ppm)**	**Velocity**
Elton Creek	11.9	7.75	10.83	high
Trout Brook	13.8	7.25	11.1	mild
North Branch Wiscoy Creek	15.1	7.25	11.8	mild to moderate
Lime Lake Outlet	15.2	7	10.95	moderate
Mongaup Creek	12.3	8	11.5	mild
Big Fishing Creek	10.65	7.88	11.55	moderate to high

Table 5.2. Metals and nutrients analyzed in sediments collected at all sites.

Aluminum	Manganese
Calcium	Molybdenum
Iron	Niobium
Potassium	Neodymium
Magnesium	Nickel
Sodium	Lead
Phosphorus	Antimony
Titanium	Scandium
Silver	Tin
Arsenic	Strontium
Gold	Tantalum
Barium	Thorium
Beryllium	Thallium
Bismuth	Uranium
Cadmium	Vanadium
Cerium	Yttrium
Cobalt	Ytterbium
Chromium	Zinc
Copper	Inorganic Carbon
Europium	Organic Carbon
Gallium	Total Carbon
Holmium	Mercury
Lanthanum	Selenium
Lithium	Sulphur

Table 5.3. Metal analyses of sediment samples. Data are presented in ppm for those metals significantly different between land cover types.

Site	Land Cover	ICPMS Ba ppm	ICPMS Ce ppm	ICPMS Cu ppm	ICPMS La ppm	ICPMS Nd ppm
Elton Creek	Deciduous	360.0	44.0	25.0	21.0	24.0
Trout Brook	Deciduous	320.0	48.0	22.0	24.0	27.0
N.B. Wiscoy Creek	D/M	360.0	42.0	24.0	20.0	23.0
Lime Lake Outlet	Coniferous	440.0	40.0	16.0	19.0	21.0
Big Fishing Creek	Coniferous	770.0	40.0	17.0	16.0	20.0
Sand	Control	19.0	7.0	5.2	3.6	3.4

D/M* = Deciduous/Meadowland

Table 5.4. Study I results including land cover, % *T. tubifex* infected, duration of TAMs released (days), the mean number of TAMs released per infected worm per day at each site (with standard error), and % lineages present.

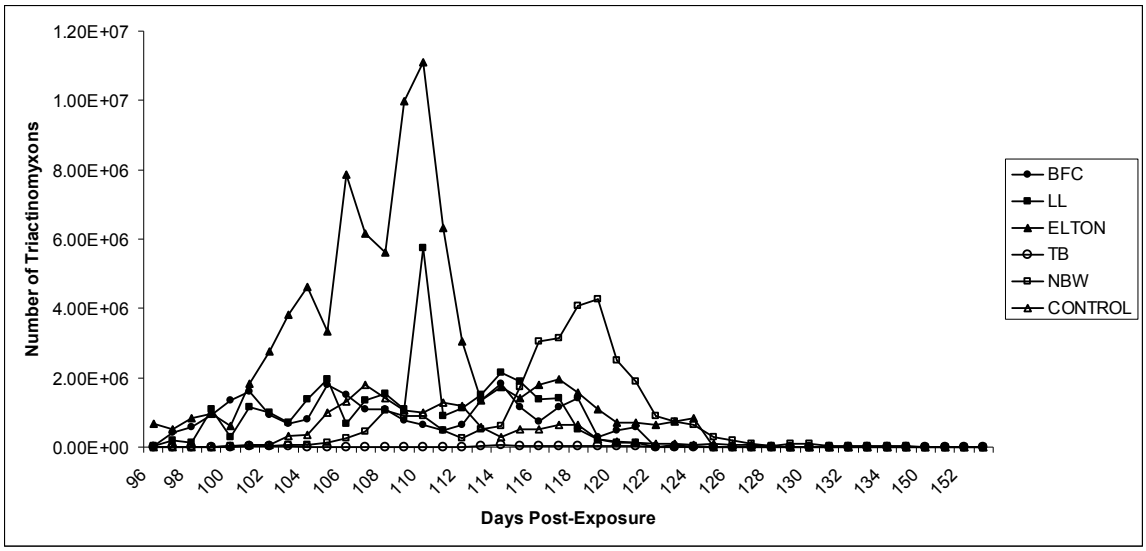
Site	Land Cover	% Infected	Duration (Days)	Mean TAMs/ Infected Worm/Day	*T. tubifex* Lineage I	*T. tubifex* Lineage III
Trout Brook	Deciduous	9	4	5.0×10^2 ($\pm 2.1 \times 10^2$)	92	8
Elton Creek	Deciduous	33	14	6.4×10^3 ($\pm 76.4 \times 10^3$)	76	24
N.B. Wiscoy Creek	D/M*	52	7	3.4×10^3 ($\pm 35.1 \times 10^3$)	74	26
Lime Lake Outlet	Coniferous	26	9	5.3×10^3 ($\pm 34.7 \times 10^3$)	77	23
Big Fishing Creek	Coniferous	23	8	5.5×10^3 ($\pm 18.5 \times 10^3$)	75	25
Sand	Control	12	12	3.2×10^3 ($\pm 28.4 \times 10^3$)	92	8

D/M* = Deciduous/Meadowland

Table 5.5. Study II results including land cover, % *T. tubifex* infected, duration of TAMs released (days), the mean number of TAMs released per infected worm per day at each site (with standard error), and % lineages present.

Site	Land Cover	% Infected	Duration (Days)	Mean TAMs/ Infected Worm/Day	*T. tubifex* Lineage I	*T. tubifex* Lineage III
Trout Brook	Deciduous	13	3	5.7×10^3 ($\pm$ 23.6 x 10^3)	77	23
North Branch	D/M*	81	7	5.1×10^3 ($\pm$ 33.9 x 10^3)	17	83
Lime Lake Outlet	Coniferous	34	12	9.5×10^3 ($\pm$ 54.5 x 10^3)	18	82
Big Fishing Creek	Coniferous	21	12	5.7×10^3 ($\pm$ 51.7 x 10^3)	80	20
Mongaup Creek	Coniferous	21	11	9.3×10^3 ($\pm$ 36.7 x 10^3)	75	25
Sand	Control	12	12	2.9×10^3 ($\pm$ 14.37 x 10^3)	81	19

D/M* = Deciduous/Meadowland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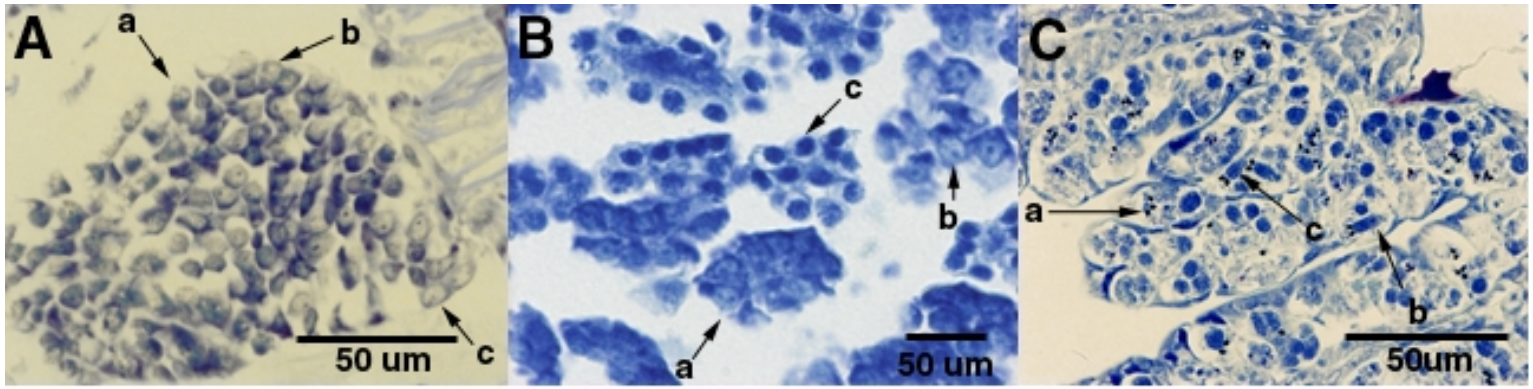
8.00E+06
6.00E+06
4.00E+06
2.00E+06
0.00E+00
Number of Triactinomyxons
96
98
100
102
104
106
108
110
112
114
116
118
120
122
124
126
128
130
132
134
150
152
Days Post-Exposure
BFC
LL
ELTON
TB
NBW
CONTROL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a
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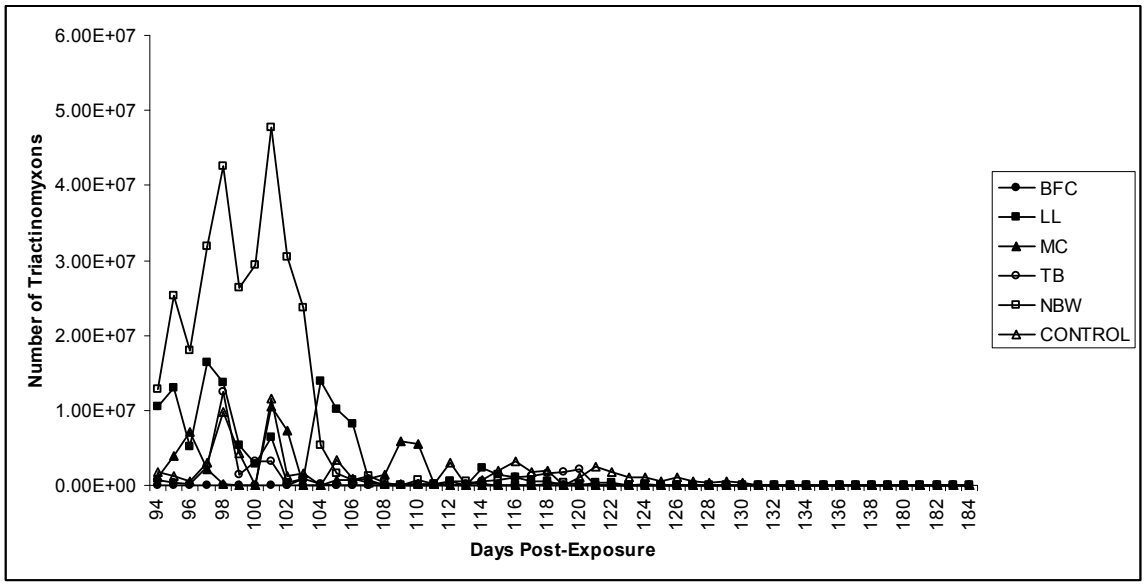
6.00E+07
5.00E+07
4.00E+07
3.00E+07
2.00E+07
1.00E+07
0.00E+00
Number of Triactinomyxons
94
96
98
100
102
104
106
108
110
112
114
116
118
120
122
124
126
128
130
132
134
136
138
180
182
184
Days Post-Exposure
BFC
LL
MC
TB
NBW
CONTROL

CHAPTER 6

CONCLUDING REMARKS

The deleterious effects of environmental stressors and anthropogenic disturbances on water quality are evident across the United States (Lafferty and Kuris 1999). Urbanization is reported to adversely affect the physical, chemical, and biological characteristics of the aquatic environment. With the adverse affects on the environment, science continuously addresses issues related to assessing and evaluating the effects of stressors on the health of aquatic ecosystems. Some of the more challenging aspects include validating ecologically significant biomarkers, determining the importance of temporal and spatial variability of physicochemical and biological factors in modifying responses to stress, and establishing cause and effect relationships between specific stressors as they relate to environmental damage (Adams 2001).

The application of parasites as bioindicators of ecosystem health is a relatively new concept (Overstreet 1997; Marcogliese 2004; Marcogliese 2005). Environmental factors that affect vertebrates also affect parasite transmission, abundance, and composition (Marcogliese 2005). Therefore, changes in the environment can influence the biology and life cycle of parasites, making them useful indicators of environmental stress.

The studies in this dissertation investigated effects of environmental stressors and anthropogenic disturbances on infections with myxozoan

parasites. Initially, field research was conducted near Birmingham, AL. I described a new myxozoan parasite, *Myxobolus stanlii* n. sp., found in largescale stonerollers (*Campostoma oligolepis*). Tissue distribution of this parasite included connective tissue of various organs, renal tubules, glomeruli, macrophage aggregates within the kidney, and nerve tissue behind the eye. Fish had increasing densities of parasite load (spores per gram fish tissue) through the lower and middle land-use gradients and a decreased density at sites with the highest urban land-use values. I concluded that at the lower and middle land-use gradient sites, water quality, eutrophication and thermal effluent can raise rates of parasitism because the associated increased productivity can increase the abundance of intermediate hosts and vectors (Lafferty and Kuris 1999), while stress on the host can decrease natural resistance to the parasites. At higher land-use gradient sites, there could be a decrease in parasitism if infected hosts have a differentially higher mortality or the parasites were more susceptible to pollution than their hosts (Lafferty and Kuris 1999). Also habitat alterations such as urbanization can affect the intermediate host populations such that their survival is lower and/or the abundance of their parasite load is reduced (Lafferty and Kuris 1999).

As myxozoan parasites have a two host life-cycle, knowledge of both hosts is important in controlled tests. The lifecycle of *M. stanlii* n. sp. is unknown and prohibited any laboratory tests. However, the life cycle of *M. cerebralis*, the causative agent of whirling disease has been described. This parasite has a complex two-host life cycle involving separate stages of sporogony in salmonid fishes and the aquatic oligochaete *Tubifex tubifex* (Markiw and Wolf 1983; Wolf et al. 1986). *Tubifex tubifex* produces the triactinomyxon (TAM) spore that infects the salmonid hosts. Population effects on wild

salmonid stocks have been reported in some geographic areas (Walker and Nehring 1995; Vincent 1996) in the Western United States. Although the parasite is endemic in other areas of the United States, similar population declines have not been observed (Hnath 1996; Modin 1998; Kaeser et al. 2006).

Previous research in our laboratory determined that substratum and temperature affect the total number of infective TAMs released by worms (Blazer et al. 2003). Further field studies were conducted in New York and Pennsylvania to evaluate the effect of riparian zone and associated stream substrata on *T. tubifex* density and infections with myxozoan parasites. Research examined *T. tubifex* and substrata from New York and Pennsylvania trout streams with coniferous (two sites), deciduous (two sites), meadowland (two sites) and mixed deciduous/meadowland (two) riparian zones. The vast majority of *T. tubifex* in the Northeast are from lineages I and III, the most susceptible lineages to *M. cerebralis* infection, yet whirling disease is not an issue for wild salmonids in this region. *Tubifex tubifex* assemblages and lineages present were affected by the dominant land cover and *M. cerebralis* infection was only prevalent in lineage III *T. tubifex* and highest at the coniferous and deciduous/meadowland sites. These findings suggest that riparian zone and surrounding land use may affect oligochaete population dynamics and lineage selection of *T. tubifex*. This finding was confirmed in subsequent laboratory studies where we examined the susceptibility of *T. tubifex* in substrate from different land covers exposed to *M. cerebralis* in a series of laboratory trials was evaluated. The first laboratory study was completed using reference worms in site specific substrate from New York and Pennsylvania. In the second laboratory study we used site specific worms in their individual substrate from New York

and Pennsylvania. *M. cerebralis* infection rates, TAM release, duration of release, populations of lineage III *T. tubifex*, and parasite load were consistently higher in *T. tubifex* maintained in coniferous and deciduous/meadowland substrate. These laboratory studies using reference worms in site specific substrate plus site specific worms in their individual substrate, supported the field observations. They also suggested that factors other than substrate may affect infectivity at some sites. The relation between *T. tubifex*, their infection with *M. cerebralis*, and land cover, may be important to the management of whirling disease.

Land cover and substrate type clearly affect resident oligochaete populations. Considering that freshwater oligochaetes are common hosts for myxozoan parasites the influences of land cover and substrate type are likely to influence the host/pathogen relationship. Certain factors such as land cover and substrate, urbanization and copper levels can be used to infer prevalence of myxozoan infection. Although different geographic sites are independent of each other, and site variability must be considered. Focusing on these factors may link a commonality in prevalence of infection with these parasites. In the case of *M. stanlii*, n. sp. high levels of urbanization decrease the severity of infection within largescale stonerollers. With the parasite *M. cerebralis*, increased severity of infection within the oligochaete host *T. tubifex* was found at sites with agricultural areas along streams, or streams with a deciduous/meadowland land cover. Apparently high copper levels also decrease *T. tubifex* abundance and lineage diversity. Although all *T. tubifex* at coniferous sites appear to have increased triactinomyxon (TAM) production, only one site (Lime Lake Outlet) had higher proportions of lineage III *T. tubifex* than the other coniferous sites. These differences at Lime Lake Outlet may be

due to the lake and anthropogenic disturbances upstream from our site. Focusing on areas with land cover and substrate or copper levels, may help in selecting sites that are a risk for increased myxozoan infection.

In conclusion, this research ascertains the importance of environmental stressors and anthropogenic disturbances on the infection of myxozoan parasites in both fish and invertebrate hosts. As discussed elsewhere, parasites complex life cycles makes them extremely valuable information units about environmental conditions, because their presence/absence tells us a great deal about their host ecology, food web interactions, biodiversity, and environmental stress (Marcogliese and Cone 1997; Overstreet 1997; Marcogliese 2002; 2003; 2004; 2005).

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