

INFLUENCE OF ENVIRONMENTAL FEATURES ON *TUBIFEX TUBIFEX* AND
MYXOBOLUS CEREBRALIS INFECTED *TUBIFEX TUBIFEX* IN YELLOWSTONE
NATIONAL PARK: IMPLICATIONS FOR WHIRLING DISEASE RISK

by

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ABSTRACT

Whirling disease (WD) is an emerging parasitic disease of salmonids that is increasing in severity and geographic range. Whirling disease is caused by the myxosporean parasite, *Myxobolus cerebralis*, and can effect significant mortality in wild and cultured salmonid populations. *Myxobolus cerebralis* was recently detected in Yellowstone National Park (YNP) where it may be causing native Yellowstone cutthroat trout (YCT) to decline. *Myxobolus cerebralis* exploits the aquatic oligochaete, *Tubifex tubifex*, as its primary host and spores released by *T. tubifex* are infective to salmonid fish. The aim of this study was to assess WD risk for YCT populations in YNP by focusing on the disease source, *T. tubifex*, which had not previously been characterized. My objectives were to characterize *T. tubifex* populations and dynamics of *M. cerebralis* infections in *T. tubifex* and to establish factors associated with *M. cerebralis* infections in *T. tubifex*. In addition, I examined relationships between infection dynamics in *T. tubifex* and transmission to fish hosts (WD risk).

In Pelican Creek, *T. tubifex* and *M. cerebralis* infected *T. tubifex* were widely distributed and abundant. Infected *T. tubifex* were most abundant in reaches characterized by intermediate geothermal influence. However, WD risk was high in all reach types, which indicated that low parasite success in the oligochaete host in reaches with high or no geothermal influence did not translate into reduced WD risk in these reaches. In tributaries throughout YNP, susceptible *T. tubifex* were widely distributed but experimental and field data suggest *M. cerebralis*-infected *T. tubifex* may be unable to survive in all tributaries where uninfected *T. tubifex* were found. In particular, environmental factors influenced by confinement, including proportions of coarse and fine substrates, may preclude establishment of *M. cerebralis* in tributaries in YNP. Thus, environmental features, rather than oligochaete host factors, may be most influential for *M. cerebralis* dynamics in *T. tubifex* and WD risk to fish in YNP. While further research is needed to identify specific mechanisms, these results suggest environmental features related to confinement may be useful for assessing WD risk at broad scales when the oligochaete host is characterized by low genetic variability.

CHAPTER ONE

INTRODUCTION TO DISSERTATION

Parasites can regulate host abundance and evolution (Anderson and May 1979, May and Anderson 1979) and affect the structure and composition of communities (Dobson and Hudson 1986, Minchella and Scott 1991, Soler et al. 2001, Mouritsen and Poulin 2002, Semple et al. 2002, Thomas et al. 2005). However, the environment, which is often overlooked, can strongly influence outcomes of host-parasite interactions (Hedrick 1998, Patz et al. 2000, 2004, Scholthof 2007). Environmental conditions may influence reproduction and development of hosts and parasites (Grainger 1992, Marcogliese 2001, Harvell et al. 2002), parasite transmission (Ewald 1983, Patz et al. 2000, Harvell et al. 2002, Walther et al. 2002, Fels and Katz 2006), host susceptibility (Blazer 1992, Harvell et al. 1999, Pearson et al. 1999, Carls et al. 2002), and the relative abundance of susceptible host genotypes (Krafsur 2003, Mitchell et al. 2005, Calvo et al. 2007). In turn, each of these factors may affect parasite success and the outcome of host-parasite interactions. Thus, environmental conditions should be considered when examining host-parasite interactions, because they may ultimately determine parasite success.

Environmental conditions may be an important determinant of parasite success during interactions between the myxozoan parasite that causes salmonid whirling disease, *Myxobolus cerebralis*, and its hosts. The life cycle of *M. cerebralis* involves two hosts, a salmonid and the freshwater oligochaete, *Tubifex tubifex*, and two environmentally transmitted spore stages, myxospores and triactinomyxons (TAMs) (Figure 1.1; Wolf and Markiw 1984, Andree et al. 1997). Infected salmonids produce myxospores that are infective

to *T. tubifex*, and *T. tubifex* produces TAMs that are infective to salmonids (Wolf and Markiw 1984).

Myxobolus cerebralis has recently become established in declining native and wild salmonid populations throughout the U.S. intermountain west (Nehring and Walker 1996, Vincent 1996, Baldwin et al. 1998, Hedrick et al. 1998, Bartholomew and Reno 2002, Downing et al. 2002, Elwell et al. 2009). The native Yellowstone cutthroat trout (*Oncorhynchus clarki bouveri*) population in Yellowstone Lake, Yellowstone National Park, is an example of a native salmonid population in the intermountain west that has declined (Koel et al. 2005, 2006). Declines have primarily been attributed to invasive lake trout (*Salvelinus namayacush*; Kaeding et al. 1996, Koel et al. 2005), but *M. cerebralis* was detected in adult Yellowstone cutthroat trout in the lake in 1998 and may also be causing the population to decline (Koel et al. 2006). *Myxobolus cerebralis* has since been detected in sentinel fish exposed in three of the lake's tributaries (Koel et al. 2006, Murcia et al. 2006) that historically supported large spawning populations of Yellowstone cutthroat trout (Pelican Creek, the Yellowstone River below the lake, and Clear Creek; Jones et al. 1982, Gresswell and Varley 1988, Gresswell et al. 1994, 1997, Koel et al. 2005). Sentinel fish are caged trout that are exposed in tributaries for 10 days and subsequently examined by molecular and histological analyses for *M. cerebralis* infection and severity (e.g., Krueger et al. 2006, Murcia et al. 2006). Sentinel fish exposed in Pelican Creek were characterized by high infection prevalence and severity (severity is assessed by scoring parasite damage in fish, Hedrick et al. 1999, Baldwin et al. 2000). Sentinel fish exposed in the Yellowstone

River and Clear Creek were characterized by comparatively low infection prevalence and severity (Koel et al. 2006).

The differences in infection prevalence and severity in sentinel fish suggested TAM production was high in Pelican Creek and low in the other tributaries. Differences in environmental conditions among tributaries may partially explain why TAM production was high in Pelican Creek relative to the other tributaries. Pelican Creek is characterized by abundant geothermal features that likely influence stream temperature, and temperature affects proliferation and transmission of *M. cerebralis* (El-Matbouli et al. 1999, Blazer et al. 2003, Kerans et al. 2005). In addition, Pelican Creek is characterized by low slope (<1%) and low confinement (unconfined streams can meander into the floodplain when flooding occurs, whereas confined streams cannot), which result in reach scale microhabitat features (e.g., substrate transport and low velocities; Hauer et al. 1997, Stewart et al. 2005), preferred by *T. tubifex*. Therefore, variation in *T. tubifex* populations related to environmental differences among tributaries may also explain differences in TAM production and whirling disease risk. Streams characterized by low slopes may be characterized by high abundances of *T. tubifex* because they provide optimal habitat (e.g., organic material or fine sediments; Brinkhurst 1971, Lazim and Learner 1987, Verdonshot 1999, 2001). Host abundance influences rates of encounter between hosts and parasites (e.g., myxospore-*T. tubifex*) and has been related to infection prevalence in *T. tubifex* as well as infection severity in sentinel fish in other systems (e.g., Rognlie and Knapp 1998, Zendt and Bergersen 2000, Krueger et al. 2006).

Genetic variation in *T. tubifex* may also contribute to differences in TAM production among the tributaries. Six genetically variable lineages of *T. tubifex* have previously been described (Sturmbauer et al. 1999), five of which occur in North America (lineages I, III-V; Beauchamp et al. 2001, 2002, Arsan et al. 2007) and susceptibility to *M. cerebralis* appears to vary among lineages. Lineages V and VI are considered resistant to *M. cerebralis* (Beauchamp et al. 2006, Elwell et al. 2006). Lineage I is also considered resistant (e.g. Arsan et al. 2007), but at least one strain within this lineage produced TAMs when experimentally infected (Kerans et al. 2005). Lineage III is considered susceptible to *M. cerebralis*, however, TAM production and parasite amplification varies among strains within this lineage (e.g., Stevens et al. 2001, Kerans et al. 2004, Baxa et al. 2008, Rasmussen et al. 2008). Genetic variants of *T. tubifex* have different environmental optima (e.g. Anlauf and Neumann 1997, DuBey and Caldwell 2004, Kerans et al. 2005), coexist (e.g. Beauchamp et al. 2005, Crottini et al. 2008, DuBey 2008), and may compete for resources and myxospores. Although the distribution and abundance of genetic variants of *T. tubifex* and their relative susceptibilities to *M. cerebralis* are unknown for tributaries in Yellowstone National Park, these factors may also influence TAM production and *M. cerebralis* success.

Overview of Dissertation

In my dissertation, I examined dynamics of *M. cerebralis* and whirling disease risk to fish in Yellowstone National Park by focusing on characterizing the ecology of *T. tubifex*. As the source of TAMs, *T. tubifex* is hypothesized to directly influence whirling disease risk to fish (Krueger et al. 2006). I was particularly interested in examining *M. cerebralis* and *T. tubifex* under different environmental conditions because the ecology of *T. tubifex* is directly

influenced by its immediate environment. Consequently, conditions in the immediate environment may strongly influence *M. cerebralis* success in *T. tubifex*.

Background information for *M. cerebralis*, *T. tubifex*, whirling disease risk, and Yellowstone cutthroat trout is provided in chapter two. In chapters three and four, I characterized *T. tubifex* ecology and examined *M. cerebralis* success in *T. tubifex* in different environments. In chapter three, I characterized the ecologies of *T. tubifex* and *M. cerebralis* in reaches of Pelican Creek. This tributary was selected because it had previously been identified with variable geothermal influence (a feature that had previously been identified as potentially influential for whirling disease risk to fish in this system; Koel et al. 2006). In chapter four, I characterized *T. tubifex* ecology and *M. cerebralis* success in reaches on tributaries to the Yellowstone River and Yellowstone Lake with variable confinement, which I hypothesized would strongly influence *T. tubifex* and interactions between *M. cerebralis* and *T. tubifex*. In chapter five, I conducted a laboratory experiment to investigate how substrate, an environmental condition predicted by confinement that appeared to influence abundance of *T. tubifex* but not *M. cerebralis* infected *T. tubifex* (chapter four), affected interactions between *T. tubifex* and *M. cerebralis*. In chapter six, I established laboratory strains of *T. tubifex* from tributaries in Yellowstone National Park, described genetic variation among the laboratory reared strains of *T. tubifex*, and tested the susceptibility of a subset of the strains to *M. cerebralis*. Finally, in chapter seven, I provided a summary of major results from each chapter and discussed how these results may influence whirling disease risk to Yellowstone cutthroat trout and the potential for their long-term survival in the Yellowstone ecosystem.

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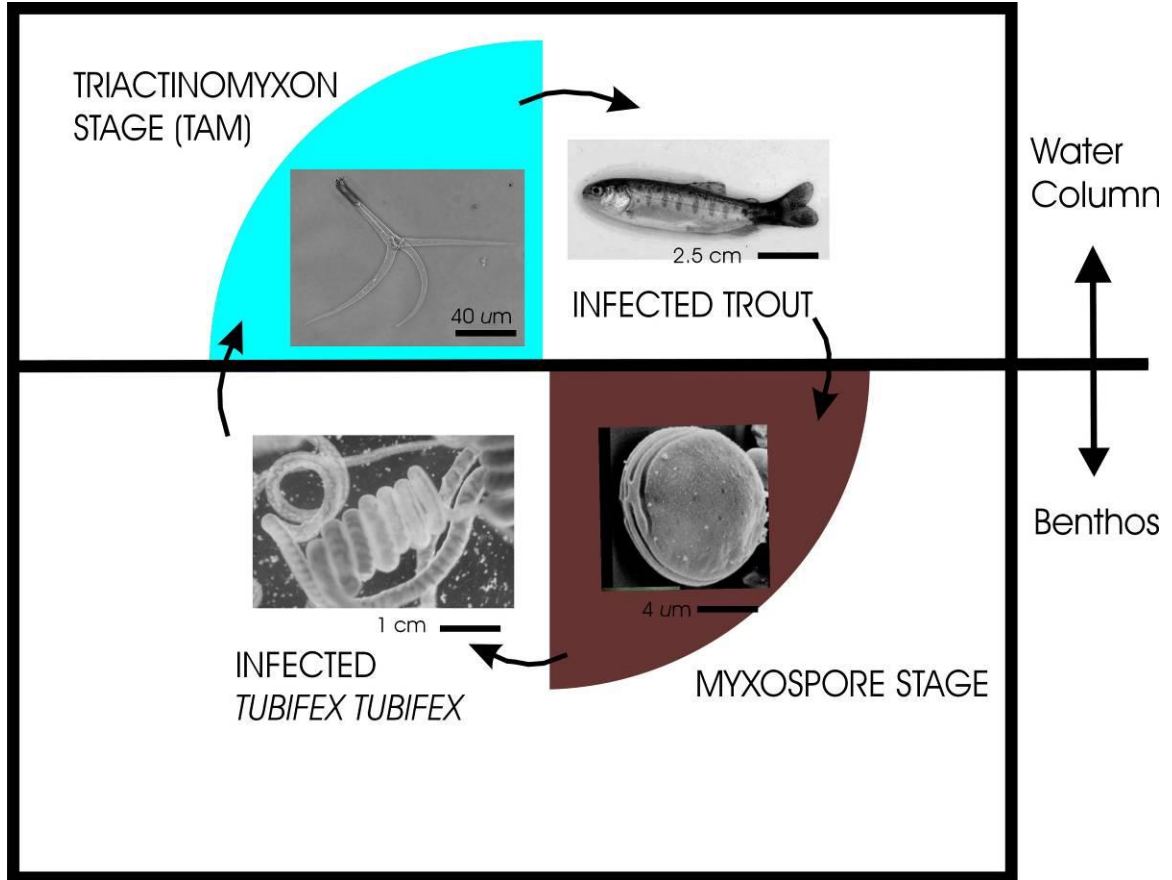


Figure 1.1. The life cycle of *Myxobolus cerebralis*. The life cycle of *Myxobolus cerebralis* involves the aquatic oligochaete, *Tubifex tubifex*, and a salmonid fish. Infected *T. tubifex* produce triactinomyxons, which float in the water column where they may encounter and infect fish. Infected fish produce myxospores, which are released after the fish decomposes. Myxospores settle in slow flowing benthic areas, where they may be encountered and ingested by *T. tubifex*. Shaded areas represent the phases in the *M. cerebralis* life cycle when spores are external to hosts and thus directly influenced by environmental conditions. Morphology of TAMs facilitates floating, thus they are influenced by environmental conditions in the water column (indicated by blue coloration). In contrast, myxospores are influenced by environmental conditions in the benthos (indicated by brown coloration) because their morphology facilitates settling.

CHAPTER TWO

BACKGROUND

Myxobolus cerebralis is a myxozoan parasite that causes salmonid whirling disease (Hofer 1903 in Bartholomew and Reno 2002). Diseased fish may exhibit a range of signs, including deformed crania and vertebrae, blackened tails, tail-chasing (whirling) behavior, and reduced growth (Halliday 1973, 1976, MacConnell and Vincent 2002). The parasite was first described in non-native rainbow trout in Europe over a century ago (*Myxosoma cerebralis* in *Oncorhynchus mykiss*; Hofer 1903 in Bartholomew and Reno 2002), and has subsequently been described in salmonids throughout much of the world (Bartholomew and Reno 2002, Bartholomew et al. 2005, Elwell et al. 2009).

Historically, whirling disease has constituted a considerable problem for salmonid culture facilities worldwide. Halted facility operations and subsequent product losses had significant economic consequences (Hoffman 1990, Bartholomew and Reno 2002). Following the discovery that an alternate host, *Tubifex tubifex*, was required to transmit *M. cerebralis* to fish, simple modifications (e.g., paving raceways and rearing ponds) that eliminated *T. tubifex* habitat served to interrupt the life cycle, and effectively eliminated the disease and *M. cerebralis* was considered a manageable pathogen (Hewitt and Little 1972, Hoffman 1974, 1990). However, sporadic and epidemic outbreaks of *M. cerebralis* in salmonid populations in different regions of the U.S., including Pennsylvania, Colorado, Montana, and California (Hoffman 1990, Nehring and Walker 1996, Vincent 1996, Hedrick 1998, Downing et al. 2002) that have occurred during previous decades suggest the opposite. In the intermountain west (CO, MT), outbreaks have been associated with significant

declines in native and wild trout populations (e.g., losses of up to 90% of some year classes of rainbow trout in MT and CO; Nehring and Walker 1996, Vincent 1996, Baldwin et al. 1998). Because the alternate host, *T. tubifex*, is found in freshwater habitats (Brinkhurst 1971, Prenda and Gallardo 1992, Anlauf and Neumann 1997, Matisoff et al. 1999), managing outbreaks in natural systems by breaking the parasite's life cycle is difficult, if not impossible. The conservation of wild and native trout is a high priority for states in the intermountain west (Nehring and Walker 1996, Vincent 1996, Nickum 1999, Sheppard et al. 2005, Rahel et al. 2008). Consequently, the outbreaks have fueled multi-agency, multi-disciplinary research collaborations focused on understanding *M. cerebralis* and factors that influence success and persistence.

The Parasite

Myxobolus cerebralis is a metazoan parasite that alternates between multiple host species and morphologically distinct spore stages during its life cycle (Wolf and Markiw 1984, Smothers et al. 1994, Andree et al. 1997, Anderson et al. 1998). This particular myxozoan, long the subject of intense debate, has played an important role in our understanding of many other significant myxozoan parasites. The breakthrough discovery by Wolf and Markiw (1984) that *M. cerebralis* had an indirect life cycle involving two host species and two morphologically distinct spore stages has affected the fields of both basic and applied science. In basic science, the elucidation of the life cycle ultimately resulted in the collapse of the once-separate Actinosporea and Myxosporea classes into one class (Kent et al. 1994, 2001, Siddall et al. 1995), and the formal addition of the Myxozoa to the phylum Metazoa. This sparked widespread debate (Smothers et al. 1994, Anderson et al. 1998) and

generated new insight into parasite evolution (e.g., Okamura and Canning 2003, Canning and Okamura 2004, Jimenez-Guri et al. 2007, Holland et al. *in press*). In applied science, the discovery prompted the discovery and description of many myxozoan life cycles (e.g., *Ceratomyxa shasta*, Bartholomew et al. 1997, Kent et al. 2001) and resulted in the ability to control and manage whirling disease in fish culture environments (Hoffman 1990).

The life cycle of *M. cerebralis* involves a salmonid (many salmonid species are susceptible, but susceptibility varies; Hofer 1903 in Bartholomew and Reno 2002, Hedrick et al. 1999a, 1999b, 2001, Vincent and MacConnell 2002) and the aquatic oligochaete, *Tubifex tubifex*, and two distinct spore stages, the myxospore and the actinospore (triacinomyxon or TAM; Wolf and Markiw 1984, El-Matbouli and Hoffman 1989, 1998). Triactinomyxons encounter and attach to fish while passively floating in the water. When TAMs contact an appropriate host, they discharge an infective sporoplasm that penetrates the salmonid epidermis and migrates to the cranial cartilage via the nervous system. Replication occurs in the cranial cartilage, which causes inflammation and lesions (El-Matbouli et al. 1992, 1998, 1999). Myxospores mature approximately three months post-infection (at 15°C, Markiw 1992, El-Matbouli et al. 1995). Myxospores are released when fish decompose and settle out in slower-flowing areas of streams where they may be encountered and ingested by foraging *T. tubifex* (Hamilton and Canning 1987, El-Matbouli and Hoffman 1991, Brinkhurst 1996, Kerans and Zale 2002). Once consumed by *T. tubifex*, myxospores discharge their sporoplasms into interstitial spaces of the intestinal lining, then divide and replicate (Wolf and Markiw 1986, El-Matbouli and Hoffman 1991, El-Matbouli and Hoffman 1998, El-Matbouli et al. 1998). Approximately three months post-infection (roughly 1300 degree days

post infection), TAMs are released from *T. tubifex* (Wolf and Markiw 1984, El-Matbouli and Hoffman 1991).

Factors Affecting *Myxobolus cerebralis* Spores

Triactinomyxons and myxospores are morphologically distinct, which reflects their different functional roles in parasite transmission. Myxospores are sphere shaped, 8-10 µm in diameter, and are characterized by high specific gravity relative to freshwater (Gates 2007). Both of these features facilitate settling out in stream bottoms, where they may have a greater probability of being encountered and ingested by *T. tubifex* (Kerans and Zale 2002). In addition, they are characterized by a hard polysaccharide capsule, which allows them to remain viable for extended periods of time (Markiw 1992). Triactinomyxons are 180-200 µm across and characterized by long processes (Figure 1.1), which allows them to float in the water column, where they have a greater probability of encountering fish hosts. Under laboratory conditions, TAM longevity is approximately 10-14 days (Markiw 1992, El-Matbouli et al. 1999b).

Little is known regarding dispersal of TAMs or myxospores; however, environmental conditions may influence the viability and transmission activity of both TAMs and myxospores. For example, stream discharge and velocity may alter myxospore deposition rates or TAM viability (MacConnell and Vincent 2002). Infected trout carcasses or myxospores may be re-suspended by high flows, which could exacerbate whirling disease risk by spreading infective spores to new areas, or flush out myxospores, making them unavailable to *T. tubifex* (Vincent 1996, Kerans and Zale 2002). Increased discharge and

velocities may also dilute or destroy TAMs, thereby reducing risk (Vincent 2002). Other environmental factors (e.g., temperature, salinity, pH, and conductivity) may also affect TAM viability (Sandell et al. 2001, Wagner et al. 2002), but probably do not affect myxospore viability (Hedrick et al. 2008).

Whirling Disease Risk

A variety of techniques are available to assess whirling disease risk. Risk may be determined directly in the environment by counting spores (e. g., Nehring et al. 2003, Lukins 2004, Lukins et al. 2007) or indirectly through infection prevalence in either or both hosts (e. g., Baldwin et al. 1998, Hiner and Moffitt 2001, Franco and Budy 2004, Koel et al. 2006, Krueger et al. 2006). Techniques to estimate myxospore abundance in stream sediments have been developed using the sodium hexametophosphate and plankton centrifuge method (Lemmon and Kerans 2001) as well as the pepsin-trypsin digest (PTD) method (Markiw and Wolf 1974a, 1974b). However, difficulties with myxospore detection (K. Gates and L. Elwell, MSU, personal communication) and test sensitivity (Lemmon and Kerans 2001) have made it impractical to measure WD risk by enumerating myxospores. In contrast, techniques to enumerate TAMs have been successfully developed as a direct measure of WD risk. Using this method, a known volume of water is strained through a filter (mesh size $\leq 80 \mu\text{m}$) and TAMs are counted (Nehring et al. 2003, Lukins et al. 2007). Specialized instruments, such as the TAM-ometer, which filters water on site (Lukins et al. 2007), have been designed to simplify detection and enumeration of TAMs in the field.

Whirling disease risk is typically quantified as *M. cerebralis* infection prevalence and severity in the fish host. Both wild and hatchery-reared (sentinel fish), are used in this method. Wild fish are collected and examined for infection. Sentinel fish are exposed to a stream in cages for a set period (24 hours to 10 days) (e. g., Hiner and Moffit 2001, Koel et al. 2006, Krueger et al. 2006). Sentinel fish are then removed and examined for infection.

Methods for detection of *M. cerebralis* in salmonids include observing fish for clinical signs, scoring parasite-induced lesions, quantifying myxospores, and the use of parasite specific molecular tests. Clinical signs include blackened caudal regions, deformed vertebrae, and whirling behavior, which may be observed 3 to 6 months post-infection (Murcia et al. 2006). Clinical signs are scored qualitatively, by presence or absence. To score parasite-induced lesions, histological slides are prepared from sections of the head or tail and stained with hematoxylin and eosin. Slides are examined for parasite-induced lesions in cartilage, and parasite damage is scored as a quantitative measure of infection severity. Parasite damage is scored on a scale of 0-5, (where a score of 0 indicates no infection and 5 indicates severe infection; e. g., the MacConnell-Baldwin scale), which is based on overall lesion number and degree of inflammation (Hedrick et al. 1999, Baldwin et al. 2000). The PTD method is used to isolate and extract myxospores from infected fish cartilage. In this method, myxospores are ‘digested’ out of fish cartilage and quantified as an estimate of myxospore load per fish (Markiw and Wolf 1974b). The *M. cerebralis*-specific PCR was designed to detect parasite DNA in host tissues. Both nested and single round PCR assays have been developed based on sequences of the *M. cerebralis* 18S ribosomal gene (Andree et al. 1998, Baldwin and Myklebust 2002). Results from PCR assays are scored qualitatively

by presence (+) or absence (-) of parasite DNA, or quantitatively by real-time, or quantitative PCR (qPCR) (Cavender et al. 2004).

Although the parasite may be detected in either host, infection measures in the oligochaete host (i.e., prevalence of infection, density of infected *T. tubifex*) have been less frequently employed as a measure of whirling disease risk than infection measures in fish. The main reason for this is that the relationship between infection in *T. tubifex* and whirling disease risk to fish is not well understood. However, at least one study showed the density of infected *T. tubifex* was positively correlated with infection severity in sentinel rainbow trout (Krueger et al. 2006). Additionally, sampling and handling oligochaetes is relatively easy compared to handling wild or sentinel fish. Thus, establishing quantitative parameters for the relationship between infection in *T. tubifex* and WD risk to fish could facilitate risk assessments in the field.

Methods for the detection of *M. cerebralis* in *T. tubifex* include observation for TAM release, histological analyses, and molecular analyses. To observe TAM release, individual oligochaetes are held in 12-well plates and periodically scanned for the presence or absence of TAMs (e. g., Krueger et al. 2006, Elwell et al. 2006). As in fish, histological slides may be prepared from infected oligochaetes. Gut epithelial tissues are stained and examined for parasite damage (e. g., El-Matbouli and Hoffman 1998). However, a scale to quantify infection severity in histological preparations of *T. tubifex* has yet to be developed. Estimates of infection prevalence in *T. tubifex* have been based on TAM release in previous studies (e. g., Krueger et al. 2006). However, recent work on the Madison River, Montana, suggests that infection prevalence may be overestimated if tubificids release TAMs that are

morphologically similar to those of *M. cerebralis* (L. Elwell, Montana State University, unpublished data, Lodh et al. *in press*). Consequently, molecular assays may be the best available method for estimating infection prevalence in *T. tubifex*. As in fish, both single round and nested *M. cerebralis*-specific PCR tests (Andree et al. 1998, Baldwin and Myklebust 2002) have successfully been used to detect *M. cerebralis* in *T. tubifex* (e.g., Zendt and Bergersen 2000).

In the present study, whirling disease risk will be quantified by infection prevalence and severity in sentinel fish deployed in each study tributary. Infection prevalence in sentinel fish will be estimated by nested PCR and infection severity will be quantified by parasite lesion severity, scored on the MacConnell-Baldwin Scale (Baldwin et al. 2000). Abundance of infected *T. tubifex* and infection prevalence in *T. tubifex* will also be quantified at multiple sites within each study tributary, and compared to infection prevalence and severity in fish. Infection in *T. tubifex* will be estimated by PCR assays. Abundance of *T. tubifex* and infected *T. tubifex* will be estimated by semi-quantitative kick net samples and from known volumes of general invertebrate samples.

The Salmonid Host

Life history diversity among salmonids may influence the risk of parasite establishment in a system because in order to complete its life cycle, *M. cerebralis* TAMs must come into contact with young fish. If timing and location of spawning, and fry emergence and rearing (most susceptible fish size and age), overlaps with TAM release by *T. tubifex* in stream systems, the risk

of disease, and thus pathogen establishment, increases significantly (e.g., Downing et al. 2002, Hubert et al. 2002, Kerans and Zale 2002).

Factors Affecting *Myxobolus cerebralis* infections in the Salmonid Host

Factors affecting infection in the salmonid host include species, strain, size and age at exposure, and parasite dose (Halliday 1976, Hedrick et al. 1999a, 1999b, Baldwin et al. 2000, MacConnell and Vincent 2002, Ryce et al. 2004, Ryce et al. 2005). Species range from highly susceptible (Yellowstone cutthroat trout; *O. clarki bouveri* or rainbow trout) to resistant (e.g., brown trout, *Salmo trutta*) to refractory (e.g., lake trout, *Salvelinus namayakush*) to *M. cerebralis* (O'Grodnick 1979, Hedrick et al. 1999a, 1999b, 2001, Vincent 2002, Murcia et al. 2006). However, at least two strains of rainbow trout exhibits resistance (Wagner et al. 2006, Miller and Vincent 2008), which suggests that susceptibility may also vary within species.

Salmonids may be infected by *M. cerebralis* at any size or age (Markiw 1992, MacConnell and Vincent 2002), but small, young fish are more susceptible than older, larger fish. Lower cartilage-to-bone ratio and a more developed epidermal barrier in the older fish (Halliday 1973, El-Matbouli et al. 1992, Markiw 1992) have been hypothesized to explain why larger fish appear to be more resistant to infection, but least one study showed the degree of skeletal ossification in young fish did not affect resistance to *M. cerebralis* (Ryce et al. 2005). Instead, a combination of size and age appear to confer resistance to *M. cerebralis*. For example, when multiple sizes of the same aged or same sizes of different aged rainbow trout were exposed to *M. cerebralis*, only fish that were both greater than 40 mm (fork

length) and older than nine weeks post-hatch exhibited increased resistance (Ryce et al. 2005).

In addition, infection and disease severity are proportional to parasite exposure, or dose (Markiw 1992, Ryce et al. 2004). For example, 8-week-old rainbow trout fry were severely infected and exhibited clinical signs when exposed to high doses (100,000 TAMs/fish), but fry exposed to low doses (<10 TAMs/fish) were not infected and did not exhibit clinical signs (Markiw 1992, Ryce et al. 2004).

The Oligochaete Host

Tubifex tubifex is a cosmopolitan species that inhabits environments characterized by abundant organic material, fine sediments (< 2mm), and low velocities (Brinkhurst and Gelder 1991, Brinkhurst 1996). Densities of *T. tubifex* tend to be low in most freshwater habitats (Milbrink 1983) but dense assemblages are common in extreme habitats (i.e., habitats that are considered marginal for other freshwater invertebrates, Bonacina et al. 1996), because this species is tolerant of extreme temperatures, desiccation, and variable oxygen regimes (e.g., Reynoldson 1987, Kaster 1980, Anlauf 1990, Brinkhurst 1996). Functionally, *T. tubifex* plays a role in nutrient cycling and organic material breakdown (Vanderbund et al. 1994, Matisoff et al. 1999, Egeler et al. 2001, Mermillod-Blondin et al. 2001, Ciutat et al. 2006).

Myxobolus cerebralis Infections in the Oligochaete Host

Tubifex tubifex is the only host known to become infected by *M. cerebralis* and produce the TAM spores that are infective to salmonids (Markiw and Wolf 1983, Wolf and Markiw 1984, El-Matbouli and Hoffman 1998, Kerans et al. 2004). The factors that affect infection in the oligochaete host are similar to those that affect infection in the fish host, but mechanisms are not as well understood. In this host, infection varies among genetically variable lineages (sometimes termed cryptic species or subspecies, Sturmbauer et al. 1999, Beauchamp et al. 2001), strains within the lineages, and by parasite dose. Infection may also vary as a function of invertebrate community composition or specific environmental conditions because these factors strongly influence the ecology of the oligochaete host.

Host Factors

Genetic variation in *T. tubifex* has been correlated with susceptibility to *M. cerebralis*. Five genetic lineages (16SrDNA lineages I, III-V, and VI) have been reported from North America (Beauchamp et al. 2001, 2002, Arsan et al. 2007) and five have been reported from Europe (I-V) (Sturmbauer et al. 1999, Crottini et al. 2008). Lineages V and VI are considered resistant (Beauchamp et al. 2006, Elwell et al. 2006). Lineage I is also considered resistant (e.g., Arsan et al. 2007), but few strains have been tested experimentally, and at least one strain belonging to this lineage produced TAMs when experimentally infected (Kerans et al. 2005). Lineage III is considered susceptible to *M. cerebralis*. However, TAM production and parasite amplification by strains of lineage III *T. tubifex* appear to be highly variable. Some strains belonging to lineage III have been shown to amplify *M. cerebralis* (e.g., many more TAMs produced than myxospores ingested; Stevens et al. 2001, Kerans et

al. 2004, Rasmussen et al. 2008), and at least one strain did not produce TAMs when experimentally infected (Baxa et al. 2008).

Community Factors

The invertebrate assemblage may influence infection in the oligochaete host because interactions within and among susceptible *T. tubifex* and resistant strains of *T. tubifex*, and other (non-compatible) oligochaetes or functionally similar invertebrates (Reno 1998, Kerans and Zale 2002) may influence infection prevalence or the outcomes of *M. cerebralis* infections in the oligochaete host. Competition for myxospores may influence *M. cerebralis* infection prevalence in, and TAM production by, susceptible *T. tubifex* (El-Matbouli et al. 1992). For example, total myxospores available to susceptible *T. tubifex* may decrease if resistant *T. tubifex* or other oligochaetes consume and deactivate myxospores. Even if myxospores are not deactivated when consumed by resistant *T. tubifex* and other oligochaetes, the encounter rate between myxospores and susceptible *T. tubifex* may be reduced if the invertebrate community is comprised of low proportions of susceptible *T. tubifex*. Beauchamp et al. (2006) reported that mixed cultures of *T. tubifex*, including lineage III (susceptible) and VI (resistant) strains produced fewer TAMs than monocultures of *T. tubifex*. In contrast, Elwell et al. (2006) did not detect a difference in infection prevalence in susceptible *T. tubifex* or in TAM production when mixed cultures, including lineage III (susceptible) and V (resistant) strains were exposed to *M. cerebralis*. This discrepancy may be explained by differences in experimental designs. Density was not held constant among exposure groups in Beauchamp et al. (2006)'s experiment, whereas densities were controlled in Elwell et al. (2006)'s experiment, and Elwell et al. (2006) observed that infection

prevalence in susceptible strains was negatively correlated with increased densities of susceptible *T. tubifex*. Thus, it is still unclear how interactions among susceptible and resistant strains of *T. tubifex* may influence *M. cerebralis*.

In addition, the host community may influence the outcomes of *M. cerebralis* infections in *T. tubifex* by influencing survival or success of infected *T. tubifex*. For example, respiration, growth, and reproduction of *T. tubifex* were positively influenced by other oligochaetes in mixed cultures (Brinkhurst 1972, Brinkhurst 1974). These types of interactions could positively affect parasite success in natural stream environments. However, the assessment of oligochaete host factors is complicated, especially in the context of the oligochaete community, by difficulty with identification because *T. tubifex* typically coexist with morphologically similar oligochaetes. Morphological identification of *T. tubifex* requires mature specimens (Kathman and Brinkhurst 1996), which typically comprise <10% of populations (e.g., Krueger et al. 2006, McGinnis 2007). Lineages are morphologically indistinguishable and must be resolved using molecular assays (Sturmbauer et al. 1999, Beauchamp et al. 2001).

Environmental Factors

Environmental conditions may influence oligochaete host distribution and ecology or community diversity (Kerans and Zale 2002), and therefore may influence interactions between *T. tubifex* and *M. cerebralis*. Variation in WD risk in other systems has been related to environmental conditions (see Reno 1998, Kerans and Zale 2002, Bartholomew et al. 2005 for review). For example, stream temperature, which influences many aspects of host and parasite ecology, has been positively correlated with WD risk in many field studies (e.g.,

Baldwin et al. 2000, Hiner and Moffitt 2001, Downing et al. 2002). In the laboratory, elevated water temperatures decreased *M. cerebralis* incubation periods, caused earlier TAM release, and increased overall *M. cerebralis* proliferation (TAM production) in *T. tubifex* (El-Matbouli et al. 1999, Blazer et al. 2003, Kerans et al. 2005). Furthermore, *M. cerebralis* proliferation and disease severity (development of clinical signs of disease and parasite replication) in fish hosts were correlated with increased temperatures (e. g., Halliday 1976, Markiw 1992, Schisler et al. 2000).

Other environmental characteristics including conductivity, pH or salinity may affect TAM or myxospore viability (Smith et al. 2002, Wagner et al. 2002, Hedrick et al. 2008). For example, Sandell et al. (2001) found WD risk was correlated with increased conductivity in the Lostine River in Oregon, and they suggested that increased conductivity increased TAM transmission success. In addition, physical habitat features which determine stream geomorphology (e.g., underlying geology or slope) may influence WD risk because substrate influences the distribution and abundance of *T. tubifex* (Brinkhurst and Jamieson 1971, Lazim and Learner 1987) and also likely influences rates of myxospore encounter and TAM release by *T. tubifex* (Blazer et al. 2003).

In addition, the distribution and abundance of mitochondrial lineages of *T. tubifex* may be related to environmental features. For example, riffle reaches were dominated by lineages VI, whereas pool habitats were dominated by *T. tubifex* from lineages I, III and IV in the San Juan River (DuBey and Caldwell 2004). Moreover, pool habitats in the San Juan River were characterized by the highest densities of infected *T. tubifex* (DuBey and Caldwell 2004), which may suggest differences in relative abundance of lineages influence *M.*

cerebralis success. In other systems, locations characterized by high whirling disease risk were dominated by lineage III *T. tubifex* (Beauchamp et al. 2002). Since the availability of suitable hosts is important for parasite transmission, whirling disease risk may be influenced by environmental features that influence the distribution and abundance of *T. tubifex*.

Whirling Disease and Yellowstone Cutthroat Trout

Native cutthroat trout have declined significantly (up to 99% of the original populations have been lost) throughout their native range (Behnke 1979, 1992, Kaeding and Boltz 2001, Ward and Ward 2004). The Yellowstone cutthroat trout population native to Yellowstone Lake in Yellowstone National Park, is considered one of the largest remaining genetically pure stocks of native interior trout (Behnke 1992), and their conservation is a high priority for Yellowstone National Park resource managers (Gresswell and Varley 1995, Koel et al. 2005, 2006).

Myxobolus cerebralis was detected in adult Yellowstone cutthroat trout in Yellowstone Lake in 1998 (Koel et al. 2006). The detection of *M. cerebralis* followed the detection of non-native lake trout (Kaeding et al. 1996). Between them, these non-natives may potentially eradicate the native Yellowstone cutthroat trout from the lake. Young Yellowstone cutthroat trout may be exposed to *M. cerebralis* while they inhabit the tributaries, and life stages that make it to the lake may be exposed to predation by, and competition with, lake trout.

Myxobolus cerebralis was detected in three tributaries to Yellowstone Lake that were historically characterized by large spawning populations of Yellowstone cutthroat trout

(Clear Creek, Pelican Creek, and the Yellowstone River downstream of Yellowstone Lake; Gresswell et al. 1994, 1997, Koel et al. 2006) using sentinel fish (caged hatchery-reared Yellowstone cutthroat trout placed *in situ* and subsequently examined by molecular and histological analyses for *M. cerebralis* infection and severity, e.g., Krueger et al. 2006, Murcia et al. 2006). Risk of disease to wild fish (assessed by scoring parasite damage to sentinel fish; Baldwin et al. 2000), was found to be high in Pelican Creek, intermediate in the Yellowstone River, and low in Clear Creek. This result suggested that *M. cerebralis* may reduce survival of young-of-the-year trout in these tributaries and may partially explain the declining population in Yellowstone Lake. The effects of the introduction of *M. cerebralis* are still unclear, but surveys suggest that the spawning population has declined significantly in at least one infected tributary (Pelican Creek; Koel et al. 2006). If not understood and managed, the combination of lake trout and *M. cerebralis* could cause further declines of Yellowstone cutthroat trout in Yellowstone Lake, and affect overall ecosystem function.

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CHAPTER THREE

CONTEXT SPECIFIC PARASITISM IN *TUBIFEX TUBIFEX* IN GEOTHERMALLY INFLUENCED STREAM REACHES IN YELLOWSTONE NATIONAL PARK

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Abstract

Parasites may regulate host abundance and influence the composition and structure of communities. However, host-parasite interactions may be context specific because environmental conditions may alter the outcome of parasitism and disease. As anthropogenic changes alter the environment, an understanding of how host-parasite interactions may change under different contexts will be useful for predicting and managing disease. We examined host-parasite ecology under different environmental contexts by describing the ecology of *Myxobolus cerebralis*, the parasite that causes whirling disease in salmonids, and its obligate host, *Tubifex tubifex* in geothermally variable stream reaches in Yellowstone National Park. We identified reaches in 4 categories of geothermal influence, which were characterized by variable substrates, temperatures, specific conductivities, and pH. We measured aspects of host ecology, including abundance, relative abundance, size, and genotype of *T. tubifex*, aspects of parasite ecology, including infection prevalence in *T. tubifex* and abundance of *M. cerebralis* infected *T. tubifex*, and whirling disease risk to fish in each reach. *Tubifex tubifex* abundance was high all in reaches characterized by geothermal influence (3 reach types), whereas abundance of *M. cerebralis* infected *T. tubifex* was high only in reaches characterized by intermediate geothermal influence (2 reach types). We suggest there may be a contextual effect of habitat on parasitism within the oligochaete host because abundance of infected hosts appears to be limited by abundance of hosts in all reach types except those characterized by high geothermal influence, where abundance of infected hosts appears to be limited by environmental conditions. In contrast to patterns of parasitism in the oligochaete host, whirling disease risk to fish was high in all reach types, which

indicated that reduced abundances of infected *T. tubifex* did not reduce whirling disease risk. This result may be important from a management perspective because it suggests whirling disease risk to fish may be high even in the absence of high abundances of infected *T. tubifex*.

Key words: *Tubifex tubifex*, *Myxobolus cerebralis*, aquatic oligochaete ecology, host-parasite relationships, salmonid whirling disease, Yellowstone National Park, context-specific parasitism.

Introduction

Parasites regulate aspects of host ecology including abundance, composition and structure of communities, and influence overall ecosystem function (Minchella and Scott 1991, Soler et al. 2001, Mouritsen and Poulin 2002, Semple et al. 2002, Thomas et al. 2005). However, interactions between hosts and parasites may be context specific because environmental conditions may alter outcomes of host-parasite interactions and disease (Walther et al. 2000). Environmental context may be particularly significant for outcomes of host-parasite interactions in freshwater systems because environmental conditions influence almost all aspects of host and parasite ecology (Patz et al. 2000, Marcogliese 2001). For example, as water temperatures increased, growth and development of microphallid trematode parasites increased in their gastropod hosts, which in turn, increased parasite transmission and success (Mouritsen and Jensen 1997). In contrast, increased temperatures decreased transmission and success of the common eye fluke, *Diplostomum scheuringi*, because the compounding effects of thermal stress and parasitic infection increased host mortality (Aho et al. 1982). As anthropogenic changes continue to disturb and alter the environment, an understanding of how host-parasite interactions may change under different environmental contexts will be useful for predicting and managing disease (Dobson and Hudson 1986, Coltman et al. 1999).

We examined host-parasite ecology under different environmental contexts using whirling disease (WD) as a model. Whirling disease is caused by the myxozoan parasite, *Myxobolus cerebralis* (Hofer 1903 in Bartholomew and Reno 2002) and is a significant emerging disease of salmonids in North America (Bartholomew and Reno 2002). *Myxobolus*

cerebralis has a complex life cycle involving a salmonid, an oligochaete host and two environmentally transmitted spore stages, the myxospore and the triactinomyxon (TAM) (Wolf and Markiw 1984, Andree et al. 1997). The oligochaete, *Tubifex tubifex*, produces TAMs that are infective to salmonids, and salmonids produce myxospores that are infective to *T. tubifex* (Wolf & Markiw 1984). Whirling disease is a good model to examine the potential for context specificity in host-parasite interactions because patterns of parasitism and disease risk vary across large (among drainages; McGinnis 2007, Anlauf and Moffitt 2008) and small (within drainages; Zendt and Bergersen 2000, Hiner and Moffitt 2001, Downing et al. 2002, DuBey and Caldwell 2004, Krueger et al. 2006) spatial scales.

Variation in patterns of parasitism and disease has been related to environmental conditions (see Hedrick 1998, Reno 1998, Kerans and Zale 2002, Bartholomew et al. 2005 for review). For example, stream temperature, which influences many aspects of host and parasite ecology, has been positively correlated with infection prevalence and severity in field exposed trout (e.g., Baldwin et al. 2000, Hiner and Moffitt 2001, Downing et al. 2002). In the laboratory, elevated water temperatures decreased *M. cerebralis* incubation periods, caused earlier TAM release, and increased *M. cerebralis* proliferation (TAM production) in *T. tubifex* (El-Matbouli et al. 1999, Blazer et al. 2003, Kerans et al. 2005).

Other environmental characteristics including conductivity, pH or salinity may affect TAM or myxospore viability (Smith et al. 2002, Wagner et al. 2002, Hedrick et al. 2008). For example, Sandell et al. (2001) found WD risk was correlated with increased conductivity in the Lostine River, and they suggested that increased conductivity increased TAM transmission success. In addition, physical habitat features related to geomorphology (e.g.,

underlying geology or slope) may influence WD risk because substrate influences the distribution and abundance of *T. tubifex* (Brinkhurst and Jamieson 1971, Lazim and Learner 1987) and also likely influences rates of myxospore encounter and TAM release by *T. tubifex* (Blazer et al. 2003).

Aspects of *T. tubifex* ecology may also influence patterns of parasitism and disease. High variability in abundances of *T. tubifex* and other oligochaetes (e.g., Brinkhurst and Jamieson 1971) may influence abundance of infected *T. tubifex* (e.g., Zendt and Bergersen 2000) because myxospores are more likely to be encountered and ingested when the obligate hosts are abundant. In addition, the relative abundance of *T. tubifex* may influence parasite success because functionally similar, but incompatible hosts may be more likely to consume myxospores, making them unavailable to *T. tubifex* when *T. tubifex* comprise a low proportion of the assemblage (Kerans and Zale 2002, Beauchamp et al. 2006).

Another aspect of *T. tubifex* ecology, host size, may influence the outcome of *M. cerebralis* infections and thereby influence WD risk. In other host-parasite interactions, host size has been positively correlated with increased availability of resources for parasite proliferation or immune defense (e.g., Altaif et al. 1989, Sousa and Grosholz 1991, Agnew and Koella 1999, Navarro et al. 2003, Krist et al. 2004, Ortego and Espada 2007). Although the relationship has not been established for *T. tubifex*, size may reflect condition or stage (e.g., Bonancina et al. 1996, Pasteris et al. 1996), or optimality of environmental conditions (Pasteris et al. 1994). Size of *T. tubifex* may influence *M. cerebralis* success because large sized *T. tubifex* presumably have more resources available for parasite proliferation (e.g.

space and energy stores) and therefore large sized *T. tubifex* may produce more TAMs than small *T. tubifex*.

Genetic variation in *T. tubifex* has also been related to patterns of parasitism and disease risk. Genetic variants of *T. tubifex* have different environmental optima (e.g. Anlauf and Neumann 1997, DuBey and Caldwell 2004, Kerans et al. 2005), coexist (e.g. Beauchamp et al. 2005, DuBey 2008), and may compete for resources and myxospores. In addition, susceptibility to *M. cerebralis* appears to be correlated with mitochondrial lineages of *T. tubifex* (16SrDNA, lineages I-VI found within North America, Beauchamp et al. 2001, Arsan et al. 2007). Lineages V and VI are considered resistant because TAMs were not produced when *T. tubifex* were experimentally infected (Beauchamp et al. 2006, Elwell et al. 2006). Lineage I is also considered resistant (e.g. Arsan et al. 2007), however, experimentally infected strains within this lineage produced TAMs but did not amplify *M. cerebralis* (Kerans et al. 2005). Lineage III is considered susceptible to *M. cerebralis*, however, TAM production and parasite amplification varies among strains within this lineage (e.g., Stevens et al. 2001, Baxa et al. 2008, Rasmussen et al. 2008).

To explore the hypothesis that host-parasite relationships are context specific, we assessed host (*T. tubifex*) and parasite (*M. cerebralis*) ecology in Pelican Creek, a Yellowstone cutthroat trout spawning tributary to Yellowstone Lake in Yellowstone National Park. We selected this tributary because it is characterized by abundant geothermal features and *M. cerebralis* was known to be present (Koel et al. 2006, Murcia et al. 2006). The specific objectives of the study were to characterize 1) environmental features, 2) host ecology (abundance, size, and genetic lineage of *T. tubifex*), 3) parasite ecology (*M.*

cerebralis infection prevalence and abundance of infected *T. tubifex*), and 4) WD risk to fish in geothermally variable stream reaches.

Methods

Study Site and Environmental Features

The field component of this study was conducted in summer months (July-September) in the Pelican Creek catchment in Yellowstone National Park, WY in 2004 (Figure 3.1). Using topographic maps, we selected 25 100 m sampling reaches based on accessibility and the presence or absence of geothermal features. The locations of specific geothermals were recorded with a handheld geographic positioning system (GPS) and we calculated distances between the upstream limits of study reaches and the nearest geothermal (Table 3.1). These distances were used to classify reaches into geothermal categories as follows: Geothermals located <5 km upstream (6 reaches), high geothermal influence; geothermals located between 5-10 km upstream (7 reaches), moderate geothermal influence; geothermal areas ≥ 10 km upstream (7 reaches), low geothermal influence; and no geothermals located upstream (5 reaches), no geothermal influence (Table 3.1, Figure 3.2).

We measured environmental variables that were previously shown to be important for parasite and oligochaete host ecology (e.g., Krueger et al. 2006). Environmental variables included channel characteristics (width, depth, water velocity, elevation and slope), sediment characteristics (substrate composition, proportion of organic material in the sediment), and physicochemical characteristics (temperature, dissolved oxygen, specific conductivity, and pH).

Channel characteristics were measured on 3 randomly selected transects in each reach. Channel width was measured once per transect. Water depth and current velocity were quantified at 3 equidistant points along each transect (Model 3000 Swoffer Instrument, Swoffer Instruments, WA, USA). Elevation was measured at the upper and lower limits of each study reach with a GPS. Reach slope was calculated as change in elevation over the 100 m reach.

Substrate composition was assessed using 2 methods. The first method apportioned substrate into fine (<2 mm) and coarse (>2 mm) particles, with a grid (a 320 x 320 mm plexi-glass square consisting of 2 mm wide intersecting grid lines drawn 40 mm apart, for a total of 49 intersecting points; Overton et al. 1997, Krueger et al. 2006) that was randomly positioned over the substrate once per transect. Substrate particles were characterized at each grid intersection. Particles visible around all 4 sides of grid intersections (>2 mm across at the sampling point) were classified as coarse and particles that were not visible around all 4 sides of the grid intersection were classified as fine. Proportions of fine and coarse sediments were calculated by averaging individual measurements for each reach. The second method determined the composition of fine (<2 mm) sediments by the hydrometer gravimetric method (Day 1965). Sediment samples (0.25-0.5 L) were collected from a haphazardly selected position on each transect with a modified core sampler, combined and frozen. During processing, samples were split (half was used to determine organic material, as described below), dried at 105 °C, sieved to remove coarse sediments (>2 mm), and proportions of sand (0.64-2 mm), silt (0.03-0.63 mm), and clay (<0.002 mm) were

determined (Day 1965). To obtain an estimate of sediment fractions at each reach, proportions were applied to counts of fines from the surface fines grid.

Proportion of organic material was determined as ash free dry mass (AFDM; Hauer and Lamberti 1996) in 2 replicate subsamples (15-30 g) in each reach using sediment retained during fine sediment processing. Proportion of organic material was calculated as $[(\text{original dry subsample weight (after 2 hr at } 105^\circ\text{C)}) - (\text{burned subsample weight (after 2 hr at } 550^\circ\text{C)})] / [\text{original dry subsample weight}]$.

Temperature ($^\circ\text{C}$), dissolved oxygen concentration (mg/L), specific conductivity (mS/cm), and pH were quantified with a Yellow Springs Instrument (Yellow Springs Instruments, Ohio, USA) once per reach.

Host Ecology

We examined aspects of *T. tubifex* ecology that were previously shown to be important for WD risk, including abundance, relative abundance (Zendt and Bergersen et al. 2000, Krueger et al. 2006), and mitochondrial (16SrDNA) lineage (e.g., Beauchamp et al. 2002, 2005). We also examined whether size of *T. tubifex* differed among reach types, maturity stages (immature, mature), or whether animals were infected with *M. cerebralis*. We expected *T. tubifex* would be larger where they were abundant because environmental conditions are likely more optimal there. We expected size differences between immature and mature individuals because mature individuals must allocate resources to functions other than growth (e.g., cocoon production). We also expected size differences between infected and uninfected individuals because of the costs of parasite proliferation or immune response (e.g., Courtney and Christensen 1991).

Tubificids were collected by kick samples (200µm mesh, Wildco, USA, 2 minute kicks) and were sorted in the field (3 persons sorting for 1 h was equivalent to 3 h of sorting time). Kick samples were repeated until 300 oligochaetes were collected or 3 h of sorting time were reached. Tubificids removed from kick samples were combined for each reach.

Up to 200 tubificids with morphology similar to *T. tubifex* per reach, confirmed under a dissecting microscope (5-50x), were preserved in Kahle's solution (18:1:1 volumes 70% ethanol: formalin: glacial acetic acid), slide mounted (CMCP-10, Polysciences, CA, USA), and identified (Kathman and Brinkhurst 1998). Immature tubificids were assigned to species based on the relative abundance of sexually mature tubificid species found in each reach (Krueger et al. 2006) because only mature species can be morphologically identified. If mature *T. tubifex* were not collected from a reach, immature tubificids were assigned to species based on DNA characteristics of a subsample (Sturmbauer et al. 1999, Beauchamp et al. 2001, see below). Abundance of *T. tubifex* was calculated as catch per unit effort (CPUE), [number of *T. tubifex* (after all immatures were assigned to species)]/(time spent sorting)/(total number of kicks) per reach. Relative abundance of *T. tubifex* was calculated as (number of *T. tubifex*)/(total tubificids collected per reach).

To assess relationships among *T. tubifex* size and reach types, maturity, and *M. cerebralis* infection, segment widths of 5 randomly selected (n=115) *T. tubifex* were measured from each reach. We used this approach because mature specimens of a similar species, *Rhyacodrilus hiemalis*, had greater segment widths than immature individuals (Ohtaka 1995), which suggested mature individuals had assimilated more resources than immature individuals. Thus, we inferred larger segment widths in *T. tubifex* would be

correlated with good host condition. In addition, width of segment five was positively correlated with individual biomass in *T. tubifex* from our laboratory cultures ($r^2=0.719$, $p<0.0001$, $n=40$, lineage III *T. tubifex* from reach 15 on Pelican Creek, Figure 3.1, Appendix A). Segment width was measured as distance from outer edges of the body wall across the point of hair chaetae insertion on segment five. Segment five was selected because of its location forward of the area covered by the clitellum in mature specimens and behind the narrow prostomium.

We also measured segment width on all sexually mature ($n=19$), and all *M. cerebralis* infected ($n=38$) *T. tubifex* to determine whether sexually mature and *M. cerebralis* infected *T. tubifex* differed in size from randomly selected *T. tubifex* among reaches where they were found. For sexually mature *T. tubifex* this included 2 reaches with high geothermal influence, 3 reaches with moderate geothermal influence, 4 reaches with low geothermal influence and 2 reaches with no geothermal influence. For *M. cerebralis* infected *T. tubifex* this included 1 reach with high geothermal influence, 6 reaches with moderate geothermal influence, 5 reaches with low geothermal influence, and 1 reach with no geothermal influence. Relative segment widths of sexually mature *T. tubifex* were calculated as (mean segment width of randomly selected *T. tubifex*)-(mean segment width of sexually mature *T. tubifex* or *M. cerebralis* infected *T. tubifex*).

To determine *T. tubifex* mitochondrial lineage, genomic DNA was extracted from the posterior segments of tubificids used for morphological identification in single and pooled samples. Single samples, 40 per reach, contained DNA from one tubificid and pooled samples, 10 per reach, contained DNA from 16 tubificids. This combination allowed us to

extract DNA from up to 200 individuals using only 50 samples, which was less expensive than 200 individual samples. The aim of preparing single samples was to be able to determine proportions of lineages, and the aim of preparing pooled samples was to maximize our ability to detect genetic variation in *T. tubifex* in each reach.

Genomic DNA was extracted as per Nucleospin tissue extraction kit protocol (Clontech Inc., CA, USA). The samples were characterized at the 16SrDNA locus (Beauchamp et al. 2001) as in Rasmussen et al. (2008) and tested using a combination of pooled and single DNA samples. In the pooled approach, DNA from up to 200 tubificids from each reach in pools of up to 32 individuals were assayed with primers for lineages I, III, V and VI, (lineages known to exist in the lower 48 states; e.g., Beauchamp et al. 2001, Arsan et al. 2007) to determine presence or absence of each lineage in each reach. In the single approach, up to 20 individual tubificids from each reach were assayed using primers for each mitochondrial lineage to determine the proportion of each lineage in that reach. The PCRs were performed on a PTC-100 thermocycler (MJ Research, Inc., MA, USA) and the products were visualized by gel electrophoresis on 2.5% agarose gels in $0.5 \times$ TAE buffer alongside standards. Samples were assigned a score of 0 or 1, to indicate absence or presence of each lineage. Tubificids that did not test positive for any *T. tubifex* lineages were considered 'other' tubificids. Proportion of each lineage was calculated as (number individuals testing positive for lineage)/(number of individuals tested) in each reach. If a lineage was detected only in pooled samples, proportion of that lineage was calculated as (number positive pooled samples)/(number of individuals in pooled samples) in that reach.

Parasite Ecology

We examined aspects of parasite ecology that were previously shown to be important for WD risk, including prevalence of infection in *T. tubifex* and abundance of *M. cerebralis* infected *T. tubifex* (Krueger et al. 2006). Infection prevalence is important because it reflects the probability that an individual *T. tubifex* is infected. However, the abundance of *M. cerebralis* infected *T. tubifex*, which reflects the number of *T. tubifex* potentially releasing TAMs, is likely a better measure of parasite success and was correlated to WD disease risk in previous studies (Krueger et al. 2006).

To determine *M. cerebralis* infection prevalence, we tested single and pooled DNA samples used for *T. tubifex* lineage assays (as described above). Infection in *T. tubifex* was determined by the nested PCR test for *M. cerebralis* (Andree et al. 1998). The PCR products were visualized by gel electrophoresis on 1.5% agarose gels in 0.5 × TAE buffer alongside positive and negative controls.

If *M. cerebralis* was detected in individually prepared samples, infection prevalence was calculated as (number infected)/(number individuals assayed). If *M. cerebralis* was detected in pooled samples, but was not detected in individually prepared samples from that reach, infection prevalence was calculated as (number infected pools)/(total number individuals in pooled samples), assuming one infected individual per *M. cerebralis* positive pool. Abundance of *M. cerebralis* infected *T. tubifex* was calculated as (*M. cerebralis* infection prevalence in *T. tubifex*) × [abundance of *T. tubifex* (CPUE, see above)] at each reach.

Whirling Disease Risk to Fish

Whirling disease risk to fish was assessed by determining *M. cerebralis* infection prevalence and severity in sentinel fish (caged hatchery reared fish placed in situ and then subsequently examined by molecular and histological analyses for *M. cerebralis* infection and severity, e.g., Krueger et al. 2006, Murcia et al. 2006) in 6 reaches during 2 10-day periods in 2004 (Figure 3.1). We selected a subset of reaches for sentinel fish cage deployment based on accessibility because it was difficult to transport fish to many reaches. Sentinel fish were obtained and transported to exposure reaches as per Murcia et al. (2006). Sentinel cages contained 60 Yellowstone cutthroat trout fry, six weeks post hatch, fork length < 2.5 in. Following the 10 day exposure period, fish were removed from their cages and transported to the Aquatic Sciences Laboratory (Montana State University-Bozeman, MT, USA) where fish from each cage were held in separate aquaria for 90 days to allow for parasite development prior to testing for *M. cerebralis* infection and severity.

Myxobolus cerebralis infection in sentinel fish was determined by nested PCR (Andree et al. 1998). Genomic DNA was extracted (Nucleospin tissue kits, Clontech Inc., CA, USA) from cranial tissue from 10 randomly selected fish per cage. Cranial tissue was obtained by bisecting fish along the sagittal line and removing a biopsy from one half of the head. The remaining half head was preserved for use in infection severity assessment, as described below. Tissue biopsies from five fish were pooled prior to DNA extraction.

Infection severity in sentinel fish was determined by histological assessment on 10 half fish heads per cage. Infection severity was scored on the MacConnell-Baldwin scale, (a score of 0 indicated no infection and a score of 5 indicated severe infection, Baldwin et al.

2000). Infection prevalence was calculated as (number of fish per pool characterized by a histology score >0)/(number of fish in pool). Infection severity was calculated as mean histological score in each reach.

Analyses

To determine if environmental features varied among reaches in geothermal categories, we tested for differences using multivariate analysis of variance (MANOVA). If the MANOVA was significant (Wilks' $\lambda < 0.05$), we used individual ANOVAs and Tukey's Honestly Significant Difference (HSD) tests to interpret results ($p < 0.1$ to maximize our ability to examine patterns).

We used the same methods (MANOVA, ANOVA, and Tukey's HSD tests) to determine if aspects of *T. tubifex* ecology including abundance, relative abundance, and size of randomly selected *T. tubifex* varied among geothermal categories. We did not include proportion of lineage III *T. tubifex* as a response variable in the MANOVA because almost all *T. tubifex* we found were lineage III. To determine if mature and *M. cerebralis* infected *T. tubifex* differed in size from randomly selected *T. tubifex*, we tested whether relative differences differed from 0 in each geothermal category with studentized t-tests.

We tested for differences in *M. cerebralis* infection prevalence and abundance of *M. cerebralis* infected *T. tubifex* using MANOVA, ANOVAs and Tukey's HSD tests to determine if aspects of parasite ecology, varied among geothermal categories.

To determine if WD risk to fish varied between reaches with high and moderate geothermal influence, we tested for differences in infection severity using studentized t-tests (replicate cages were in high and moderate geothermal categories). We did not test for

differences in infection prevalence in sentinel fish among geothermal categories because infection prevalence was $100\% \pm 0.00\%$ in all reaches except reach 3.

Results

Environmental Features

Environmental features varied among geothermal categories (Wilks' $\lambda=0.005$, $F_{42,24.5}=2.90$, $p=0.0032$, Tables 3.2 and 3.3, Figure 3.3, refer to Appendix B for reach data). The environments of reaches with intermediate geothermal influence were characterized by similar features, but reaches with no and high geothermal influence differed from other reach types. Reaches with no geothermal influence had higher proportions of coarse sediments and lower proportions of sand and clay sediments than reaches with high geothermal influence. Reaches with no geothermal influence also had higher proportions of coarse sediments and lower proportions of sand than reaches with low geothermal influence. In addition, reaches with no geothermal influence had lower specific conductivities than other reach types and higher pH than reaches with high geothermal influence. Reaches with high geothermal influence were characterized by higher specific conductivities than other reach types. Reaches with high geothermal influence also had higher stream temperatures than reaches with moderate geothermal influence and lower pH than reaches with no geothermal influence.

Host Ecology

Immature tubificids identified as *T. tubifex* were collected from 22 reaches (tubificids were not found in reaches 21 or 25 and oligochaetes collected in reach 14 were naids),

Figure 3.1). Sexually mature *T. tubifex* were collected from 14 reaches (reaches 1-5, 7-8, 15, 17-19, 20, 22, 24, Figure 3.1).

Tubifex tubifex ecology varied among reach types (Wilks' $\lambda = 0.157$, $F_{9,41.5} = 5.27$, $p < 0.001$, Table 3.4). Abundances of *T. tubifex* were lower in reaches with no geothermal influence than in reaches with high, moderate or low geothermal influence (Table 3.4, Figure 3.4a). We did not detect differences in relative abundances of *T. tubifex* among reach types (Table 3.4, Figure 3.4b).

Randomly selected *T. tubifex* (all immature) were larger in reaches with no geothermal influence than in reaches with high geothermal influence (Table 3.4, Figure 3.4c). Mature *T. tubifex* were measured from reaches 1 (n=1), 2 (n=2), 3 (n=3), 4 (n=1), 15 (n=3), 17 (n=1), 18 (n=1), 19 (n=1), 20 (n=1), 22 (n=1), and 24 (n=4). Infected *T. tubifex* were measured from reaches 1 (n=3), 2 (n=1), 4 (n=1), 5 (n=7), 8 (n=4), 9 (n=2), 10 (n=1), 15 (n=15), 16 (n=1), 17 (n=3), 18 (n=3). Infected *T. tubifex* were also collected in reaches 12 (n=2) and 24 (n=1), but could not be measured because they were part of pooled samples. We did not detect differences in relative segment widths of *M. cerebralis* infected *T. tubifex* or mature *T. tubifex* in any geothermal category (Table 3.5, all t-stats < 5.25, $p > 0.12$).

We detected only lineage III *T. tubifex* in individual and pooled samples from all reaches where tubificids were collected except reaches 14, 15, and 18. Tubificids from reach 14 did not test positive for any *T. tubifex* lineage and were likely not *T. tubifex*. We detected *T. tubifex* belonging to lineage VI in two pooled samples from reach 15 (proportion lineage VI: 0.0064) and reach 18 (proportion lineage VI: 0.0357). All sexually mature and *M. cerebralis* infected *T. tubifex* were lineage III.

Parasite Ecology

Myxobolus cerebralis ecology varied among reach types (Wilks' $\lambda=0.441$, $F_{6,36}=3.03$, $p=0.007$, Table 3.6). *Myxobolus cerebralis* infected *T. tubifex* were detected at all reaches where tubificids were collected except reaches 14, 20, 22, and 23. Infection prevalence was higher in *T. tubifex* from reaches with low and moderate geothermal influence than reaches with no geothermal influence (Figure 3.5a). Abundance of infected *T. tubifex* was higher in reaches with moderate and low geothermal influence than reaches with high or no geothermal influence (Figure 3.5b). *Myxobolus cerebralis* ecology varied among reach types (Wilks' $\lambda=0.441$, $F_{6,36}=3.03$, $p=0.007$, Table 3.6). *Myxobolus cerebralis* infected *T. tubifex* were detected in all reaches where tubificids were collected except reaches 14, 20, 22, and 23. Infection prevalence was higher in reaches with moderate geothermal influence than reaches with high or no geothermal influence, and higher in reaches with low geothermal influence than reaches with no geothermal influence (Figure 3.5a). Abundances of infected *T. tubifex* were high in reaches with low and moderate geothermal influence, and low in reaches with high and no geothermal influence (Figure 3.5b).

Whirling Disease Risk to Fish

Myxobolus cerebralis infection prevalence in sentinel fish was high in reaches with no (100%, $n=1$ cage), low (100%, $n=1$ cage), moderate ($100\% \pm 0.000$, $n=2$ cages), and high ($90\% \pm 10.000$, $n=2$ cages) geothermal influence. Infection severity in sentinel fish was high in reaches with no (mean histology score: 4.900, $n=1$), low (mean histology score: 4.825, $n=1$), moderate (mean histology score: 4.725 ± 0.225 , $n=2$), and high (mean histology score: 4.125 ± 0.375 , $n=2$) geothermal influence. We did not detect differences in infection severity

in sentinel fish between reaches with high and moderate geothermal influence ($t_2=1.33$, $p=0.315$).

Discussion

Our objectives were to describe environmental features, *T. tubifex* and *M. cerebralis* ecology, and WD risk in reaches with variable geothermal influence. We examined our objectives in the Pelican Creek catchment, a geothermally influenced tributary to Yellowstone Lake in Yellowstone National Park, which was previously shown to have high WD risk (Koel et al. 2006, Murcia et al. 2006). We identified 4 categories of geothermal influence in stream reaches. In general, the environments of reaches with high geothermal influence were characterized by unique physicochemical conditions (e.g. conductivity and pH), reaches with no geothermal influence were characterized by unique substrates, and reaches with intermediate geothermal influence (low and moderate) were characterized by intermediate features. Host (*T. tubifex* abundance) and parasite (prevalence of *M. cerebralis* infection and abundance of *M. cerebralis* infected *T. tubifex*) abundance varied among reach types. Hosts were abundant in reaches characterized by geothermal influence (high, moderate, and low) and low in reaches without geothermal influence. Patterns of parasite abundance (abundance of *M. cerebralis* infected *T. tubifex*) were similar to those of host abundance, except in reaches characterized by high geothermal influence, where they were low relative to host abundance. Interestingly, widths of the 5th segment in *T. tubifex* from non-geothermal reaches were larger than those of *T. tubifex* from reaches with high geothermal influence but we did not detect differences in other aspects of host ecology examined (relative abundance and genetic variation). Despite detecting different patterns of

parasitism in the oligochaete host among reach types, we did not detect differences in WD risk, which was high in all reach types. This result may be important from a management perspective because it suggests that even low abundances of infected *T. tubifex* (e.g., in reaches with high and no geothermal influence) can produce high WD risk to fish.

Environmental conditions may explain why host (abundances of *T. tubifex*) and therefore parasite (abundances of *M. cerebralis* infected *T. tubifex*) characteristics were low in reaches with no geothermal influence. In general, non-geothermal reaches were characterized by unique physicochemical (specific conductivity and pH) and substrate conditions. Non-geothermal reaches had lower specific conductivities than all other reach types. Conductivity typically increases with stream order and productivity (e.g., Sandell et al. 2001) but geothermals clearly influenced conductivity in Pelican Creek. In addition, the pH of non-geothermal reaches was more basic than the pH of reaches with high geothermal influence, which was probably also related to geothermal influence; geothermal discharges are alkaline in this region of Yellowstone (Fournier 1989). Non-geothermal reaches also had higher proportions of coarse sediments and lower proportions of fine sediments than geothermally influenced reaches. The higher proportions of fine sediments in geothermally influenced reaches could be related to geothermals (oxidation activity and mineral precipitation, Nordstrom et al. 2005), or to location. Although we did not detect differences in elevation and reach slope among reach types, non-geothermal reaches were located on lower order tributaries and many geothermally influenced reaches were located on higher order tributaries, which likely influenced substrate composition.

The environments of non-geothermal reaches may be marginal for *T. tubifex* relative to other reach types. High proportions of coarse substrate and low conductivity values in non-geothermal reaches may have limited abundance of *T. tubifex*, which in turn limited abundance of infected *T. tubifex*. Bacterial production is positively correlated with conductivity (Nordstrom et al. 2005), thus non-geothermal reaches, which were characterized by low conductivity, may not support adequate bacterial growth for *T. tubifex*. In addition, the coarse substrates found in these reaches may limit *T. tubifex* abundance (*T. tubifex* feeds on organic matter on fine particles; Brinkhurst and Jamieson 1971, Lazim and Learner 1987, Sauter and Gude 1996, Rodriguez et al. 2001).

Environmental conditions may explain why parasite (infection prevalence and abundances of *M. cerebralis* infected *T. tubifex*) characteristics were low even though hosts were abundant in reaches with high geothermal influence. In general, reaches with high geothermal influence were characterized by high proportions of clay, specific conductivities, and stream temperatures, and low pH. Although clay substrate provides ideal conditions for *T. tubifex* (Brinkhurst and Jamieson 1971, Sauter and Gude 1996, Matisoff et al. 1999), the conditions resulting from the combination of high specific conductivities, water temperatures and low pH are likely not optimal for most freshwater organisms. *Tubifex tubifex* is tolerant of poor environmental conditions and may survive in reaches with high geothermal influence because of a combination of release from pressure (e.g., competition with or predation) with other organisms that are not able to exist in such conditions and tolerance to environmental stress (Sarkka 1996, Khangarot et al. 2003, Rathore and Kangarot 2003). However, the additional stress of parasitic infection by *M. cerebralis* could negatively affect *T. tubifex*

survival in these reaches and may explain why infection prevalence and abundance of infected hosts were low even though hosts were moderately abundant in these reaches.

Low myxospore availability may also explain why parasite (infection prevalence and abundances of *M. cerebralis* infected *T. tubifex*) characteristics were low in reaches with no and high geothermal influence because myxospore dose influences infection prevalence in *T. tubifex* and TAM production (Elwell et al. 2009). Low myxospore availability could be a result of low myxospore abundance (e.g. infected fish don't inhabit these reach types) or low myxospore viability (e.g., myxospores are deposited but do not remain viable).

Other aspects of *T. tubifex* ecology (relative abundance and abundance of susceptible lineages) probably did not influence differences in parasite characteristics, because they did not vary among reach types, with the exception of host size. *Tubifex tubifex* from reaches with high geothermal influence were smaller than individuals from reaches with low geothermal influence. However, the size differences were probably related to differences in stage (e.g., Poddubnaya 1980, Pasteris et al. 1996) rather than differences in environmental optima or parasite characteristics.

Despite having detected differences in abundances of infected *T. tubifex* among reach types, we did not detect differences in patterns of WD risk, which were high in all reach types. We expected TAM production and WD risk would be higher in reaches with high abundances of infected *T. tubifex* than in reaches with low abundances of infected *T. tubifex* because previous work by Kruger et al. (2006) showed that WD risk was correlated with abundance of *M. cerebralis* infected *T. tubifex*.

Why patterns of WD risk did not differ among reach types and were not similar to patterns of parasite characteristics may be explained by the locations of sentinel cages or environmental conditions. Sentinel cages were located in lower portions of the catchment (limited access) and TAMs produced in upstream reaches may have influenced WD risk in downstream reaches (Kerans and Zale 2002, Krueger et al. 2006). On the other hand, TAM production or transmission may differ among reach types. For example, Sandell et al. (2001) hypothesized that conductivity influenced host recognition and TAM attachment activity. If conductivity does influence TAM transmission to fish, high conductivity could offset the potentially lower TAM production in reaches where abundances of infected *T. tubifex* are low, which could explain the high WD risk in reaches with high geothermal influence. Substrate conditions could also influence TAM production and WD risk. *Tubifex tubifex* select small particles when foraging (Sauter and Gude 1996, Rodriguez et al. 2001) and may be more likely to consume myxospores ($< 10 \mu\text{m}$) when coarse particles comprise a high proportion of the substrate (e.g., in non-geothermal reaches). Increased myxospore consumption could result in increased TAM production (e.g., Elwell et al. 2009) and may explain why WD risk was high in reaches with no geothermal influence despite low abundances of infected hosts.

To examine common patterns among host and parasite factors, we considered relative mean host and parasite success in each reach type relative to the mean values across all reach types (Figure 3.6). We suggest abundance of *T. tubifex* is host success, and parasite success may be split into potential parasite success; abundance of infected *T. tubifex* (which may produce TAMs) and realized parasite success; WD risk to fish. Host success ((mean

abundance of *T. tubifex* in each reach type-overall mean abundance)/overall mean abundance) explains potential parasite success ((mean abundance of infected *T. tubifex* in each reach -overall mean abundance)/overall mean abundance) in reaches with moderate and low geothermal influence, where abundance of hosts and parasites were above average, and in reaches with no geothermal influence, where abundance of hosts and parasites were below average. In reaches with high geothermal influence, abundance of hosts was average and abundance of parasites was below average, which suggests host success does not explain parasite success. In contrast, patterns of realized parasite success ((mean WD risk-overall mean WD risk)/overall mean WD risk) did not differ among reach types and were actually opposite patterns of potential parasite success (abundance of *M. cerebralis* infected *T. tubifex*) in reaches with high and no geothermal influence (Figure 3.6). This disconnect may be important from a management perspective because it suggests that even low abundances of infected *T. tubifex* (e.g., in reaches with high and no geothermal influence) effect high WD risk. Thus, previously proposed management options for WD, such as targeted substrate and *T. tubifex* removal (Koel et al. 2006), would probably not be effective for reducing the impact of disease in this system.

We acknowledge the potential importance of differences in myxospore availability or viability among reach types for differences in the abundances of *M. cerebralis* infected *T. tubifex*. However, trout were observed (not sampled) in all reach types, which suggests myxospores are probably deposited and not limiting. Instead, we offer the idea that the outcome of *M. cerebralis* infections in *T. tubifex* may be context specific (e.g. Khangarot et al. 2003) because reaches with high geothermal influence may be suboptimal for *T. tubifex* to

begin with, and may be even less optimal for *T. tubifex* when infected by *M. cerebralis*.

Thus, parasitized *T. tubifex* may tolerate a narrower range of environmental conditions than unparasitized *T. tubifex* because of the additional stress of infection.

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Table 3.1. Categories of geothermal influence, distance to geothermals, number of sites in category, and reach number.

Category of thermal influence	Distance (km) to most proximal thermal input (± 1 S.E)	n	Reaches
high	1.65(± 0.61)	7	3,6,7,11,12,20,21
moderate	7.98 (± 0.36)	7	1,2,5,10,13,15,16
low	16.53(± 1.20)	6	4,8,9,17,18,19
none	-	5	14,22,23,24,25

Table 3.2. Environmental features of geothermal categories. Values are means (± 1 S.E.), n=number of reaches. Boldface type indicates environmental characteristics that varied among categories and letters indicate Tukey's HSD results (see Table 3.3).

Environmental characteristics		Geothermal category			
		High (n=7)	Moderate (n=7)	Low (n=6)	None (n=5)
Sediment characteristics	Proportion coarse	0.29 (0.05) A	0.52 (0.10) AB	0.27 (0.09) A	0.75 (0.13) B
	Proportion sand	0.56 (0.07) A	0.38 (0.10) AB	0.62 (0.08) A	0.21 (0.10) B
	Proportion clay	0.08 (0.01) A	0.05 (0.01) AB	0.05 (0.01) AB	0.02 (0.01) B
	Proportion silt	0.06 (0.03)	0.06 (0.01)	0.06 (0.01)	0.02 (0.01)
	Proportion organic material	0.02 (0.01)	0.08 (0.04)	0.02 (0.01)	0.02 (0.01)
Channel characteristics	Width (m)	10.25 (2.63)	10.23 (2.65)	11.88 (4.08)	3.20 (0.56)
	Velocity (m/s ³)	0.44 (0.14)	0.55 (0.28)	0.25 (0.06)	0.11 (0.02)
	Depth (m)	0.24 (0.03)	0.33 (0.08)	0.29 (0.09)	0.22 (0.04)
	Elevation (m)	2398.67 (6.73)	2405.16 (12.06)	2403.17 (5.73)	2437.15 (19.35)
	Reach slope	0.07 (0.03)	0.05 (0.03)	0.03 (0.02)	0.03 (0.01)
Physico-chemical characteristics	Temperature (°C)	21.15 (1.18) A	15.66 (1.68) B	17.72 (1.43) AB	15.98 (1.33) AB
	Dissolved oxygen (mg/L)	5.59 (1.21)	6.37 (1.21)	7.74 (1.01)	7.51 (0.30)
	Specific conductivity (mS)	0.47 (0.10) A	0.24 (0.02) B	0.23 (0.03) B	0.07 (0.01) C
	pH	6.50 (0.85) A	8.03(0.10) AB	7.91 (0.26) AB	8.30 (0.08) B

Table 3.3. Analysis of variance results for differences in environmental characteristics among geothermal categories. Transforms, if used, are indicated in parentheses below response variables and significant results ($p < 0.10$) are shown in boldface.

Environmental characteristic		Source of variation	df	SS(III)	F	P-value
Sediment characteristics	Proportion coarse	Model	3	0.86	5.65	5.00x10⁻³
		Error	21	1.07		
	Proportion sand	Model	3	0.60	4.05	0.02
		Error	21	1.04		
	Proportion silt	Model	3	0.01	1.28	0.31
		Error	21	0.04		
	Proportion clay	Model	3	0.01	4.36	0.02
		Error	21	0.02		
	Proportion organic material (ln)	Model	3	1.77	1.84	0.17
		Error	21	6.74		
Channel characteristics	Width (ln)	Model	3	4.23	2.36	0.10
		Error	21	12.52		
	Depth (ln)	Model	3	0.03	0.67	0.58
		Error	21	0.28		
	Velocity (sqrt)	Model	3	0.39	1.75	0.19
		Error	21	1.55		
	Elevation	Model	3	5.02x10 ⁻⁰³	2.13	0.13
		Error	21	2.15x10 ⁻⁰⁴		
	Reach slope	Model	3	9.11x10 ⁻⁰³	0.77	0.52
		Error	21	0.08		
Physicochemical characteristics	Temperature	Model	3	126.97	2.52	0.09
		Error	21	352.98		
	Dissolved oxygen	Model	3	19.26	0.87	0.48
		Error	21	155.83		
	Specific conductivity (ln)	Model	3	9.01	25.08	1.00x10⁻⁴
		Error	21	2.52		
	pH	Model	3	12.75	2.73	0.07
		Error	21	32.75		

Table 3.4. Analysis of variance results for differences in *T. tubifex* abundance, relative abundance of *T. tubifex*, and segment widths of immature *T. tubifex* among geothermal categories. Transforms, if used, are indicated in parentheses below response variables and significant results are shown in boldface.

<i>Tubifex tubifex</i> characteristic	Source of variation	df	SS(III)	F	P-value
Abundance	Model	3	37.744	14.00	<0.0001
(ln)	Error	19	17.074		
Relative abundance	Model	3	0.208	0.46	0.715
(arcsin)	Error	19	2.877		
Width of fifth segment of	Model	3	0.051	2.75	0.071
randomly selected immatures	Error	19	0.117		

Table 3.5. Mean relative segment widths of mature *T. tubifex* and *M. cerebralis* infected *T. tubifex* (± 1 S.E.) by geothermal category, n=number of reaches.

Relative difference from randomly selected <i>T. tubifex</i>	Geothermal category			
	High	Moderate	Low	None
Mature <i>T. tubifex</i>	0.002 (0.074) n=2	-0.071 (0.051) n=3	0.013 (0.062) n=4	-0.042 (0.008) n=2
<i>M. cerebralis</i> infected <i>T. tubifex</i>	n=0	-0.006 (0.009) n=6	-0.005 (0.030) n=5	n=0

Table 3.6. Analysis of variance results for differences in prevalence of *M. cerebralis* infection in *T. tubifex* and abundance of *M. cerebralis* infected of *T. tubifex* among geothermal categories. Transforms, if used, are indicated in parentheses below response variable and significant results are shown in boldface.

Parasite characteristic	Source of variation	df	SS(III)	F	P-value
Infection prevalence in <i>T. tubifex</i>	Model	3	0.223	5.52	0.007
(sqrt)	Error	19	0.254		
Abundance of infected <i>T. tubifex</i>	Model	3	15.230	7.41	0.002
(ln)	Error	19	13.009		

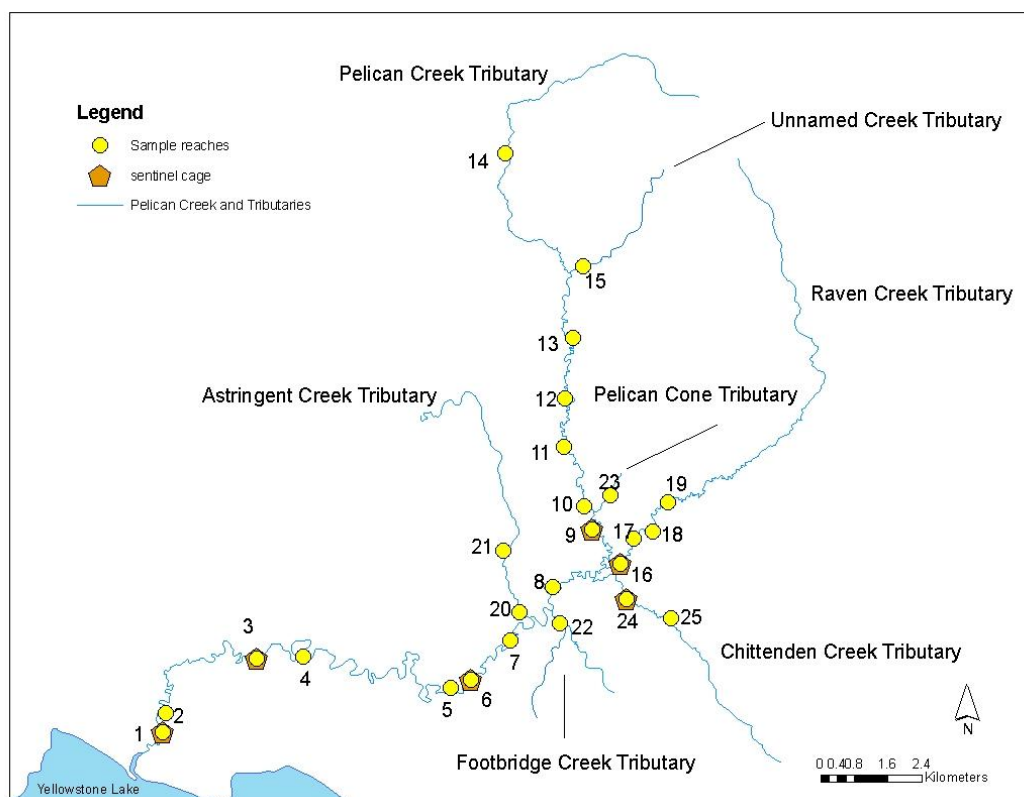


Figure 3.1. Tubificid sampling reaches (n=25) and locations of sentinel cages (n=6) on Pelican Creek and tributaries to Pelican Creek in Yellowstone National Park.

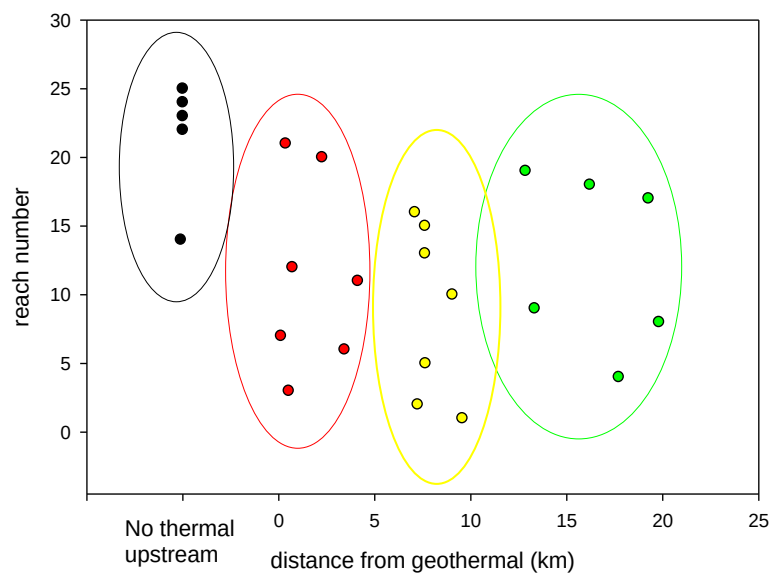


Figure 3.2. Reach distance to geothermals and geothermal categories. Reaches classified as having 1) *high geothermal influence* were located 0-5 km downstream from geothermal features, 2) *moderate geothermal influence* were located 5-10 km downstream from geothermal features, 3) *low geothermal influence* were located greater than 10 km downstream from geothermal features, and 4) *no geothermal influence* had no geothermal features located upstream.

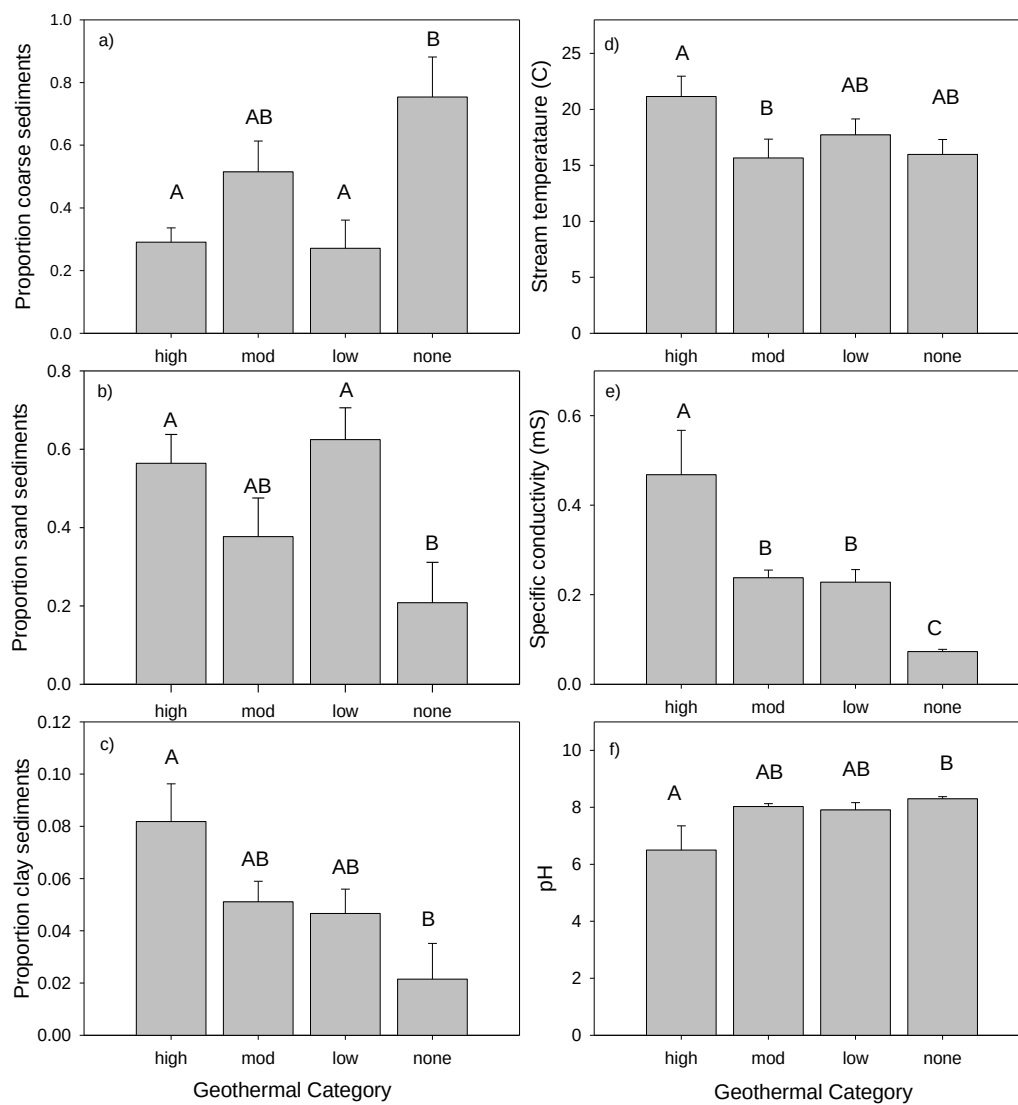


Figure 3.3. Environmental features that varied among geothermal categories. a) proportion coarse sediments, b) proportion sand sediments, c) proportion clay sediments, d) water temperature, e) specific conductivity, and f) pH. Letters represent Tukey's HSD results, $p < 0.10$.

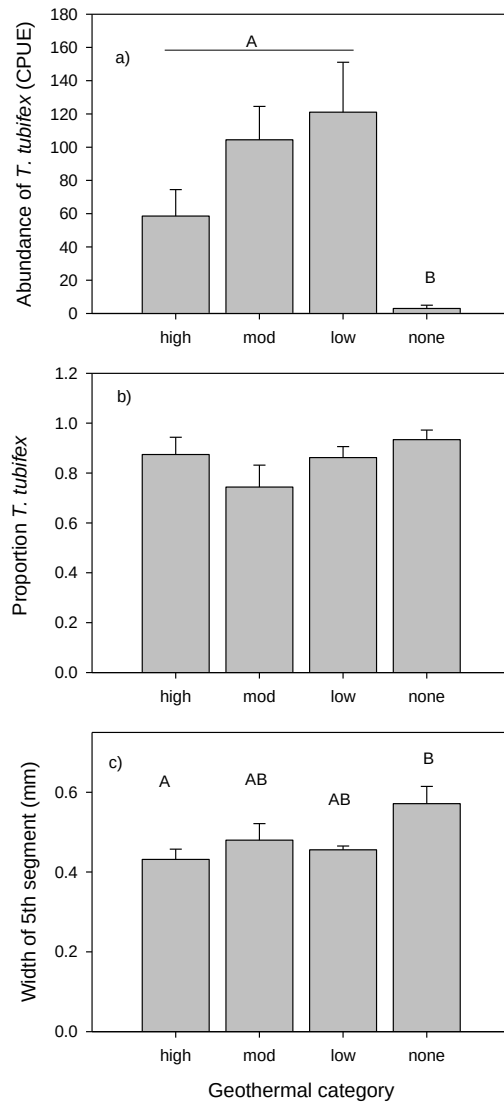


Figure 3.4. Host factors shown by geothermal category, a) abundance of *T. tubifex*, b) relative abundance of *T. tubifex*, and c) widths of 5th segment of randomly selected *T. tubifex*. Letters represent Tukey's HSD results, $p < 0.10$.

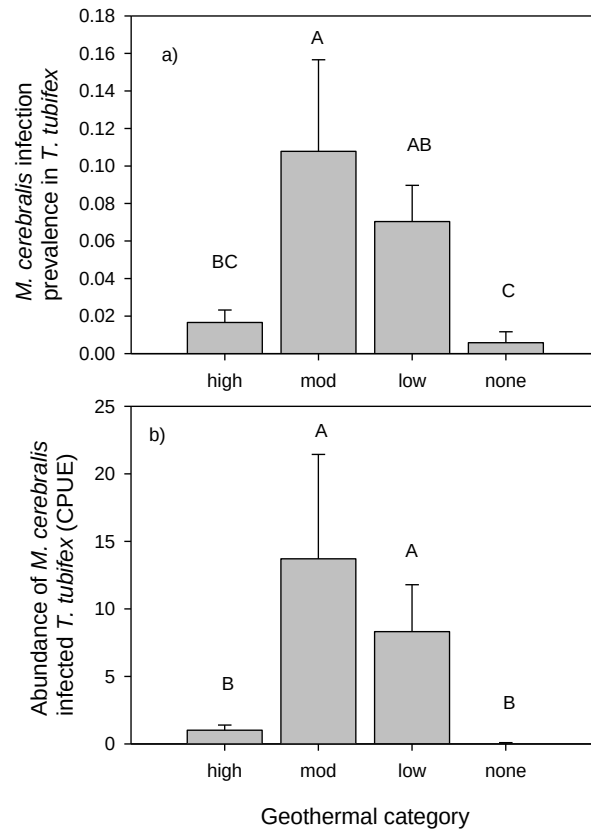


Figure 3.5. Parasite factors shown by geothermal category, a) prevalence of *M. cerebralis* infection in *T. tubifex* and b) abundance of *M. cerebralis* infected *T. tubifex*. Letters represent Tukey's HSD results, $p < 0.10$.

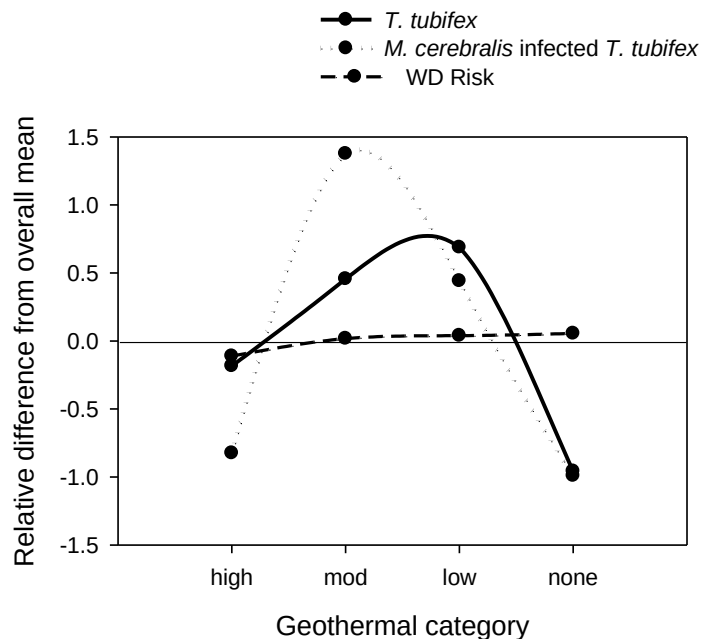


Figure 3.6. Patterns of relative host (represented as relative difference from overall mean abundance of *T. tubifex*), potential parasite (represented as relative difference from overall mean abundance of infected *T. tubifex*), and realized parasite success (represented as relative difference from overall mean WD risk) are represented in reaches with variable geothermal influence. We suggest potential parasite success was context specific: Host success explains potential parasite success in reaches with moderate and low geothermal influence, where abundance of hosts and parasites were above average, and in reaches with no geothermal influence, where abundance of hosts and parasites were below average. Abundance of hosts was average and abundance of parasites was below average in reaches with high geothermal influence, which suggests host success does not explain potential parasite success.

CHAPTER FOUR

HABITAT AND PARASITE SUCCESS: INFLUENCE OF HOST ENVIRONMENT ON *MYXOBOLUS CEREBRALIS* IN TRIBUTARIES IN YELLOWSTONE NATIONAL PARKAbstract

Environmental conditions that influence host abundance, the viability of parasitized hosts, or parasite stages released from hosts may influence parasite success under different environmental conditions, while the host environment may influence parasite success. The invasive parasite *Myxobolus cerebralis* has established and caused whirling disease-related population declines in native and wild salmonid populations throughout the intermountain west of the United States. Interactions between the environment and the obligate oligochaete host, *Tubifex tubifex*, may influence the success of *M. cerebralis*. This chapter examines the hypothesis that, primarily through effects on oligochaete host ecology, environmental conditions related to tributary confinement may influence *M. cerebralis* success in tributaries in Yellowstone National Park.

Confinement describes tributaries in terms of floodplain connectivity, and as such, is a proxy for physical habitat features (e.g., bedform, flow, substrate) that may influence benthic invertebrates, including *T. tubifex*. We tested for environmental differences among tributaries characterized by variable confinement. We characterized the invertebrate community (abundance and diversity), oligochaete host ecology (abundance of *T. tubifex* and genetic diversity), and parasite success (infection prevalence and abundance of infected *T. tubifex*, and infection prevalence and severity in sentinel fish) in depositional reaches of

tributaries, and examined relationships among these factors and confinement. The environments of unconfined reaches were characterized by more sand, silt, and clay, and fewer coarse substrates than environments of confined reaches. Differences in abundance and diversity of invertebrates were not detected among confinement types. In contrast, the oligochaete host, *T. tubifex*, was strongly influenced by environmental factors related to confinement (primarily substrate); abundance of *T. tubifex* and susceptible *T. tubifex* were higher in unconfined habitat types than in intermediate or confined habitats. However, parasite success was not related to abundance of oligochaete hosts. We did not detect differences in infection prevalence in or abundances of infected *T. tubifex* or infection prevalence and severity in fish, although *M. cerebralis* was never detected in *T. tubifex* or in sentinel fish in confined reaches.

Introduction

Environmental conditions (e.g., flow regime; Cattaneo 2005) that select for functional characteristics in species assemblages (Townsend and Hildrew 1994, Thorp et al. 2006) may be useful for predicting the success of invasive species. Invasive species pose a serious threat to native animal populations and are considered one of the world's most challenging environmental threats (Vitousek et al. 1996, Wikelski et al. 2004, Causton et al. 2006). For example, the invasive parasite *Plasmodium relictum* exacerbated the decline of native Hawaiian honeycreepers, many of which are now extinct (van Riper III et al. 1986, Atkinson et al. 1995, Atkinson et al. 2000). Thus, the search for a predictive subset of characteristics that may confer success to invasive species continues (Sakai et al. 2001, Piementel et al. 2005).

The invasive parasite *Myxobolus cerebralis*, which causes whirling disease (Hofer 1903 in Bartholomew and Reno 2002), has become established in native and wild salmonid populations throughout the U.S. intermountain west (Bartholomew and Reno 2002, Elwell et al. 2009b). The life cycle of *M. cerebralis* involves two hosts, a salmonid and an oligochaete, and two spore stages, myxospores and triactinomyxons (TAMs) (Wolf and Markiw 1984, Andree et al. 1997). Infected salmonids produce myxospores that are infective to the oligochaete, *Tubifex tubifex*, and in turn, *T. tubifex* produces TAMs that are infective to salmonids (Wolf & Markiw 1984).

Environmental conditions influence the success of *M. cerebralis* at various points during the life cycle and thus may be useful for predicting where this parasite may become established. Water velocity may influence transmission success by flushing myxospores

from streams (Vincent 1996) or reducing TAM attachment (Hallett and Bartholomew 2008). Water temperature influences proliferation (El-Matbouli et al. 1999, Schisler et al. 2000, Kerans et al. 2005). Other environmental conditions also appear to influence *M. cerebralis* success (e.g., Downing et al. 2002, Hiner and Moffitt 2002, Blazer et al. 2003, Krueger et al. 2006), but appear to do so indirectly, via the invertebrate host. For example, confinement influences substrate composition (*confinement* describes tributary connectivity with the adjacent floodplain and provides a proxy for physical habitat, e.g., bedform, flow, and substrate; Gordon et al. 2004), and substrate composition influences the distribution of invertebrates, including oligochaetes (Brinkhurst 1971, Lazim and Learner 1987, Prenda and Gallardo 1992, Martinet et al. 1993, Yates et al. 1993, Juget and Lafont 1994, Storeid 1994, Verdonchot 1999, 2001). Thus, confinement may influence *M. cerebralis* success by influencing the abundance and distribution of the oligochaete host.

Because *T. tubifex* is the only oligochaete species that is compatible with *M. cerebralis* (Markiw and Wolf 1983, Kerans et al. 2004), intraspecific interactions among *T. tubifex* and other oligochaete species (e.g., Brinkhurst et al. 1972, Elwell et al. 2009), or other functionally similar invertebrates (e.g., other gathering collectors; Vanderbund et al. 1994, Hiner and Moffitt 2001) may influence rates of encounter between *T. tubifex* and myxospores, and thus may influence parasite transmission. Myxospore dose is proportional to infection prevalence in *T. tubifex* (Elwell et al. 2009) and presumably TAM production.

Environmental conditions may also influence the genetic composition of *T. tubifex* populations, which may influence *M. cerebralis* success. Genetically variable *T. tubifex* ‘lineages’ (mitochondrial DNA lineages I-VI found within North America; Beauchamp et al.

2001, Arsan et al. 2007) exhibit variable susceptibilities to *M. cerebralis*. *Tubifex tubifex* belonging to lineages I, V, and, VI are considered resistant (Beauchamp et al. 2006, Elwell et al. 2006, Arsan et al. 2007 but see Kerans et al. 2005). *Tubifex tubifex* belonging to lineage III are susceptible, but susceptibility varies among strains within lineages and among lineages (e.g., Baxa et al. 2008, Rasmussen et al. 2008). In addition, lineages co-exist (DuBey and Caldwell 2004, Beauchamp et al. 2005, Crottini et al. 2008) and may have different environmental optima (DuBey et al. 2005, Kerans et al. 2005).

In Yellowstone National Park, *M. cerebralis* has established in at least two tributaries where it may be causing native Yellowstone cutthroat trout to decline. Previous research in this system suggests parasite success is high in Pelican Creek (Koel et al. 2006, Murcia et al. 2006, Alexander et al. submitted), which is characterized by low confinement. We hypothesize that the success of *M. cerebralis* in this tributary and others in Yellowstone may be indirectly influenced by environmental conditions related to confinement, primarily through effects on the invertebrate host, *T. tubifex*. The objectives were to characterize i) environments, ii) invertebrate assemblages, including *T. tubifex*, and iii) parasite success in tributaries in Yellowstone National Park, and to iv) examine relationships between environments and these variables.

Methods

To characterize the environment, we measured reach-scale environmental features. To characterize the invertebrate community, we measured abundance and diversity. To characterize oligochaete host ecology, we measured abundance of *T. tubifex* and abundance of *T. tubifex* lineages. To examine parasite ecology, we measured i) *M. cerebralis* infection

prevalence in *T. tubifex*, ii) abundance of infected *T. tubifex*, and iii) whirling disease risk to fish (infection prevalence and severity in sentinel fish). To examine the influence of the environment on the invertebrate community, host ecology, and parasite ecology, we examined relationships between confinement and these factors.

Study Site and Environmental Features

This study was conducted in summer months (late July-early September) in six tributaries to Yellowstone Lake (lower Pelican Creek mainstem, the Chittenden tributary of Pelican Creek, Upper Pelican Creek, Clear, Arnica, Beaverdam and Bridge Creeks and the Upper Yellowstone River) and six tributaries to the Yellowstone River (Trout, Alum, Elk Antler, Thistle, Otter, and Slough Creeks) in Yellowstone National Park, WY (Figure 4.1). Slough Creek was the only study tributary located downstream from the falls of the Yellowstone River. Selected tributaries were historically used by spawning Yellowstone cutthroat trout and were characterized by a range of confinement (NPS 1987, Koel et al. 2006, Table 4.1). Confinement describes the degree of connectivity between a tributary and its floodplain. An unconfined tributary would meander freely, flooding during high flows and cutting new banks and creating a new channel, which allows for fine sediment retention (hypothesized to be ideal for *T. tubifex*). A confined tributary would have limits, such as steep valley walls, prohibiting lateral movement, which does not allow for fine sediment retention (hypothesized to be unsuitable for *T. tubifex*). Confinement provides a proxy for bedform, flow, derived sediment particle sizes, and sediment transport regimes (Gordon et al. 2004). These factors may influence invertebrate host ecology and parasite success at the reach scale, but are not easily determined at the catchment level. Confinement provides

proxy for these variables and can be easily determined from topographic maps. Each reach was assigned a score to reflect elevation change on either side of the reach, which were averaged for the reach (Table 4.1). Elevation change on land adjacent to the tributary (within 0.8 km) was scored as follows: A change of <7 m was assigned a confinement score of 0, 7-14 m was assigned a confinement score of 1, 14-21 m was assigned a confinement score of 2, >21 m was assigned a score of 3. Tributaries with an overall score of ≤ 1.5 were classified as unconfined, between 1.5-2.5, inclusive, were classified as intermediate, and >2.5 were classified as confined.

Twenty-eight 100 m sampling reaches were selected based on relative reach length and accessibility (Figure 4.1). Two study reaches were allocated to each catchment with the exception of Pelican Creek, which was assigned six reaches because it is large in comparison to the majority of the other tributaries included in this study. On Pelican Creek, two reaches were located in the lower third of the catchment, two reaches were located in the middle third of the catchment, and two reaches were located in the upper third of the catchment.

To examine the influence of catchment scale features, we tested for differences in environmental features among reaches that were characterized by variable geomorphic confinement (confined, unconfined, or intermediate). We measured environmental variables that were previously shown to be important for oligochaete host and parasite ecology (e.g., Krueger et al. 2006). Environmental variables included channel characteristics (width, depth, water velocity, elevation, and slope), substrate characteristics (sediment composition, proportion of organic material in sediment), and physicochemical characteristics (temperature, dissolved oxygen, specific conductivity, and pH).

Channel characteristics were measured on three randomly-selected transects in each reach. Channel width was measured once per transect. Water depth and current velocity were quantified at three equidistant points along each transect (Model 3000 Swoffer Instrument, Swoffer Instruments, WA, USA). Elevation was measured at the upper and lower limits of each study reach with a GPS device. Reach slope was calculated as change in elevation over the 100 m reach.

Substrate composition was assessed using two methods. The first method apportioned substrate into fine (<2 mm) and coarse (>2 mm) particles, with a grid (a 320x320 mm Plexiglas square consisting of 2 mm-wide intersecting grid lines drawn 40 mm apart, for a total of 49 intersecting points; Overton et al. 1997, Krueger et al. 2006) that was randomly positioned over the substrate once per transect. Substrate particles were characterized at each grid intersection. Particles visible around all four sides of grid intersections (> 2mm across at the sampling point) were classified as coarse and particles that were not visible around all four sides of grid intersections were classified as fine. Proportions of fine and coarse sediments were calculated by averaging individual measurements for each reach. The second method determined the composition of fine (<2 mm) sediments by the hydrometer gravimetric method (Day 1965). Sediment samples were collected from a haphazardly selected position once per transect with a modified core sampler (a sawed off 500 mL Nalgene bottle pushed 20-30 cm deep in the substrate), combined for each reach, and frozen. During processing, samples were split (half was used to determine organic material, as described below), dried at 105 °C, sieved to remove coarse sediments (>2 mm), and proportions of sand (0.64-2 mm), silt (0.03-0.63 mm), and clay

(<0.002 mm) were determined (Day 1965). To obtain an estimate of sediment fractions in each reach, proportions were applied to counts obtained from the surface fines grid.

Proportion of organic material was determined as ash-free dry mass (AFDM; Hauer and Lamberti 1996) in two to four replicate subsamples (15-30 g) in each reach using sediment retained during fine sediment processing. Proportion of organic material was calculated as [original dry subsample weight (after 2 hrs at 105 °C)]-[burned subsample weight (following 2 hrs at 550 °C)]/[original dry subsample weight].

Temperature (°C), dissolved oxygen concentration (mg/L), specific conductivity (mS/cm), and pH were quantified with a Yellow Springs Instrument (Yellow Springs Instruments, Ohio, USA) once per reach.

We tested for differences in environmental features among confinement types using multivariate analysis of variance (MANOVA) (PROC GLM, SAS Institute, V.9.2). Significant effects ($p < 0.05$) were examined using individual analysis of variance (ANOVAs) and Tukey's Honestly Significant Difference (HSD) tests.

Invertebrate Community

To characterize the invertebrate community, we measured abundance and diversity of invertebrate families. Invertebrates were collected using a kick net (200 µm mesh, Wildco, FL, two minute kicks). One quarter of each kick sample was retained for invertebrates, preserved, and combined with other quartered kick samples from each reach. The remaining $\frac{3}{4}$ of each kick sample was sorted in the field to collect oligochaetes that were morphologically similar to *T. tubifex* for molecular analyses (see below, Host Ecology).

Invertebrate samples were preserved in Kahle's solution (18:1:1 volumes 70% ethanol:formalin:glacial acetic acid) for two weeks, rinsed over a 500 μm sieve, and the fraction $\geq 500 \mu\text{m}$ was retained and stored in 70% ethanol. Invertebrates were sorted and identified to family under a dissecting microscope (5-50x) (Merritt and Cummings 1996), with the exception of oligochaetes, which were slide-mounted and identified with a phase contrast compound microscope (100-400x). Non-tubificid oligochaetes (e.g., Lumbriculidae) were identified to family. Tubificids were identified to species when mature (Kathman and Brinkhurst 1998) and immature tubificids were assigned to species based on the relative abundance of sexually mature tubificid species found in each reach (Krueger et al. 2006). Abundance of invertebrate families were calculated as catch per unit effort (CPUE) as [total number of invertebrates a family/total number of kick samples]*[split conversion factor (4)] in each reach. Abundance of tubificid species were calculated as above, CPUE, except that the total number of tubificids in each species was used in place of total number of invertebrates in a family.

Invertebrate diversity was assessed in each reach using a modified Shannon-Weaver index of diversity. The index was modified by calculating diversity based on the number of invertebrates within individual families and number of families represented, rather than species (Siemann and Haarstad 1997).

Principal components analysis (PCA) was used to describe abundance of invertebrate families in each reach (PROC PRINCOMP, SAS Institute, NC). Characteristics with eigenvectors $\geq \pm 0.25$ were considered influential features of the PCA axes. Many invertebrates co-vary in streams, and PCA produces independent axes from which we could

examine relationships among environments (see below, Relationships). Data were transformed prior to inclusion in the analysis when non-normally distributed.

Host Ecology

To examine host ecology, we measured abundance of *T. tubifex* and determined the abundance of *T. tubifex* belonging to different lineages. Abundance of *T. tubifex* was calculated based on the proportion of tubificids identified as *T. tubifex* in invertebrate samples as catch per unit effort (CPUE), using the formula [total number of *T. tubifex* (mature *T. tubifex* and immature tubificids)/total number of kick samples]*[split conversion factor (4)] in each reach. (For a comparison of abundance estimates used herein and in Chapter 3, refer to Appendix C).

To determine abundance of *T. tubifex* belonging to different lineages, we assayed individual tubificids by molecular methods. Tubificids used for molecular assays were collected from the remaining $\frac{3}{4}$ of the kick samples collected at each reach (see above, Invertebrate Community). Each kick sample was sorted in the field (three persons sorting for one hour was considered equal to three hours of sorting time) to collect oligochaetes that were morphologically similar to *T. tubifex* for molecular analyses. Kick samples were repeated until 300 oligochaetes were collected or three hours of sorting time were reached. Oligochaetes removed from kick samples were combined for each reach.

The anterior segments from up to 200 tubificids with morphology similar to *T. tubifex* per reach (confirmed under a dissecting microscope, 5-50x) were preserved in Kahle's, slide mounted, and identified (Kathman and Brinkhurst 1996). Immature tubificids were assigned to species based on the proportion of tubificid species that had been morphologically

identified, as above. If mature *T. tubifex* were not collected from a reach, immature tubificids were assigned to species based on the proportion of individuals that tested positive for previously described *T. tubifex* lineages by PCR (Sturmbauer et al. 1999, Beauchamp et al. 2001).

DNA was extracted from the posterior segments (corresponding to slide-mounted anterior segments) of tubificids in single and pooled samples using Nucleospin tissue extraction kits (Clontech Inc., CA, USA). Single samples (100 per reach) contained DNA from one tubificid and pooled samples (up to 4 per reach) contained DNA from up to 25 tubificids. The combination of pooled and individually prepared samples enabled us to extract DNA from up to 200 individuals using fewer (up to 104) samples, which was less expensive than processing 200 individual samples. The aim of using the pooled samples was to maximize our ability to detect i) genetic variation and ii) *M. cerebralis* infection (see below, Parasite Ecology) in *T. tubifex* in each reach. The aim of using the single samples was to be able to determine the proportion i) of each lineage and ii) *M. cerebralis*-infected *T. tubifex* in each reach.

Proportions of *T. tubifex* lineages in each reach were determined using two methods. In the first approach, up to 20 randomly-selected tubificids were assayed for lineage to determine proportion of each lineage in each reach. Samples were assayed using previously developed primers for lineages I, III, V and VI at the 16S mtDNA locus (Beauchamp et al. 2001, 2002, Rasmussen et al. 2008). The PCRs were performed on a PTC-100 thermocycler (MJ Research, Inc., MA) and products were visualized by gel electrophoresis on 2.5%

agarose gels in $0.5 \times$ TAE buffer run next to positive controls. Samples were assigned a score of 0 or 1, to indicate absence or presence of each lineage.

In the second approach, pooled samples were assayed to determine if each lineage was present. The aim of using this approach was to maximize detection ability for rare lineages by testing the DNA of every tubificid prepared for that reach. In this approach, DNA from tubificids from each reach were assayed, as above, except that DNA pooled from up to 25 individuals was assayed.

Proportion of each lineage was calculated as [(number individuals testing positive for that lineage)/(number of individuals tested)] in each reach. If a lineage was detected only in pooled samples, proportion of that lineage was calculated as [(number positive pooled samples)/(number of individuals in pooled samples)] in that reach. Abundance of each lineage was calculated as (proportion of lineage) $\times$ [*T. tubifex* abundance (CPUE, see above)] in each reach. Tubificids that did not test positive for any *T. tubifex* lineages were considered not to be *T. tubifex* unless they were morphologically identified as *T. tubifex*.

Parasite Ecology

To examine parasite ecology, we measured infection in *T. tubifex* and whirling disease risk to fish. We measured infection prevalence in *T. tubifex* and calculated the abundance of infected *T. tubifex* as measures of parasite success, because abundance of *M. cerebralis*-infected *T. tubifex* is correlated with whirling disease risk to fish (Krueger et al. 2006). We measured whirling disease risk to fish because infection in the oligochaete host does not directly provide information regarding parasite transmission, which is what directly influences whirling disease risk to fish.

To determine prevalence of *M. cerebralis* infection in *T. tubifex*, we tested single and pooled DNA samples assayed for *T. tubifex* lineage (as described above, Host Ecology). Infection in *T. tubifex* was determined using the nested PCR test for *M. cerebralis* (Andree et al. 1998). The PCR products were visualized by gel electrophoresis on 1.5% agarose gels in $0.5 \times$ TAE buffer alongside positive and negative controls (refer to Appendix D for validation of PCR results with DNA sequences). Infection prevalence was calculated as $[(\text{number infected})/(\text{number individuals assayed})]$. If *M. cerebralis* was detected in pooled samples but was not detected in individually prepared samples from that reach, infection prevalence was calculated as $[(\text{number infected pools})/(\text{total number individuals in pooled samples})]$, assuming one infected individual per *M. cerebralis*-positive pool. Abundance of *M. cerebralis*-infected *T. tubifex* was calculated as (*M. cerebralis* infection prevalence in *T. tubifex*) $\times$ [abundance of *T. tubifex* (CPUE, see above)] in each reach.

Whirling disease risk to fish. Whirling disease risk to fish was assessed by determining *M. cerebralis* infection prevalence and severity in sentinel fish (caged hatchery-reared fish placed in situ and then subsequently examined by molecular and histological analyses for *M. cerebralis* infection and severity, e.g., Krueger et al. 2006, Murcia et al. 2006). Cages were located < 1 km upstream from the mouths of study tributaries during 10-day periods in 2005-2007 (Figure 4.1, Table 4.1) because it was difficult to transport fish to study reaches. We assumed TAMs released in upper parts of the catchment would infect sentinel fish exposed at the tributary mouth.

Sentinel fish were transported to exposure reaches in aerated coolers (Murcia et al. 2006). Sentinel cages contained 60 Yellowstone cutthroat trout fry, six weeks post hatch,

fork length < 2.5 in. Following the 10-day exposure period, fish were removed from cages and transported to the Aquatic Sciences Laboratory (Montana State University, Bozeman, MT, USA). Fish from each cage were held in separate aquaria for 90 days prior to testing for *M. cerebralis* infection and severity, to allow for parasite development.

Infection prevalence in sentinel fish was determined by nested PCR (Andree et al. 1998). Genomic DNA was extracted (Nucleospin tissue kits, Clontech Inc., CA, USA) from cranial tissue from 10 randomly-selected fish per cage. Cranial tissue was obtained by bisecting fish along the sagittal line and removing a biopsy from one half of the head. The remaining half-head was preserved for assessment of infection severity, as described below. Tissue biopsies from five fish were pooled prior to DNA extraction.

Infection severity was determined by histological assessment of 10 fish per cage. Infection severity was scored as parasite damage on a scale of 0 to 5, where a score of 0 indicated no infection and a score of 5 indicated severe infection (Baldwin et al. 2000). Infection prevalence was calculated as [(number of fish per pool characterized by a histology score > 0)/(number of fish in pool)]. Infection severity was calculated as mean histological score in each tributary.

Confinement and Invertebrates

To examine relationships among confinement types and the invertebrate community, we tested for differences in invertebrate family abundance (principal components axes describing abundances of invertebrates) using MANOVAs. Significant effects were examined as before (ANOVAs and Tukey's HSD tests). We tested for differences in invertebrate diversity among reach types using ANOVA.

Confinement Types and Host Ecology

To examine relationships among confinement types and host ecology, we tested for differences in abundance of *T. tubifex* and lineage III *T. tubifex*. We planned to test for differences in abundance of other lineages among confinement types, but we did not detect many *T. tubifex* belonging to lineages other than lineage III. We tested for differences in abundance of *T. tubifex* and lineage III *T. tubifex* with MANOVAs. Significant effects ($p < 0.05$) were examined as before (ANOVAs and Tukey's HSD tests).

Confinement Types and Parasite Ecology

To examine relationships among confinement types and parasite ecology, we tested for differences in prevalence of infection and abundance of infected *T. tubifex* using MANOVAs. Significant effects ($p < 0.05$) were examined as before (ANOVAs and Tukey's HSD tests). We did not include whirling disease risk to fish in the MANOVA because infection prevalence and abundance of *T. tubifex* were assessed in 28 reaches and whirling disease risk was assessed in 14 reaches (the most downstream reach in each tributary). We planned to test for differences in whirling disease risk among confinement types by testing for differences in infection prevalence and severity in sentinel fish. However, infection prevalence highly non-normal; either 0% (uninfected cages) or 100% (infected cages), so we only tested for differences in infection severity. We tested for differences in infection severity among confinement types with ANOVA, followed by Tukey's HSD when significant ($p < 0.05$) effects were detected.

Results

Environmental Features

Environmental features differed among confinement types (marginally significant, Wilks' $\lambda=0.131$, $F_{2,26}=1.76$, $p=0.078$). The environments of confined reaches were characterized by higher slopes and proportions of coarse sediments and lower proportions of silt and clay sediments than unconfined reaches (Tables 4.2, 4.3, Figure 4.2). Reaches with intermediate confinement were characterized by substrates that were intermediate between those of confined and unconfined reaches (Figure 4.2). Differences in other environmental features were not detected among confinement types (Table 4.3).

Invertebrate Ecology

Four major PCA axes described 60.5% of the variation in invertebrate abundance among reaches (Table 4.4). Principal component one differentiated reaches characterized by Chironomidae, Tipulidae, Acari, and Ostracoda from reaches characterized by tubificids. Principal component two differentiated reaches characterized by Chloroperlidae, Almidiae, Amphizoidae, and Syrphidae from reaches characterized by Ceratopogonae, Psychodidae, and Perlodidae. Principal component three discriminated reaches characterized by Hyalellidae, Gammaridae, and Amphipoda (unknown family) from reaches characterized by Sialidae, Tabanidae, and Ptychopteridae. Principal component four discriminated reaches characterized by Hydroptimildae and Amphipoda (unknown family) from reaches characterized by Haliplidae, Cyclopodia, Branchiopoda, Rhyacodrilinae, and Tubificidae. However, relationships among confinement types and principal components were not detected (Wilks' $\lambda=0.644$, $F_{8,44}=1.35$, $p=0.245$). Invertebrate diversity was similar among

confinement types (Table 4.5) and we did not detect differences among confinement types ($F_{8,44}=1.35$, $p=0.245$).

Oligochaete Host Ecology

Tubifex tubifex were identified from all reaches except 6, 8, 21, 22 and 23. Abundance of *T. tubifex* was higher in unconfined reaches than confined and intermediate reaches (Tables 4.6 and 4.7, Figure 4.3). Lineage III *T. tubifex* were detected in all unconfined (9 out of 9) reaches, 8 of 11 intermediate reaches (no lineage IIIs were detected in reaches 8, 21-22 and *T. tubifex* were not morphologically identified from any of these reaches) and 3 of 8 confined reaches (no lineage IIIs were detected in reaches 6, 7, 10, 23, 27; however, *T. tubifex* were morphologically identified from reaches 7 and 27). Lineage I *T. tubifex* were detected in one unconfined reach (12). Lineage VI *T. tubifex* were detected in four reaches (intermediate reaches: 5 and 15, unconfined reaches: 17 and 19). No lineage could be assigned to morphologically-identified *T. tubifex* from four reaches (confined reaches: 7, 11, unconfined reaches 11, 12). The abundance of lineage III *T. tubifex* was higher in unconfined reaches than in confined and intermediate reaches (Tables 4.6, 4.7, Figure 4.3).

Parasite Ecology

Infected *T. tubifex* were collected from seven reaches (unconfined reaches: 1, 2, 19, 20, intermediate reaches: 3, 5, 17). Neither infection prevalence, nor abundance of infected *T. tubifex*, differed among reach types (Tables 4.8, 4.9, Wilks' $\lambda=0.821$, $F_{4,34}=0.88$, $p=0.484$), which was a function of an extremely high prevalence (27.1%) in reach 5. When reach 5 was excluded from the analysis, infection prevalence and abundance of infected *T. tubifex*

differed among confinement types (Wilks' $\lambda=0.551$, $F_{4,32}=2.78$, $p=0.043$). *Myxobolus cerebralis* was detected in sentinel fish in five tributaries including lower Pelican Creek (below reaches 1-2), the Chittenden tributary to Pelican Creek (below reaches 3-4), upper Pelican Creek (below reaches 5-6), Elk Antler Creek (below reaches 17-18), and Trout Creek (below reaches 19-20). Infection prevalence was either 0: fish in cages where *M. cerebralis* was not detected, or 100: fish in cages where *M. cerebralis* was detected (Table 4.8). Infection severity did not differ among reach types ($F_{2,11}=2.45$, $p=0.132$).

Discussion

The goal of this study was to test the hypothesis that, primarily through their effects on invertebrate host ecology, environmental features may be useful predictor variables for *M. cerebralis* success. We tested this hypothesis in tributaries to Yellowstone Lake and River in Yellowstone National Park, where whirling disease risk has previously been described in some tributaries, but patterns within and among invertebrate assemblages, including *T. tubifex* and *M. cerebralis*-infected *T. tubifex*, had not previously been examined (Koel et al. 2006, Murcia et al. 2006). In Yellowstone National Park, aspects of the *M. cerebralis* life cycle involving *T. tubifex* are not well understood, and establishing how specific environmental conditions may influence the oligochaete host may be crucial for understanding whirling disease in this system.

We identified three categories of confinement, which were characterized by unique substrates and slopes. Confined reaches were dominated by coarse (>2 mm) sediments, whereas unconfined reach types were dominated by fine (≤ 2 mm) sediments. Benthic invertebrates are strongly influenced by environmental conditions (e.g., Vannote et al. 1980,

McAuliffe 1983), but we did not detect differences in abundances or diversity of invertebrates among confinement types. This may have occurred because, regardless of confinement, every tributary is characterized by depositional areas, and these areas were targeted during invertebrate collections.

In contrast, we detected differences in abundances of *T. tubifex* among confinement types. This suggests that reaches characterized by increased proportions of fine sediments composed of sand, silt, and clay particles may be optimal for *T. tubifex*, while those characterized by coarse substrate may be unsuitable for *T. tubifex*. Lineage III *T. tubifex* were more abundant in unconfined reach types than in confined reach types, but the proportion of lineage III did not differ among reach types. However, we detected lineage III in all unconfined and intermediate reaches where *T. tubifex* was morphologically identified, but not in all confined reaches where *T. tubifex* was morphologically identified. We also detected *T. tubifex* belonging to lineages I and VI, which are considered resistant to *M. cerebralis*, but they were rare, which suggests they probably do not exert important effects on parasite success. We suggest the morphologically-identified *T. tubifex* that did not amplify any lineage during molecular assays may represent a new lineage (Chapter 6).

Physical geography in Yellowstone National Park may explain why the relative abundance of lineage III *T. tubifex* was high in all reach types. The Yellowstone region is geographically isolated, and lineage III, which is characterized by a widespread distribution in the U.S. (Beauchamp et al. 2001), may have been the most likely to have dispersed into the system. Possibly, because of a lack of competition from other lineages, lineage III *T. tubifex* from Yellowstone National Park may occupy a greater range of environmental

conditions than would be the case in more genetically diverse populations. In this system, this may be important for parasite success because lineage III *T. tubifex* are considered to exhibit moderate-to-high susceptibility to *M. cerebralis* in comparison with other lineages (Kerans et al. 2004, 2005, Beauchamp et al. 2005, but see Baxa et al. 2008), whereas other lineages are considered to have low-to-no susceptibility.

We did not detect differences in the prevalence of *M. cerebralis* infection in *T. tubifex* or the abundances of *M. cerebralis* infected *T. tubifex* or whirling disease risk among reach types. Substrate differences among confinement types may partially explain this result because it may influence the probability *T. tubifex* will encounter and ingest *M. cerebralis* myxospores by altering myxospore availability. Myxospore encounter may be increased in environments dominated by silt-sized particles, <63 μm in diameter, because myxospores adhere more strongly to small particles than large particles (Lemmon and Kerans 2001) and *T. tubifex* actively selects small particles when foraging (Rodriguez et al. 2001). In contrast, myxospore encounter may be decreased in environments dominated by coarse substrate particles, which could act as sinks because myxospores, which are <10 μm in diameter, may settle in interstitial spaces and thus be less likely to be ingested by *T. tubifex*.

Although we did not detect differences in whirling disease risk among confinement types, sentinel fish exposed in confined reaches were never infected by *M. cerebralis*. Why patterns of whirling disease risk did not differ among unconfined and intermediate reach types may be explained by considering that a single infected *T. tubifex* may produce enough TAMs to cause high whirling disease risk.

Although this study is highly specific to this system, the results may have broader application. Variation in environmental conditions, hosts, and parasites have been examined in other systems (e.g., Hiner and Moffitt 2001, Downing et al. 2002, Kreuger et al. 2006, Hallett et al. 2009) but have not proven useful for predicting parasite success, which may relate to differences in relative abundances of susceptible oligochaete hosts. The differential abundances of hosts in the study tributaries suggest interactions between environmental features and hosts may importantly influence parasite success during transmission from myxospore to TAM. We suggest that investigating how substrate may influence i) *T. tubifex* and ii) *M. cerebralis*-infected *T. tubifex* would be helpful for our understanding of infection dynamics in the oligochaete host and whirling disease risk to fish. If substrate does influence host fitness and success and the outcomes of *M. cerebralis* infections in *T. tubifex*, this environmental feature might have broad application in controlled systems (e.g., dammed rivers) and contribute to our understanding of context specificity in host-parasite relationships.

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Table 4.1. Study site. Drainage, tributary name, status of *M. cerebralis* (from Koel et al. 2006, Murcia et al. 2006), reach number, confinement category, and years sentinel fish were exposed in each tributary. *= sentinel fish exposed in Clear Creek tested positive for *M. cerebralis* once (Koel et al. 2006) but the parasite has not been detected since.

Drainage	Tributary	<i>M. cerebralis</i> status:	Reach	Confinement	Year(s) sentinel fish sampled
Yellowstone Lake	Pelican Creek	+	1	Unconfined	2005-2007
	Mainstem		2	Unconfined	.
	Chittenden Creek	+	3	Intermediate	2005-2006
			4	Intermediate	.
	Upper Pelican Creek	+	5	Intermediate	.
			6	Confined	.
	Clear Creek	-*	7	Confined	2006-2007
			8	Intermediate	2006-2007
	Beaverdam Creek	-	9	Unconfined	2007
			10	Confined	.
	Upper Yellowstone River	-	11	Unconfined	2007
			12	Unconfined	.
	Arnica Creek	-	13	Confined	2006-2007
			14	Confined	.
	Bridge Creek	-	15	Intermediate	2006-2007
			16	Intermediate	.
Yellowstone River	Elk Antler Creek	Unk	17	Unconfined	2005-2006
			18	Unconfined	.
	Trout Creek	Unk	19	Unconfined	2005-2006
			20	Unconfined	.
	Alum Creek	Unk	21	Intermediate	2006-2007
			22	Intermediate	.
	Otter Creek	Unk	23	Confined	2005-2007
			24	Intermediate	.
	Slough Creek	Unk	25	Confined	2006-2007
			26	Intermediate	.
	Thistle Creek	Unk	27	Confined	2006-2007
			28	Intermediate	.

Table 4.2. Environmental features of confinement categories. Values are means (± 1 S.E.), n=number of reaches. Boldface type indicates environmental characteristics that varied among categories (see Table 4.3).

Environmental characteristic		Confinement Category		
		Confined n=8	Intermediate n=11	Unconfined n=9
Channel characteristics	Width (m)	7.38 (2.68)	7.60(2.71)	15.80(4.79)
	Depth (m)	0.24(0.05)	0.40(0.12)	0.33(0.07)
	Velocity (m/s)	0.11(0.05)	0.17(0.05)	0.23(0.07)
	Elevation (m)	2338.16 (66.17)	2365.47 (39.87)	2369.82 (3.90)
	Reach slope (%)	5.18(1.49)	2.44(0.75)	1.69(0.36)
Substrate characteristics	Proportion coarse	0.71(0.09)	0.48(0.09)	0.30(0.05)
	Proportion sand	2.55*10-01 (7.81*10-02)	4.29*10-01 (8.15*10-02)	4.87*10-01 (4.31*10-02)
	Proportion silt	9.04*10-03 (3.47*10-03)	4.53*10-02 (1.60*10-02)	1.37*10-01 (5.00*10-02)
	Proportion clay	2.17*10-02 (6.31*10-03)	4.95*10-02 (8.74*10-03)	7.80*10-02 (1.62*10-02)
	Proportion organic matter	9.96*10-01 (1.90*10-01)	1.09 (1.67*10-01)	1.69(6.74*10-01)
	Temperature (°C)	12.94(2.05)	15.25(2.28)	15.16(1.93)
Physicochemical characteristics	Dissolved oxygen mg/L	7.21(0.76)	6.09(0.66)	8.28(0.89)
	Specific conductivity (mS)	142.88(32.20)	223.84(98.98)	161.93(32.74)
	ph	7.61(0.31)	7.84(0.26)	7.83(0.24)

Table 4.3. Analysis of variance results for differences in environmental characteristics among confinement types. Transforms, if used, are indicated in parentheses below response variables and significant results ($p < 0.05$) are shown in boldface

Environmental characteristic		Source of variation	df	SS(III)	F	P-value
Channel characteristic	Width	Model	2	420.23	1.83	0.1806
		Error	25	2864.26		
	Depth (ln)	Model	2	1.15	1.20	0.318
		Error	25	11.95		
	Velocity	Model	2	0.0524	0.82	0.450
		Error	25	0.794		
	Reach slope	Model	2	0.0366	3.73	0.038
		Error	25	0.122		
Substrate characteristics	Proportion coarse	Model	2	0.733	5.94	0.008
		Error	25	1.54		
	Proportion sand	Model	2	0.244	2.53	0.098
		Error	25	1.21		
	Proportion clay	Model	2	0.259	6.66	0.005
		Error	25	0.487		
	Proportion silt (sqrt)	Model	2	0.0135	5.72	0.009
		Error	25	0.0294		
Physico-chemical characteristics	Temperature	Model	2	29.42	0.34	0.713
		Error	25	1074.27		
	Dissolved oxygen	Model	2	23.86	2.17	0.135
		Error	25	137.23		
	Specific conductivity (ln)	Model	2	3.51*10+04	0.36	0.700
		Error	25	1.21*10+06		
	pH	Model	2	0.28	0.21	0.811
		Error	25	16.71		

Table 4.4. Results of principal components analysis for invertebrate abundance. Invertebrate characteristics with eigenvectors $\geq \pm 0.25$ were considered influential features of the PCA axes and are indicated in boldface.

Invertebrate Family	Principal component 1	Principal component 2	Principal component 3	Principal component 4
Ephemereilidae	0.127	0.109	-0.169	0.169
Baetidae	0.192	0.243	0.029	0.016
Chloroperlidae	0.222	0.279	0.082	0.124
Perlodidae	0.221	-0.314	0.063	-0.076
Limnephilidae	0.087	0.115	-0.131	-0.049
Hydroptilidae	-0.124	-0.070	0.244	0.316
Corixidae	-0.146	-0.020	0.182	-0.072
Gerridae	0.110	0.193	0.097	-0.080
Syrphidae	0.135	0.256	0.132	0.000
Dytiscidae	0.046	0.155	0.102	-0.075
Elmidae	0.220	0.278	0.076	0.175
Haliplidae	-0.062	0.061	0.117	-0.398
Amphizoidae	0.192	0.280	0.164	-0.009
Sialidae	0.007	0.007	-0.264	0.127
Chironomidae	0.287	-0.209	0.074	-0.010
Tipulidae	0.276	-0.238	0.066	-0.016
Ceratopogonidae	0.221	-0.308	0.077	-0.075
Tabanidae	-0.022	0.000	-0.270	0.073
Simuliidae	0.169	0.243	0.163	0.101
Psychodidae	0.231	-0.297	0.096	-0.062
Ptychopteridae	0.054	0.011	-0.318	0.186
Acari	0.295	-0.165	0.102	-0.061
Hyaellidae	-0.153	-0.027	0.342	0.230
Gammaridae	-0.171	-0.074	0.280	0.224
Amphipoda-unknown	-0.184	-0.085	0.290	0.269
Cyclopoida	0.009	0.197	0.191	-0.269
Harpacticoida	-0.010	0.007	-0.160	0.023
Ostracoda	0.280	-0.118	0.165	0.088
Branchiopoda	-0.059	0.071	0.103	-0.416
Sphaeriidae	-0.060	0.031	0.041	0.092
Lymnaeidae	-0.108	-0.002	0.141	-0.091
Hirudinea	-0.132	-0.013	0.182	-0.157
Ryacodrilinae	-0.068	0.007	0.000	-0.277
Enchytraeidae	-0.145	-0.046	0.135	0.086
Tubificidae	-0.277	-0.061	-0.095	-0.832

Table 4.5. Modified Shannon-Weaver index of diversity values for invertebrates among confinement types. Values are means (± 1 S.E.), n=number of reaches.

	Confinement category		
	Confined n=8	Intermediate n=11	Unconfined n=9
Modified Shannon-Weaver Index of Invertebrate Diversity	-1.675 (0.168)	-1.516 (0.174)	-1.363 (0.189)

Table 4.6. *Tubifex tubifex* characteristics including abundance of *T. tubifex* and abundance of lineage III *T. tubifex* among confinement types. Values are means (± 1 S.E.), n=number of reaches. Boldface type indicates variables that differed among categories (see Table 4.9).

Host characteristic	Confinement category		
	Confined n=8	Intermediate n=11	Unconfined n=9
Abundance of <i>T. tubifex</i>	6.51(2.54)	23.36(12.04)	90.42(49.02)
Abundance of lineage III <i>T. tubifex</i>	1.12(0.53)	1.53(0.51)	3.46(0.48)

Table 4.7. Analysis of variance results for *T. tubifex* characteristics among confinement types. Transforms, if used, are indicated in parentheses below response variables and significant results ($p < 0.05$) are shown in boldface.

Host characteristic	Source of variation	df	SS(III)	F	P-value
Abundance of <i>T. tubifex</i> (ln+1)	Model	2	21.73	5.04	0.015
	Error	24	51.72		
Abundance of mtDNA lineage III <i>T. tubifex</i>	Model	2	26.99	5.68	0.010
	Error	24	57.02		

Table 4.8. Parasite characteristics including prevalence of infection in *T. tubifex*, abundance of infected *T. tubifex*, prevalence of infection in sentinel fish, and severity of infection in sentinel fish among confinement types. Values are means (± 1 S.E.), n=number of reaches. Boldface type indicates variables that varied among categories (see Table 4.9).

Parasite characteristic	Confinement category		
	Confined n=8	Intermediate n=11	Unconfined n=9
Prevalence of infection in <i>T. tubifex</i> (%)	0.00(0.00)	3.80(3.35)	3.22(1.00)
Abundance of infected <i>T. tubifex</i> (CPUE)	0.00(0.00)	4.37(4.36)	3.04(1.17)
Prevalence of infection in sentinel fish (%)	0.00(0.00)	100.00(0.00)	100.00(0.00)
Infection severity score in fish (0-5 scale)	0.00(0.00)	1.64(1.01)	2.28(0.94)

Table 4.9. Analysis of variance results for differences parasite characteristics among confinement types including prevalence of infection in *T. tubifex* and abundance of infected *T. tubifex*. Transforms, if used, are indicated in parentheses below response variables and significant results are shown in boldface. Reaches 21, 22, 23, 27, 4, 6, 10 were excluded because we did not detect *T. tubifex*.

Parasite characteristic		Source of variation	df	SS(III)	F	P-value
Oligochaete host	Infection	Model	2	0.06	1.47	0.256
	prevalence in <i>T. tubifex</i>	Error	18	0.34		
	Abundance of	Model	2	3.21	1.58	0.234
	infected <i>T. tubifex</i>	Error	18	18.23		

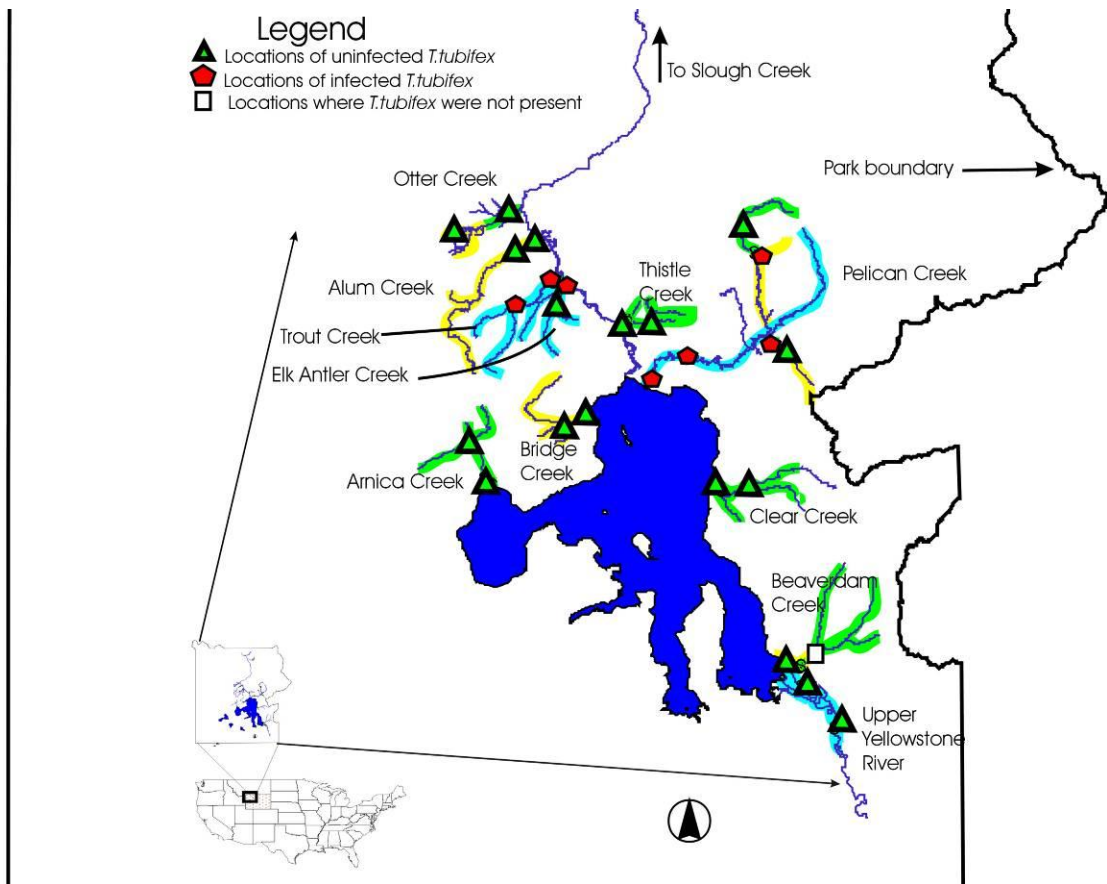


Figure 4.1. Study site, confinement types, and invertebrate collection reaches in Yellowstone National Park in 2005. Tributary names appear in black, unconfined reaches are highlighted in blue, intermediate reaches are highlighted in yellow, and confined reaches are highlighted in green. Slough creek is not shown (lower reach is confined, upper reach is intermediate). Invertebrate sampling locations are indicated by triangles (uninfected *T. tubifex*), hexagons (infected *T. tubifex*), and squares (no *T. tubifex*).

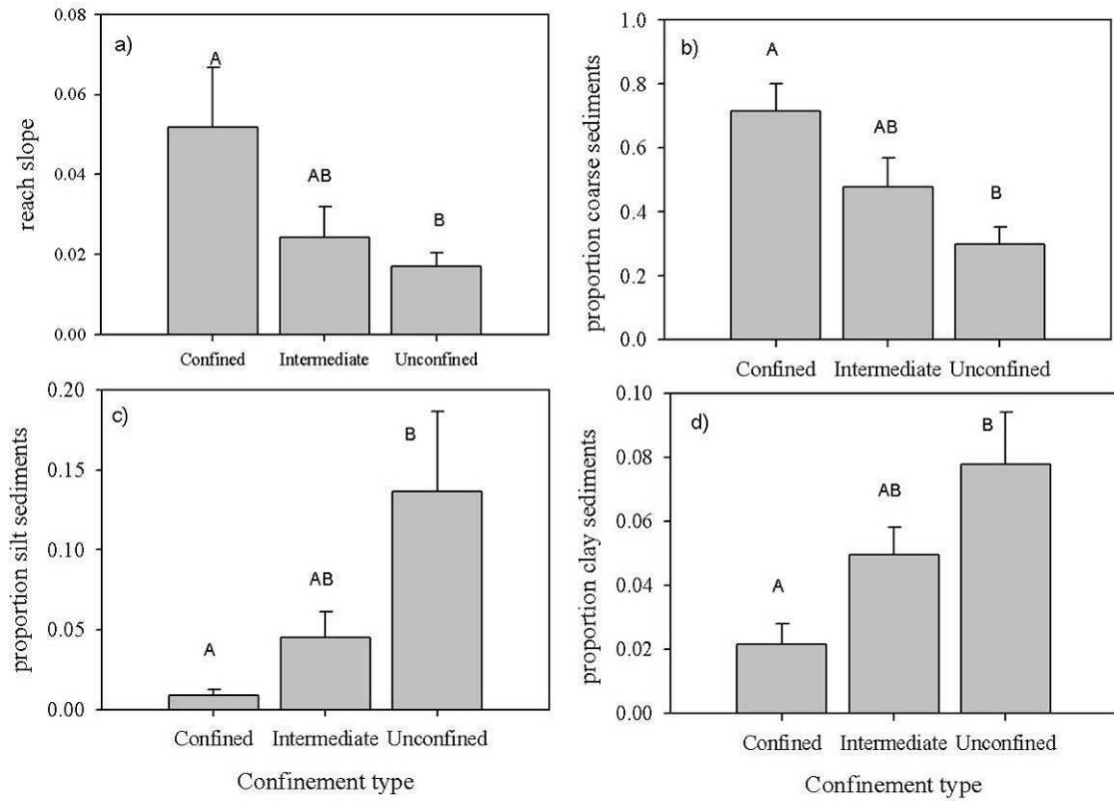


Figure 4.2. Environmental variables that differed among confinement types, including a) reach slope, b) proportion coarse sediments, c) proportion silt sediments, d) proportion clay sediments. Letters represent significant Tukey's HSD results $p < 0.05$.

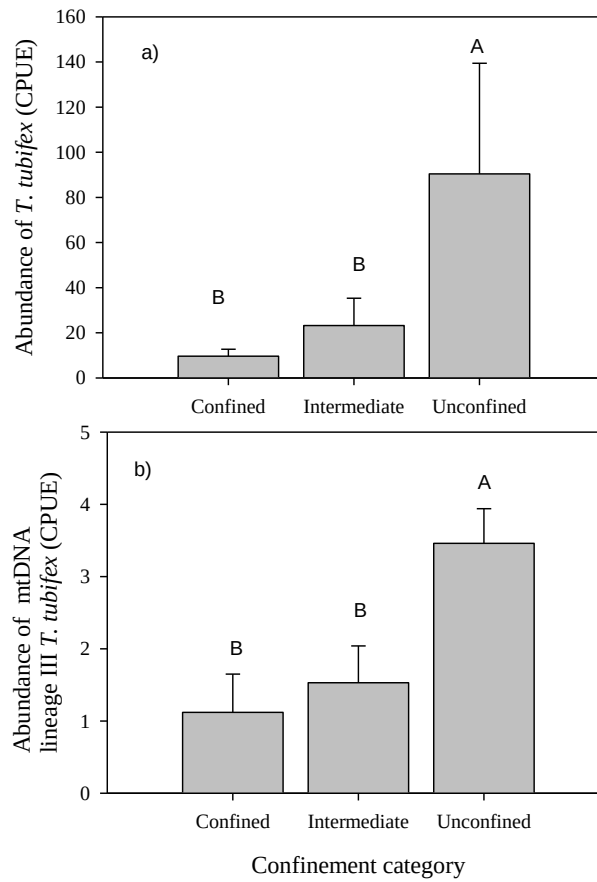


Figure 4.3. Host factors shown by confinement category. Abundance of a) *T. tubifex* and b) lineage III *T. tubifex*. Letters represent Tukey's HSD results, $p < 0.05$.

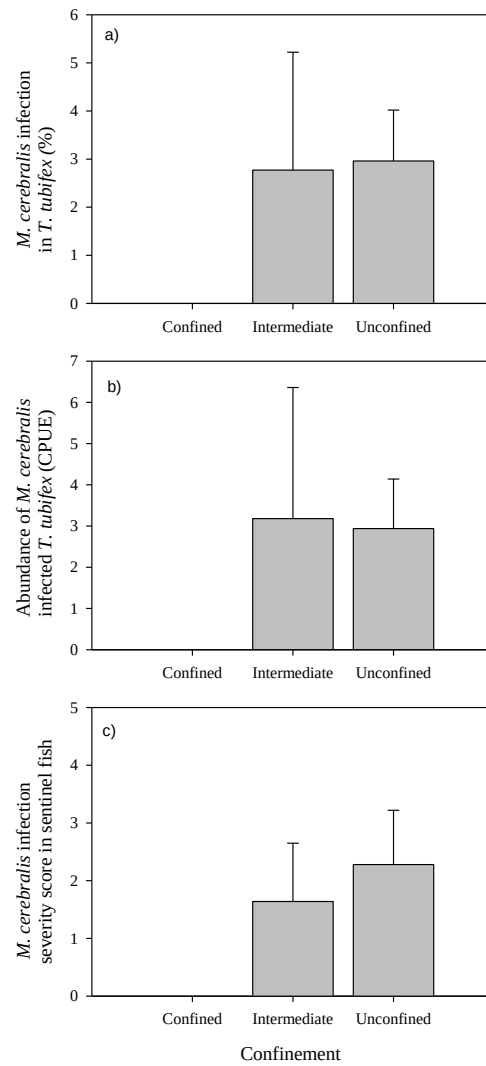


Figure 4.4. Parasite factors shown by confinement category. Mean a) *M. cerebralis* infection prevalence in *T. tubifex*, b) abundance of *M. cerebralis* infected *T. tubifex*, and c) *M. cerebralis* infection severity score in sentinel fish.

CHAPTER FIVE

EFFECTS OF SUBSTRATE ON *TUBIFEX TUBIFEX* AND THE OUTCOME OF *MYXOBOLUS CEREBRALIS* INFECTIONS

Contribution of Authors and Co-Authors

Manuscripts in Chapter 3, Chapter 5, and Chapter 6.

Chapter 5:

Author: Julie D. Alexander

Contributions: Designed the study and conducted the experiment, analyzed data, and wrote the manuscript.

Co-author: Billie L. Kerans

Contributions: Obtained funding and assisted with experimental design and analyses and discussed the results and implications and edited the manuscript at all stages.

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Abstract

Environmental conditions that affect host or parasite success can significantly influence outcomes of host-parasite interactions. We determined the influence of environmental conditions on host and parasite success by examining outcomes of interactions between the aquatic oligochaete, *Tubifex tubifex*, and the myxozoan parasite, *Myxobolus cerebralis*, under different substrate conditions. Substrate has been correlated with the successful parasitism of *T. tubifex* by *M. cerebralis* in natural systems but it is unclear whether substrate directly influences rates of host-parasite encounter, or whether parasite success is influenced by host factors responding to environmental conditions. We used a split-plot laboratory experiment to examine the influence of three substrates, coarse sand, fine sand, and silt on interactions between *T. tubifex* and *M. cerebralis*. The experiment was conducted in two periods; an exposure period (5 days), the aim of which was to determine if substrate influenced rates of host-parasite encounter, followed by a rearing period (143 days), the aim of which was to determine if substrate influenced 1) parasite spore (TAM) production, and 2) measures of host success (population growth, mortality, and progeny production, as well as individual adult growth, progeny growth, and food availability).

Infection prevalence did not differ among *T. tubifex* exposed to *M. cerebralis* on different substrates, which suggested exposure substrate did not influence rates of host-parasite encounter. TAM production did not differ among different substrates. However, all *T. tubifex* reared on coarse sand that were exposed to *M. cerebralis* died, and therefore did not produce TAMs (low parasite success).

Population growth rates were influenced by exposure and rearing substrates, and by *M. cerebralis* infection. In uninfected *T. tubifex*, population growth rates were low when reared on coarse sand and fine sand, except when initially exposed on silt (intermediate) and high on silt, except when exposed on coarse sand (intermediate). Population growth rates of infected *T. tubifex* were low in comparison to uninfected *T. tubifex*, but the same trend was evident. Mortality was highest on coarse substrate, irrespective of dose. Progeny production, was highest in uninfected *T. tubifex* reared on silt. Progeny biomass was highest when produced by uninfected *T. tubifex* exposed and reared on silt, and interestingly, lowest when produced by infected *T. tubifex* exposed and reared on silt. These results suggested coarse sand may be suboptimal for uninfected *T. tubifex*, and silt and fine sand may be more optimal. Combined with infection prevalence, which did not differ among substrates, these results suggested that the probability of parasite production by *T. tubifex* would be low when environments are dominated by coarse substrates because infected hosts cannot survive long enough for parasite transmission to occur.

Introduction

Environmental conditions influence host-parasite interactions and may ultimately determine parasite success (Harvell et al. 2002, Patz et al. 2004, Fels and Katz 2006, Calvo et al. 2007). For example, environmental conditions influence abundance of hosts and parasites (Sousa and Grosholz 1991, Krashnov et al. 1997, Marcogliese 2001, Mouritsen et al. 2003), which in turn may influence rates of host-parasite encounter (Anderson and May 1988, Hedrick 1998, Agnew and Koella 1999, Gerber et al. 2005). Rates of host-parasite encounter influence the probability that an individual parasite will infect a host and be transmitted to the next host (parasite success; Anderson and May 1988, Hedrick 1998, Agnew and Koella 1999, Gerber et al. 2005).

Environmental conditions influence other aspects of host ecology that may also influence parasite success (e.g., Blazer 1992, Lafferty and Kuris 1999, Marcogliese 2001, Lafferty 2009), including resource availability, which may influence the outcome of host-parasite interactions. For example, *Vavraia culicis* (microsporidian parasite) proliferation and spore production in *Aedes aegypti* mosquitoes are proportional to host food availability (Bedhomme et al. 2004). This result suggests resource availability influenced the outcome of interactions between *V. culicis* and *A. aegypti*. The influence of resource availability on parasite success has also been observed in other host-parasite interactions (e.g., Crompton 1987, Jokela et al. 2005, Zanette and Clinchy *in press*).

We investigated the effects of environmental conditions on parasite success by examining interactions between the aquatic oligochaete, *Tubifex tubifex*, and the myxozoan parasite, *Myxobolus cerebralis*. *Myxobolus cerebralis* causes whirling disease in salmonids

(Hofer 1903 in Bartholomew and Reno 2002). The life cycle of this complex parasite involves two hosts, a salmonid and *T. tubifex*, and two environmentally transmitted spore stages, myxospores and triactinomyxons (TAMs) (Markiw and Wolf 1983, Wolf and Markiw 1984, Andree et al. 1997). *Tubifex tubifex* produces TAMs, which are infective to salmonids, and salmonids produce myxospores, which are infective to *T. tubifex* (Wolf and Markiw 1984, El-Matbouli and Hoffman 1989, Markiw 1992). Triactinomyxons float passively in the water column where they encounter and infect salmonids, whereas myxospores settle out in stream benthos where they are consumed by *T. tubifex* feeding on substrate particles and associated organic matter (Kerans and Zale 2002).

Substrate is an important physical component of natural benthic environments that may influence the outcome of interactions between *T. tubifex* and *M. cerebralis*. In the laboratory, TAM production by *T. tubifex* was inversely proportional to substrate size (Blazer et al. 2003). In the Madison River, MT, Krueger et al. (2006) found that abundance of *M. cerebralis* TAM spore stages, measured indirectly as risk to fish, was higher in environments characterized by high proportions of fine sediments than in those characterized by low proportions of fine sediments. Environments characterized by high proportions of fine sediments were also characterized by high densities of *T. tubifex* so it is unclear if TAM abundance was a function of environmental conditions, or host or parasite ecology, or interactions among these factors. Although the mechanism is not clear, these results suggest substrate may influence *M. cerebralis* success.

Substrate could influence the success of *M. cerebralis* in several ways. First, substrate may influence the distribution and abundance of *T. tubifex*, also influencing rates of

host-parasite encounter and the probability a myxospore will be encountered and consumed by a susceptible host. *Tubifex tubifex* inhabits a range of freshwater environments (Brinkhurst 1971, Timm 1980, Holmquist 1983, Anlauf 1994), but is often abundant in habitats characterized by fine sediments and not abundant in habitats characterized by coarse sediments (Brinkhurst 1971, Sauter and Gude 1996, Verdonchot, 1999, 2001, Krueger et al. 2006, Anlauf and Moffitt 2008). Consequently, *M. cerebralis* success may be higher when environments are characterized by fine substrates than coarse substrates because myxospores may be more likely to be encountered and ingested when *T. tubifex* is abundant.

Second, the physical properties of substrates may influence the probability a myxospore will be encountered and consumed by *T. tubifex*. Myxospores adhere to small particles (Lemmon and Kerans 2001) and *T. tubifex* actively selects small particles when foraging (McMurtry et al. 1983, Rodriguez et al. 2001), so the rate of myxospore encounter by *T. tubifex* may be high in environments dominated by small, silt sized (<63µm in diameter) particles. In contrast, the rate of myxospore encounter by *T. tubifex* may be low in environments dominated by coarse substrate particles because myxospores, which are <10µm in diameter, may settle in interstitial spaces where they may be less frequently encountered and ingested by *T. tubifex* during foraging. Myxospore dose is directly proportional to infection in *T. tubifex* (Elwell et al. 2009) so rates of myxospore encounter should influence prevalence of infection (parasites per group) in *T. tubifex* and severity of infection (parasites per individual) in *T. tubifex*, which are both factors that may influence *M. cerebralis* success.

Finally, substrate may influence food availability for *T. tubifex*, which may influence the outcome of infections in *T. tubifex* and thus may influence parasite success. Resources

that would otherwise be available for reproduction or survival (e.g., reduced fecundity in infected *T. tubifex*; Elwell et al. 2006) are diverted towards parasite proliferation of *M. cerebralis*. *Tubifex tubifex* feed by ingesting substrate particles and digesting the associated organic material (Binkhurst 1971, Rodriguez et al. 2001). Smaller particles have relatively more surface area available for bacteria and other organic material than larger particles (Shepard and Minshall 1984, Stratzner and Higler 1986). This property, which may also affect myxospore adsorption, likely makes smaller particles optimal for foraging *T. tubifex*, and may explain why they select silt and clay (Rodriguez et al. 2001). In addition, growth and reproduction in *T. tubifex* are positively correlated with increased organic material (>2%; Kaster 1980, <2% is common in headwater streams, Wallace et al. 1999). Thus, infected *T. tubifex* in environments characterized by increased food availability may produce more TAMs than equally infected *T. tubifex* inhabiting environments characterized by decreased food availability because they may have additional resources available for parasite proliferation and success.

The aim of this experiment was to investigate the potential for substrate to influence the success of the parasite, *M. cerebralis*. We examined host factors that may influence parasite success including population growth rates, components of population growth, including mortality and progeny production. We also examined progeny biomass, individual adult growth, and food availability. We hypothesized that substrate would either compound (coarse substrate) or mitigate (silt substrate) costs of *M. cerebralis* infection in *T. tubifex*, which would be detected in measures of host success (Figure 5.1a). We examined parasite factors that influence parasite success, including infection prevalence and TAM production.

We hypothesized that infection prevalence and TAM production would be higher on silt than other substrates due to higher rates of myxospore encounter (Figure 5.1a). We also hypothesized that infected *T. tubifex* would produce more TAMs when reared on silt than equally infected *T. tubifex* reared other substrates because we expected silt would be the most optimal habitat for *T. tubifex* and infected *T. tubifex* reared on optimal substrate would have more resources available for TAM production.

Methods

Experimental Design

Tubifex tubifex laboratory cultures were established in 2007 with *T. tubifex* collected from Pelican Creek in Yellowstone National Park, (Koel et al., 2006, Chapter 3) and propagated as described in Stevens et al. (2001). The *T. tubifex* from this culture were susceptible to *M. cerebralis* in a previous experiment (Chapter 6).

We used a split plot design to test the effects of substrate on the outcomes of interactions between *M. cerebralis* and *T. tubifex* (Figure 5.1b). The experiment was conducted in two periods, a short exposure period and a longer rearing period. During the exposure period, we manipulated substrate and parasite exposure (doses of 0 or 500 myxospores per worm) to test the effects of substrate (coarse sand (500-2000 μm), fine sand (125-250 μm), and silt (<63 μm)) on infection prevalence in *T. tubifex* (infection prevalence was assumed to reflect myxospore encounter). In addition, we examined how myxospore encounter influenced mortality and individual growth over the short term. During the rearing period we moved subsets of *T. tubifex* from exposure period groups onto one of the three substrate types (coarse sand, fine sand, or silt; Figure 5.1b) to examine the effects of substrate

on 1) on population growth rates of *T. tubifex* and *M. cerebralis* infected *T. tubifex* and 2) TAM production by infected *T. tubifex* (Figure 5.1c). We examined differences in organic material among substrate treatments at the end of the rearing period to determine if food availability differed among substrates (Figure 5.1c).

Exposure Period. We used six combinations of substrate type and myxospore dose (exposure combinations) including coarse sand with no myxospores added (coarse+0), coarse sand with 17500 (500 myxospores x 35 *T. tubifex*) myxospores added (coarse+500), fine sand with no myxospores added (fine+0), fine sand with 17500 myxospores added (fine+500), silt with no myxospores added (silt+0), and silt with 17500 myxospores added (silt+500), and three replicates for each exposure combination for a total of 18 containers. Each exposure container held 50mL of one of the three substrates. Substrate fractions were obtained by separating commercially available masonry sand through a series of five stacked 12 in. brass sieves including 2.00 mm (#10), 500 μ m (#35), 250 μ m (#60), 125 μ m (#120), and 63 μ m (#230) (ATM testing sieves, BenMeadows, WI). Coarse sand included material that passed through the 2.00 mm sieve but was retained on the 500 μ m sieve. Fine sand included material that passed through the 250 μ m sieve but was retained on the 125 μ m sieve. Silt included material that passed through the 63 μ m sieve. Substrate fractions were burned in a muffle furnace for two hours at 550° C prior to the experiment to remove any organic material.

Prior to the exposure period, *T. tubifex* were removed from culture and held without substrate for 24 hrs to equalize hunger levels. Groups of 35 *T. tubifex* were wet weighed to the nearest mg and randomly assigned to an exposure container. We used 35 *T. tubifex* in

each container to ensure that we would have at least 30 *T. tubifex* for the rearing period. A suspension of 17500 myxospores (500 myxospores per *T. tubifex*) or an equivalent volume of spore-free emulsion (0 myxospores per *T. tubifex*) was added to each exposure container. Myxospores were extracted from three laboratory infected rainbow trout *Onchorhynchus mykiss* obtained from the National Fish Health Research Laboratory, USGS, Kearneysville, WA using the continuous plankton centrifuge method (O'Grodnick 1975, Elwell et al. 2009). Extracted myxospores were enumerated in 1 μ L suspensions on hemacytometer under a compound microscope (400x). The total number of myxospores extracted was determined as (mean number of myxospores in three hemacytometer counts) x (total volume extracted). Spore-free emulsion was extracted from disease free laboratory reared rainbow trout by the same method. The master suspensions were mixed with a magnetic stirrer during dose administration to ensure even distribution.

Containers were incubated at 15°C on a 12:12 light dark cycle without air for 24h to allow myxospores to settle and then supplied with air stones and maintained for an additional five days. This combination of myxospore dose and exposure time was chosen because it produced infection in previous experiments (Elwell et al. 2009).

After six days, surviving *T. tubifex* were counted and wet weighed to the nearest mg in groups and returned to their original container. Mortality was calculated as [(number of *T. tubifex* pre-exposure period - number of *T. tubifex* post exposure period) / number of *T. tubifex* pre-exposure period]. Individual growth was calculated as [weight of surviving *T. tubifex* post-exposure period / total number of surviving *T. tubifex* post-exposure period - weight of pre-exposure period *T. tubifex* / 35]. Progeny were not produced during this period so we did

not examine progeny production or population growth. Although *T. tubifex* were exposed to *M. cerebralis* only during the exposure period, infection prevalence in *T. tubifex* was determined following the rearing period because prevalence was assayed as the proportion of *T. tubifex* releasing TAMs (see below, assays for infection), which takes approximately 90 days.

Rearing Period. Thirty *T. tubifex* were randomly selected from individuals remaining in each exposure container at the end of the exposure period and wet weighed to the nearest mg in groups of 10. Each group of 10 was assigned to a rearing container, which held 20 mL of fresh substrate so that *T. tubifex* from each exposure container were moved to one rearing container with coarse sand, one with fine sand, and one with silt, for a total of 54 rearing containers (Figure 5.1). Rearing containers were randomly assigned to one of two incubators and maintained as above for 150d. Water was changed by removing and replacing ~90% of the water once a week. *Spirulina spp.* (0.125 g) was added to each container following weekly water changes to stimulate the growth of bacteria. Containers were randomly re-assigned to incubators following weekly water changes.

At the end of the rearing period surviving *T. tubifex* were identified as adults or progeny, counted, and wet weighed to the nearest mg. In general, distinguishing between adults and progeny was not difficult because progeny were much smaller than *T. tubifex* at the beginning of the experiment (e.g., Elwell et al. 2006). Population growth rate was calculated as $[\ln(\text{abundance of } T. \text{ tubifex post rearing period} - \text{abundance of } T. \text{ tubifex pre rearing period}) / \text{days in rearing period}]$.

We examined factors that may directly influence population growth rate including adult mortality and progeny production. Adult mortality was calculated as [(number of *T. tubifex* pre rearing period - number of *T. tubifex* post rearing period)/ number of *T. tubifex* pre rearing period]. Number of progeny produced per initial adult was calculated as [number of progeny/number of initial adult *T. tubifex* pre rearing].

We also examined other factors that may affect population growth rate including individual growth and progeny biomass. Individual adult growth was calculated as [weight of *T. tubifex* post rearing period/number of *T. tubifex* post rearing period]-[weight of *T. tubifex* pre rearing period/number of *T. tubifex* pre rearing period]. Progeny biomass produced per initial adult was calculated as [weight of progeny/total number of initial *T. tubifex* pre rearing].

Organic Material. We determined the percent of organic material retained on substrate types post rearing period by ash free dry mass (AFDM; Hauer and Lamberti 1996) to assess potential differences in food availability among substrate types. At the end of the rearing period substrate was removed from each container and two randomly selected subsamples (~15g each) were dried, weighed, and burned for 2h at 550°C, and the proportion organic material was calculated as [(dry wt-burned wt)/dry wt*100]. Negative (substrate and dechlorinated water) and positive (substrate, dechlorinated water, and *Spirulina spp.*) controls created post experiment were held in incubators at 15°C for three months with weekly water changes and processed as above.

Infection Assays. Beginning 60d post exposure (900 degree days), we examined water collected from each replicate during weekly water changes for the presence of TAMs. Water was filtered through a sieve made from a section of PVC pipe fitted with 20 μm mesh (Nytex, Wildco, FL). Sieved contents were backwashed into a 50 mL centrifuge tube with 10-15mL water and three 100 μL subsamples were placed on 1mm glass slides that were air dried prior to examination (phase contrast 200x). Triactonomyxons were quantified as [mean TAMs /100 μL x volume of sample in centrifuge tube]. Water collections were combined across replicates until TAMs were detected. Once TAMs were detected, water samples were filtered individually.

We calculated infection prevalence and total TAMs produced. Infection prevalence was determined by observing surviving adults for TAM release over a four day observation period beginning on day 148 (Krueger et al. 2006, Elwell et al. 2009). Adults were placed in 12 well tissue culture plates for 48 h on day 148. Adults were transferred to new 12 well plates with fresh water for 48 h on day 150. Well plates were dried and examined with a inverted compound microscope (phase contrast 200x) to determine presence or absence of TAMs in each well. Infection prevalence was calculated as the number of surviving adults that produced TAMs while held in tissue culture plates/number of surviving adults. Total TAMs produced was calculated per replicate as sum of TAMs produced from days 60-148.

Data Analysis

Exposure Period. We used one way ANOVAs (PROC GLM, SAS) to test for differences in pre-exposure weights, mortality, and individual growth among exposure combinations. Residuals were examined for normality and significant effects were followed

with Tukey's honestly significant difference (HSD) tests. We used the Bonferroni correction to correct for multiple comparisons (Holm 1979).

Rearing Period. We used mixed model ANOVAs (PROC GLM, SAS) to test for differences in population growth rates, mortality of *T. tubifex*, and organic material. In the model, exposure container, which was nested within exposure combination (coarse+0, coarse+500, fine+0, fine+500, silt+0, and silt+500) and rearing substrate were tested over the subplot error term and exposure combination was tested over the whole plot error term using appropriate degrees of freedom. Residuals were examined for normality and significant effects were followed with Tukey's HSD tests. We used the Bonferroni correction to correct for multiple comparisons (Holm 1979). We tested for differences in individual adult growth and progeny number and biomass produced per initial adult using mixed model ANOVAs as above except replicates reared on coarse sand were removed from the analysis because of high mortality in this group. We did not analyze infection prevalence because all surviving *T. tubifex* that were exposed to myxospores released TAMs. We used mixed model ANOVA (PROC GLM, SAS) to test for differences in total TAM production as above, except all +0 dose replicates were removed from the analysis.

Results

Exposure Period

We did not detect differences in pre-exposure weights or mortality among exposure combinations (Tables 5.1 and 5.2). Individual growth was affected by exposure combination

(Table 5.2) and was highest in the fine+0 and silt+0 and lowest in the fine+500 and silt+500 exposure combinations (Figure 5.2).

Rearing Period

Population growth was affected by rearing substrate and exposure combination (Table 5.3). Population growth was negative in replicates reared on coarse sand, regardless of exposure combination (Figure 5.3). Population growth was negative in replicates that were reared on fine sand except those from the silt+0 exposure combinations. Population growth was positive in replicates that were reared on silt except those from the coarse+0, coarse+500, or fine+500 exposure combinations, which were negative.

Adult mortality was also influenced by both rearing substrate and exposure combination (Table 5.4). Adult mortality was highest in *T. tubifex* reared on coarse substrate except in those from the silt+0 exposure combination (Figure 5.4). Adult mortality was low in replicates reared on fine sand when from silt+0 exposure combination and in replicates reared on silt when from the fine+0 and silt+0 exposure combinations (Figure 5.4).

Progeny were produced by replicates reared on fine sand from silt+0 exposure combinations (n=3/3) and by replicates reared on silt from coarse+0 (n=3), fine+0 (n=3), silt+0 (n=3/3), and silt+500 (n=2/3) exposure combinations. Rearing substrate and exposure combination had an effect on number of progeny produced per initial adult but we did not detect a container effect (Table 5.4). Replicates reared on silt from silt+500 and silt+0 exposure combinations produced high numbers of progeny and replicates reared on fine sand from the silt+0 exposure combinations and those reared on silt from the coarse+0 exposure combinations produced low numbers of progeny (Figure 5.5).

Individual growth did not differ among treatments (Table 5.5, Figure 5.6). Rearing substrate and exposure combination had a significant effect on progeny biomass produced per initial adult but we did not detect a container effect (Table 5.5). Replicates reared on silt from silt+0 exposure combinations produced the greatest progeny biomass, and in contrast to trends in the number of progeny produced per initial adult, replicates reared on silt from the silt+500 exposure combinations produced the lowest progeny biomass (Figure 5.7).

Organic Material. Organic material retained on substrates post rearing period differed among rearing substrates, but not among exposure combinations (Table 5.6). The amount of organic material in replicates reared on fine sand was higher than organic matter in replicates reared on coarse sand and in the replicates from the 500 dose exposure combinations that were reared on silt (Figure 5.8). However, we did not detect differences in organic material among replicates reared on silt from any of the 0 dose exposure combinations and coarse sand.

Infection Assays. All surviving adults released TAMs, which demonstrated that prevalence of infection was 100% in surviving individuals. *Triactinomyxon* production did not differ among rearing substrate, exposure combination or container (Table 5.7, Figure 5.9). Although not significant, rearing substrate clearly influenced parasite success because infected *T. tubifex* reared on coarse sand all died and therefore did not produce TAMs.

Discussion

We examined the potential for environmental conditions to influence parasite success by examining interactions between *T. tubifex* and *M. cerebralis*, under different substrate conditions. We hypothesized that substrate influenced the transmission of myxospores to *T.*

tubifex and subsequent production of TAMs. We tested our hypotheses in two objectives conducted in two periods, an exposure period and a rearing period. We separated the rearing period from the exposure period in order to be able to determine whether potential differences in TAM production were a function of the rate of myxospore encounter by *T. tubifex* or the level of TAM production on different substrates.

During the exposure period, we were interested in the effects of substrate on rate of myxospore encounter by *T. tubifex*, which we measured as infection prevalence in *T. tubifex*. We hypothesized encounter would be highest on silt and lowest on coarse sand. *Tubifex tubifex* exposed to *M. cerebralis* on silt and fine sand lost weight (negative adult growth) and *T. tubifex* that were not exposed to *M. cerebralis* gained weight (positive adult growth) on silt and fine sand. This result suggested *T. tubifex* on fine sand and silt were becoming infected and experiencing a cost associated with infection, perhaps related to an attempt at mounting an immune response. In contrast, we did not detect differences in individual adult growth among *T. tubifex* on coarse sand during the exposure period, and although not significant, trends in individual adult growth suggested *T. tubifex* on coarse sand that were exposed to *M. cerebralis* gained weight. Since coarse sand is likely poor habitat for *T. tubifex* and low potential for myxospore encounter (which we will argue below), this result may indicate that *T. tubifex* exposed to *M. cerebralis* under poor conditions derive some nutritional benefit from light exposure and infection.

During the rearing period we hypothesized population growth would be highest when *T. tubifex* and infected *T. tubifex* were reared on silt and lowest on coarse sand. However, we expected groups of *T. tubifex* exposed to the 500 myxospore dose of *M. cerebralis* would

have lower rates of population growth than groups exposed to the 0 myxospore dose because infection is known to reduce fecundity of *T. tubifex* (Elwell et al. 2006). We measured population growth rates of *T. tubifex* because abundance of hosts is influential for short and long term parasite success. Positive population growth rates were observed only in treatments that were reared on silt or fine sand that had not been exposed to *M. cerebralis*. This result suggested that silt and fine sand are optimal and coarse sand is poor habitat for *T. tubifex*.

Negative population growth rates were influenced by high adult mortality during the rearing period. Adult mortality was high during the rearing period in all treatments involving coarse substrate, regardless of whether it was during the short (six day) exposure period or the long (142 day) rearing period. The high adult mortality was not surprising when coarse substrate was involved during the rearing period because *T. tubifex* probably were unable to feed sufficiently over the 142 day period. *Tubifex tubifex* feed by ingesting substrate particles and digesting the associated organic material (Binkhurst 1971, Rodriguez et al. 2001) and mean and maximum diameter of particles observed in the digestive tract of *T. tubifex* were <63 μm and <130 μm , respectively (Juget, 1979, Juget and Lafont 1994). Coarse sand would be suboptimal for *T. tubifex* if they are unable to ingest particles greater >130 μm .

In addition, we observed high adult mortality during the rearing period for *T. tubifex* that were exposed to coarse substrate during the six day exposure period. These results suggest that only six days of exposure to poor environmental conditions was sufficiently detrimental to cause high mortality at a later time. Mortality was high in groups that were

reared on silt when they had been on coarse sand during the exposure period. Interestingly, when groups were exposed on coarse sand to *M. cerebralis* we observed the opposite (low mortality during the rearing period), which may also suggest *T. tubifex* either benefited from or experienced a lower cost of infection when conditions were poor.

Positive population growth rates were driven by high progeny production during the rearing period. With one exception, the only groups that produced progeny were replicates that had not been infected with *M. cerebralis*. The exception was the treatment group reared on silt from the silt+500 exposure combination (n=2 replicates). Interestingly, the replicates from this treatment group produced the greatest number of progeny during the experiment but progeny were characterized by low biomass compared to progeny produced by replicates from other treatment groups. Assuming the progeny from these replicates were produced sexually, this result could represent an adaptive strategy for parasite escape and may support our hypothesis that myxospore encounter by *T. tubifex* was highest on silt. Alternatively, this result could be further evidence that silt substrate is optimal for *T. tubifex* because no other groups of infected *T. tubifex* produced progeny.

We assessed organic matter retained in rearing containers at the end of the rearing period as a measure of food availability because potential differences in food availability could explain potential differences in host factors or parasite success among substrates. We hypothesized that percent organic material would be negatively correlated with substrate size and that substrates with the higher proportions of organic material would be optimal for *T. tubifex* and infected *T. tubifex*. Our results showed fine sand retained more percent organic material than coarse sand and that silt retained more organic material than fine or coarse sand

in the 0 dose groups only. We observed the expected negative relationship between organic material and substrate size in the controls (although we did not test for differences among the organic material controls). Thus, it was surprising that fine sand and silt from only the 0 dose exposure combinations retained the highest proportions of organic material. This result may explain why *T. tubifex* were able to survive on the sub-optimal fine sand even when infected by *M. cerebralis*. Others have shown that impact of parasites on host fitness and survival may be inversely proportional to the optimality of environmental conditions (e.g., DeLope et al. 1998).

We assessed the potential for substrate to influence *M. cerebralis* success by examining prevalence of infection in *T. tubifex* as a measure of rate of myxospore encounter. We had expected infection prevalence would be high in *T. tubifex* exposed to *M. cerebralis* on silt and fine sand and low in *T. tubifex* exposed to *M. cerebralis* on coarse sand because we observed differences in individual growth that suggested the frequency of myxospore encounter was increased on silt and fine sand. In fact, our results suggested substrate did not influence the rate of myxospore encounter by *T. tubifex* because we observed 100% infection prevalence in all surviving *T. tubifex* exposed to *M. cerebralis*. This result is consistent with a previous study that demonstrated a dose of 50 myxospores caused 100% infection prevalence in *T. tubifex* (at densities approximately twice the densities we used in this experiment, Elwell et al. 2009). The threshold dose of myxospores that causes infection in *T. tubifex* is unknown and it is unlikely that all infected *T. tubifex* were equally infected because a single myxospore may have produced infection. In future experiments, this could be resolved using molecular tools designed to assess parasite load (e.g., qPCR, Cavender et al.

2004). In natural systems, substrate probably most strongly influences the encounter rate between *T. tubifex* and myxospores by influencing abundance of *T. tubifex* because infection prevalence is typically low (<10%).

We also assessed the potential for substrate to influence *M. cerebralis* success by examining the total number of TAMs produced by *T. tubifex*. We hypothesized that TAM production would be highest in *T. tubifex* on silt (assuming equally infected *T. tubifex* because Blazer et al. (2003) showed a trend, although non-significant, that suggested TAM production by infected *T. tubifex* was increased in mud, which was probably composed of silt and clay sized particles, when compared with sand substrates.

Distribution and abundance of *T. tubifex* have been correlated with fine sediments in many systems (Verdonschot 2001, Burkhardt and Hubert 2005, Anlauf and Moffitt 2008). However, ‘fine sediments’ is often a catch-all term used to refer to substrates < 2mm in diameter (but see Krueger et al. 2006). The results from our experiment suggest that individual fractions within the ‘fine sediments’ substrate type may importantly influence population growth and mortality in *T. tubifex*, and that under certain substrate conditions (e.g., high proportions of coarse sand rather than silts as fine sediments), *M. cerebralis* infected *T. tubifex* would not be a problem because they cannot survive.

The results from our experiment suggest that environmental factors may exert an important influence on parasite success through the host, *T. tubifex*. Thus, the manipulation of substrate could create a natural ‘breaking point’ for the life cycle in natural systems because *M. cerebralis* infected *T. tubifex* likely cannot survive and produce TAMs in environments where ‘fine sediment’ is composed dominated by larger grain sizes (>250 µm).

This may bolster arguments for management practices targeting riparian conservation and re-vegetation or other practices that may reduce the load of fine substrates reaching streams inhabited by *T. tubifex* and *M. cerebralis*. In addition, our results underscore the importance of actually determining composition of fine sediments (e.g., Krueger et al. 2006), especially the proportions of silt and clay when conducting field studies.

Applied whirling disease research has largely focused on identifying factors that limit the abundance of *M. cerebralis* without investigating the possible mechanisms.

Consequently, we lack complete understanding of how infection and disease may change under different environmental contexts. Many environmental conditions cannot effectively be manipulated in natural environments and thus have limited management applicability for reducing parasite success, and ultimately, disease. However, substrate can be manipulated in natural systems (Osborne and Kovacic 1993, Schofield et al. 2004) and may therefore be useful for applied management and disease control strategies in the context of parasites that are dependent on benthic invertebrates at some point during their life cycle.

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Table 5.1. Exposure period means (± 1 SE) for pre-exposure weights and mortality by exposure combination. Exposure combinations included coarse sand with no myxospores added (coarse+0), coarse sand with 17500 (500 myxospores x 35 *T. tubifex*) myxospores added (coarse+500), fine sand with no myxospores added (fine+0), fine sand with 17500 myxospores added (fine+500), silt with no myxospores added (silt+0), and silt with 17500 myxospores added (silt+500), and three replicates for each exposure combination for a total of 18 containers.

Exposure period variable	Exposure Combination					
	Coarse +0	Coarse +500	Fine +0	Fine +500	Silt +0	Silt +500
Pre-exposure weight	0.20 (0.002)	0.19 (0.002)	0.20 (0.002)	0.20 (0.002)	0.20 (0.0003)	0.20 (0.001)
Mortality	0.04 (0.03)	0.12 (0.03)	0.06 (0.03)	0.01 (0.01)	0.08 (0.04)	0.06 (0.00)

Table 5.2. Exposure period ANOVAs for the effects of exposure combination (coarse+0, coarse+500, fine+0, fine+500, silt+0 and silt+500) on pre-exposure weights, mortality, and individual adult growth. Transforms, if used, are indicated in parentheses below response variable and significant results are shown in boldface.

Exposure period variable	Source of variation	SS(III)	F	df	P-value
Pre-exposure weight	Exposure combination	6.6×10^{-7}	1.48	5	0.268
	Error	1.1×10^{-4}		12	
Mortality	Exposure combination	0.022	1.99	5	0.152
	Error	0.027		12	
Individual growth	Exposure combination	7.296×10^{-6}	11.19	5	0.0003
	Error	1.564×10^{-6}		12	

Table 5.3. Rearing period mixed model ANOVA results for the effects of exposure combination, rearing substrate, and initial container number on population growth rate in *T. tubifex*. Transforms, if used, are indicated in parentheses below response variable and significant results are shown in boldface.

<i>Rearing period variable</i>	<i>Source</i>	<i>DF</i>	<i>Type III SS</i>	<i>F</i>	<i>P-value</i>
Population growth rate (ln final abundance of <i>T. tubifex</i> -ln initial abundance of <i>T. tubifex</i>)	container(exposure-dose combination)	12	8.48x10 ⁻⁰⁴	1.03	0.45
	rearing substrate	2	4.27 x10⁻⁰³	31.00	<0.0001
	Error (subplot)	34	2.34 x10 ⁻⁰³		
	exposure-dose combination	5	2.01 x10⁻⁰³	5.68	0.0065
	Error (whole plot)	12	8.48 x10 ⁻⁰⁴		

Table 5.4. Rearing period ANOVA results for the effects of exposure combination, rearing substrate, and initial container number on components of population growth in *T. tubifex*, including adult mortality and number of progeny produced per initial adult. Transforms, if used, are indicated in parentheses below response variable and significant results are shown in boldface.

<i>Rearing period variable</i>	<i>Source</i>	<i>DF</i>	<i>Type III SS</i>	<i>F</i>	<i>P-value</i>
Proportion adult mortality	container(exposure-dose combination)	12	0.53	0.54	0.871
	rearing substrate	2	2.68	16.44	<0.0001
	Error (subplot)	34	2.77		
	exposure-dose combination	5	3.56	16.07	<0.0001
	Error (whole plot)	12	0.53		
Number of progeny produced per initial adult (ln)	container(exposure-dose combination)	12	0.24	0.51	0.874
	rearing substrate	1	0.61	15.13	0.0021
	Error (subplot)	12	0.48		
	exposure-dose combination	5	0.88	8.24	0.0008
	Error (whole plot)	14.15	0.30		

Table 5.5. Rearing period ANOVA results for the effects of exposure combination, rearing substrate, and initial container number on individual adult growth and progeny biomass produced per initial adult. Transform is indicated in parentheses below response variable and significant results are shown in boldface. All coarse sand replicates were excluded due to high mortality so rearing substrate df=1.

<i>Rearing period variable</i>	<i>Source</i>	<i>DF</i>	<i>Type III SS</i>	<i>F</i>	<i>P-value</i>
Individual adult growth (sqrt)	container(exposure-dose combination)	12	2.11 x10 ⁻⁰⁸	2.59	0.059
	rearing substrate	1	3.05 x10 ⁻¹⁰	0.44	0.518
	Error (subplot)	12	8.23 x10 ⁻⁰⁹		
	exposure-dose combination	5	1.22 x10 ⁻⁰⁸	1.43	0.281
	Error (whole plot)	12.5	2.12 x10 ⁻⁰⁸		
Progeny biomass produced per initial adult (sqrt)	container(exposure-dose combination)	12	0.0029	0.50	0.902
	rearing substrate	1	0.0056	10.45	0.0063
	Error (subplot)	12	0.0062		
	exposure-dose combination	5	0.011	8.26	0.0007
	Error (whole plot)	14.35	0.0034		

Table 5.6. Rearing period ANOVA results for the effects of exposure combination, rearing substrate, and initial container number on organic material. Transform is indicated in parentheses below response variable and significant results are shown in boldface.

<i>Variable</i>	<i>Source</i>	<i>DF</i>	<i>Type III SS</i>	<i>F</i>	<i>P-value</i>
% organic material (ln)	container(exposure-dose combination)	12	0.16	0.80	0.649
	rearing substrate	2	3.13	92.91	<0.0001
	Error (subplot)	33	0.55		
	exposure-dose combination	5	0.19	2.89	0.061
	Error (whole plot)	12.12	0.16		

Table 5.7. Rearing period ANOVA results for the effects of exposure combination, rearing substrate, and initial container number on total TAM production. Transform is indicated in parentheses below response variable and significant results are shown in boldface. Groups reared on coarse substrate did not produce TAMs but were excluded from the ANOVA due to mortality.

<i>Variable</i>	<i>Source</i>	<i>DF</i>	<i>Type III SS</i>	<i>F</i>	<i>P</i>
Total TAMs produced (ln)	container(exposure- dose combination)	6	51.37	1.42	0.315
	rearing substrate	1	0.11	0.02	0.894
	Error (subplot)	8	48.29		
	exposure-dose combination	2	22.93	1.34	0.331
	Error (whole plot)	6	51.37		

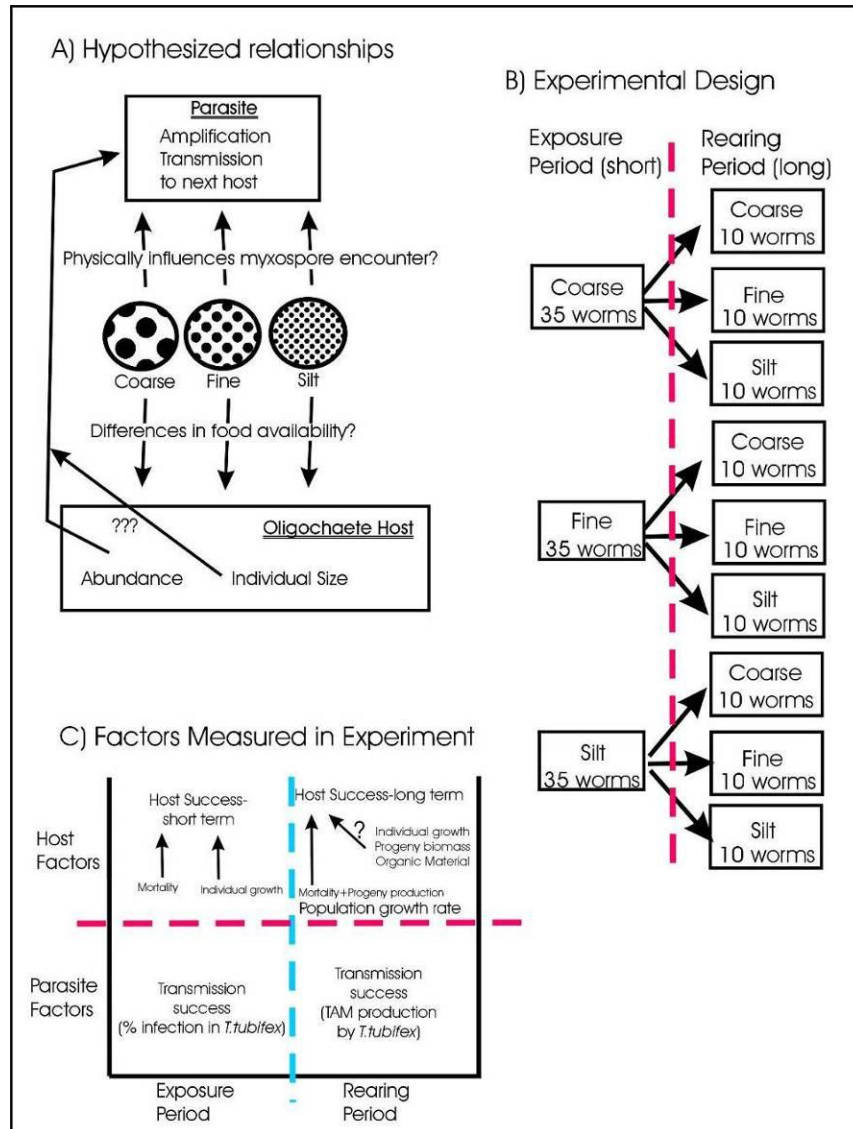


Figure 5.1. Schematic of experiment including A) Hypotheses, B) Experimental design. In the exposure period (left of dotted line) we manipulated substrate type including coarse sand (500-2000 μm), fine sand (125-250 μm), and silt (<63 μm) and *M. cerebralis* myxospore dose (0 or 500 myxospores per individual, not shown) for a total of six exposure categories (coarse+0, fine+0, silt+0, coarse+500, fine+500, and silt+500, with three replicates for each exposure category). At the beginning of the rearing period (below dotted line) subsets of *T. tubifex* were moved from exposure period groups onto one of three substrate types (coarse sand, fine sand, or silt) for a total of 18 rearing categories, and C) Host and parasite factors measured during the experiment including infection prevalence and TAM production (parasite factors), and population growth rate and factors that may influence population growth rate (e.g., progeny production).

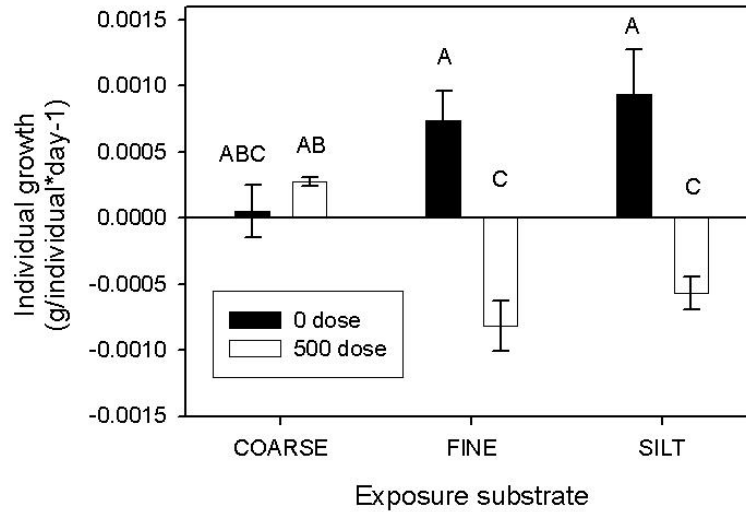


Figure 5.2. Individual growth during the exposure period when exposed to either 0 or 500 *M. cerebralis* myxospores per individual on coarse sand, fine sand, or silt substrate. Letters represent groups that differed (Tukey's post hoc tests).

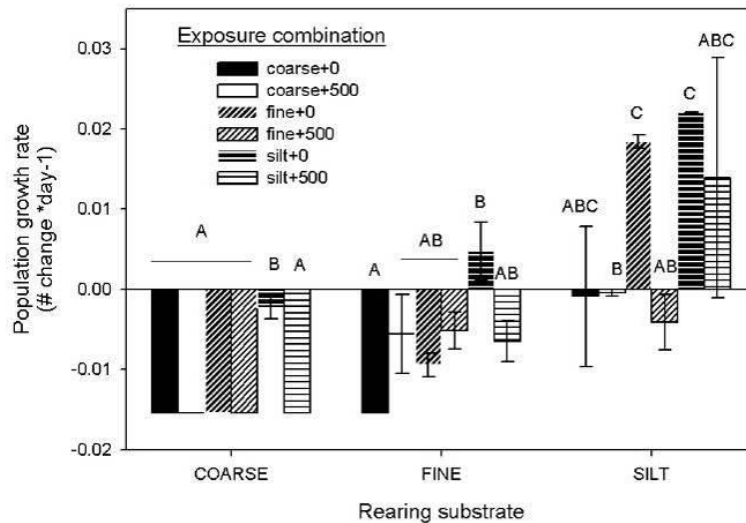


Figure 5.3. Rearing period rate of population growth in *T. tubifex* by rearing substrate and exposure combination. The six exposure combinations (coarse+0, fine+0, silt+0, coarse+500, fine+500, and silt+500), were reared on each of the three substrates (coarse sand, fine sand, and silt, for a total of 18 exposure-rearing combinations. Letters represent groups that differed (Tukey's post hoc tests).

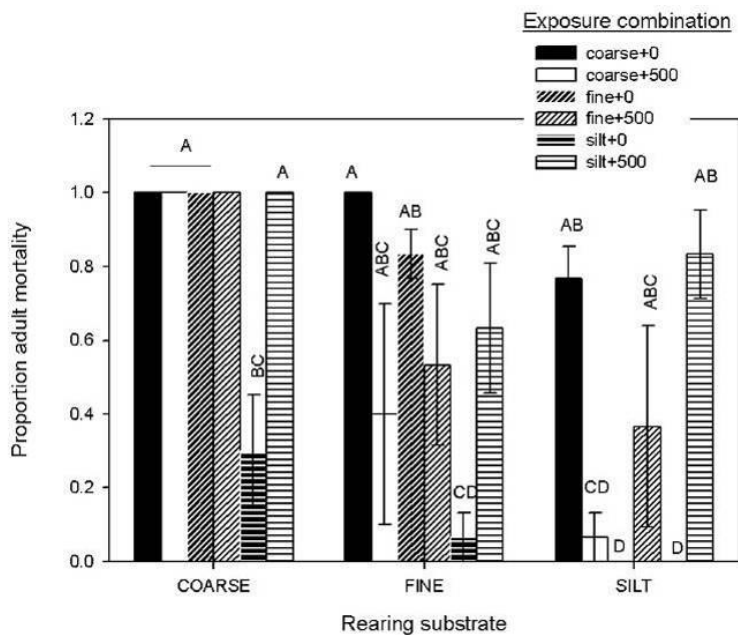


Figure 5.4. Rearing period proportion adult *T. tubifex* mortality by rearing substrate and exposure combination. The six exposure combinations (coarse+0, fine+0, silt+0, coarse+500, fine+500, and silt+500), were reared on each of the three substrates (coarse sand, fine sand, and silt, for a total of 18 exposure-rearing combinations. Letters represent groups that differed (Tukey's post hoc tests, $p < 0.05$).

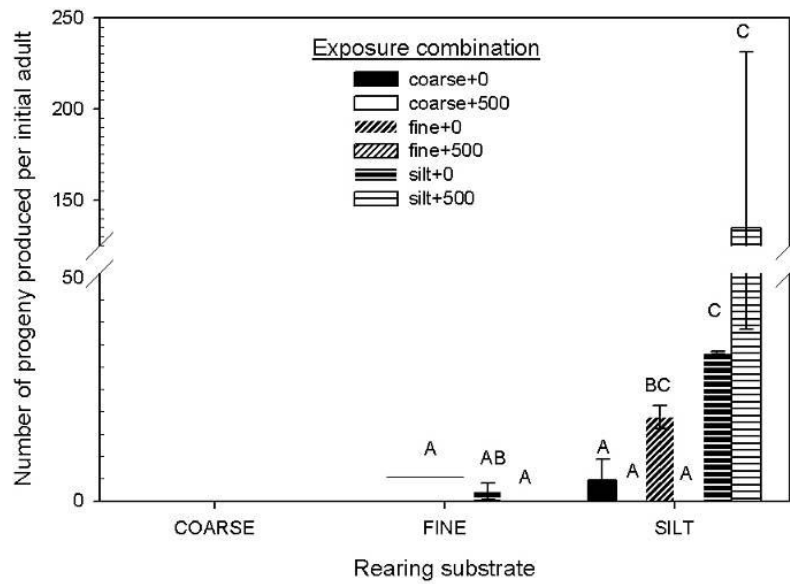


Figure 5.5. Number of progeny produced per initial adult *T. tubifex* during the rearing period shown by rearing substrate and exposure combination. Number of progeny produced per initial adult was affected by rearing substrate and exposure combination. Letters represent groups that differed (Tukey's post hoc tests). All *T. tubifex* in replicates reared on coarse sand were excluded from the analysis due to mortality.

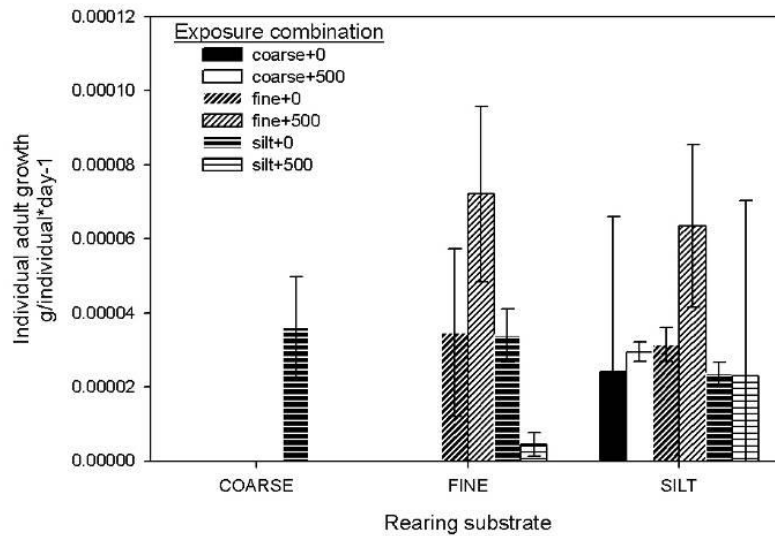


Figure 5.6. Rearing period individual adult growth by rearing substrate and exposure combination. We did not detect effects of rearing substrate or exposure combination. Replicates reared on coarse substrate were excluded from the ANOVA because of high mortality. For exposure combinations reared on coarse sand $n=0$ except silt+0 ($n=3$). For exposure combinations reared on fine sand $n=3$ except coarse+0 ($n=0$) and coarse+500 ($n=2$). For exposure combinations reared on silt $n=3$ except silt+500 ($n=2$).

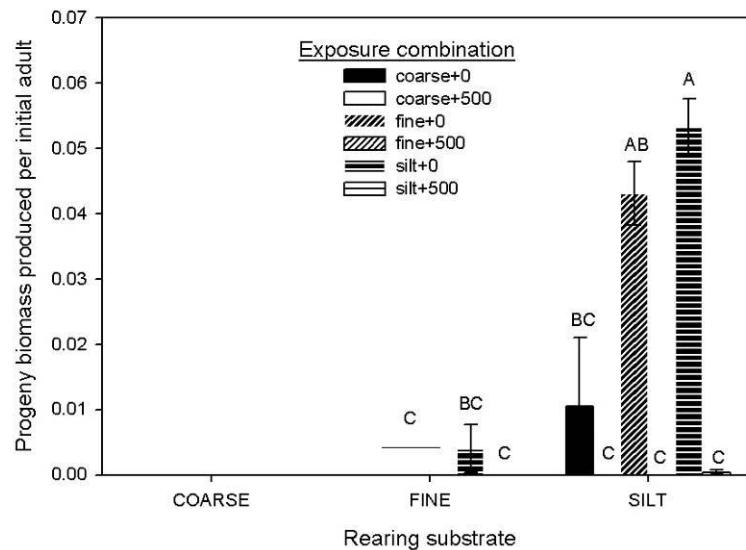


Figure 5.7. Rearing period progeny biomass produced per initial adult *T. tubifex* by rearing substrate and exposure combination. We detected effects of rearing substrate and exposure combination. Letters represent groups that differed (Tukey's post hoc tests). All *T. tubifex* in replicates reared on coarse sand were excluded from the analysis due to mortality.

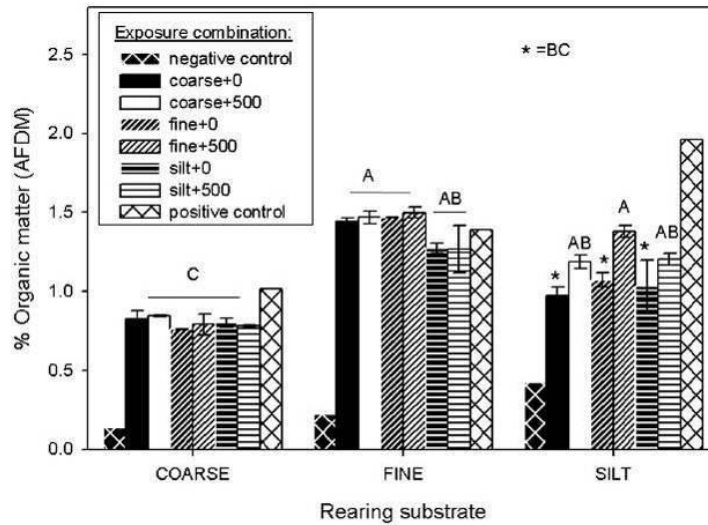


Figure 5.8. Percent organic material retained on rearing substrate post rearing period. Amount of organic material in replicates reared on fine sand was higher than organic matter in replicates reared on coarse sand except when from the silt+500 exposure combination but we did not detect differences in organic matter between replicates reared on coarse sand and silt. Negative and positive controls were not included in the analyses.

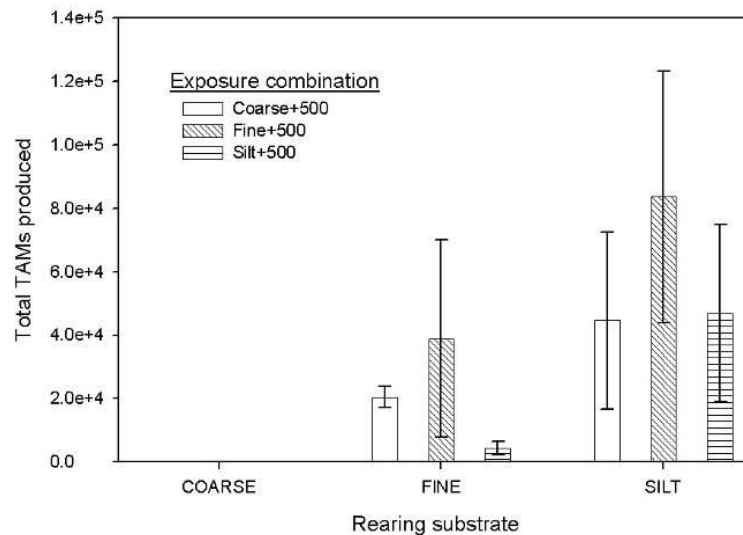


Figure 5.9. Rearing period total number of TAMs produced by rearing substrate and exposure combination. The three exposure combinations (coarse+500, fine+500, and silt+500), were reared on each of the three substrates (coarse sand, fine sand, and silt, for a total of 18 exposure-rearing combinations). All *T. tubifex* in replicates exposed to *M. cerebralis* that were reared on coarse sand died during this period. Letters represent groups that differed (Tukey's post hoc tests).

CHAPTER SIX

TUBIFEX TUBIFEX FROM YELLOWSTONE NATIONAL PARK AND
SUSCEPTIBILITY TO *MYXOBOLUS CEREBRALIS*

Contribution of Authors and Co-Authors

Manuscript in Chapter 6:

Author: Julie D. Alexander

Contributions: Designed the study, conducted the experiment, collected and analyzed output data, and wrote the manuscript. Sequencing was performed by L. Stevens (University of Vermont).

Co-author: Billie L. Kerans

Contributions: Obtained funding and assisted with experimental design and analyses and discussed the results and implications and edited the manuscript at all stages.

Co-author: Todd M. Koel

Contributions: Obtained funding, assisted with field collections, and discussed the results and implications of the study.

Co-author: Charlotte Rasmussen

Contributions: Discussed the results and implications and edited the manuscript.

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Abstract

Genetic variation within hosts may influence the success of invasive parasites. Genetic variation in the oligochaete *Tubifex tubifex* has been correlated with susceptibility to the invasive parasite *Myxobolus cerebralis*, and with the severity of salmonid whirling disease. In Yellowstone National Park, genetic variation in *T. tubifex* may partially explain *M. cerebralis* success in native Yellowstone cutthroat trout spawning tributaries. Therefore, we sequenced a portion of mitochondrial 16S rDNA to assess genetic variation and compare with *T. tubifex* sequences from other geographic regions. Additionally, we examined the susceptibility to *M. cerebralis* and the effects of infection on *T. tubifex* collected from tributaries in Yellowstone National Park by exposing five cultured strains of *T. tubifex* belonging to lineage III and a previously undescribed lineage, and one cultured strain from California (as a positive control) to *M. cerebralis* in a dose challenge experiment.

Tubifex tubifex belonged to three lineages; lineage I (one tributary), for which susceptibility is considered low, lineage III (11 tributaries), for which susceptibility is considered moderate to high, and one lineage that had not previously been described (five tributaries), for which susceptibility was unknown. All *T. tubifex* exposed to *M. cerebralis* were susceptible and we did not detect differences in susceptibility among culture strains. This result suggests variable *M. cerebralis* dynamics in Yellowstone National Park is probably not influenced by genetic variation in the oligochaete host. We also did not detect differences in population growth or relative biomass change among strains or between doses. However, parasite proliferation (TAM production) was negatively correlated with relative

biomass change, which suggested *T. tubifex* experienced a cost of being highly infected. In addition, parasite proliferation was marginally positively correlated with population growth rate, which suggested severe infections may trigger *T. tubifex* reproduction. Our results suggest that *M. cerebralis* success has the potential to be high in many tributaries in Yellowstone National Park because the majority of *T. tubifex* belonged to lineage III, which are susceptible to *M. cerebralis*. In addition, we have described a novel lineage of the host, *T. tubifex*, which was also susceptible to *M. cerebralis*.

Key words: Tubifex tubifex, lineage III, Myxobolus cerebralis, salmonid whirling disease, mitochondrial DNA, dose challenge experiment, Yellowstone National Park, phylogenetic relationships, effect of parasitic infections, Yellowstone cutthroat trout, invasive parasite.

Introduction

Host genotype influences parasite success in natural host populations (e.g., Hamilton 1980, Ebert et al. 1998, Jaenike 1998, Ebert et al. 2000, Keeling and Grenfell 2000). Parasite success may be high when host populations are dominated by genetically similar, susceptible hosts, (Schmid-Hempel 1994, 1998, Acevedo-Whitehouse et al. 2003, Spielman et al. 2004). Conversely, parasite success may be low when host populations are comprised of genetically variable hosts characterized by variable susceptibilities (Little and Ebert 1999, 2000, Carius et al. 2001, Kover and Schaal 2002). Host genetic variation may be particularly influential for the success of invasive parasites. During invasion, founder effects and population bottlenecks can reduce genetic variation (Sakai et al. 2001, Hanfling et al. 2002, Voisin et al. 2005). These factors may limit parasite success if low genetic variation constrains their compatibility with novel hosts.

Myxobolus cerebralis, an invasive parasite that causes salmonid whirling disease, has had high success as demonstrated by dramatic and widely publicized population declines in several species of native and wild salmonids in the intermountain west of the U.S. (Nehring and Walker 1996, Hedrick et al. 1998, Baldwin et al. 1998, Bartholomew and Reno 2002). The parasite is transmitted between salmonids, which produce myxospores, and the oligochaete *Tubifex tubifex* (definitive host), which produces triactinomyxons (TAMs) (Wolf and Markiw 1984, El-Matbouli et al. 1995, Andree et al. 1997, El-Matbouli and Hoffman 1998, Hedrick et al. 1998). However, the success of *M. cerebralis* varies within (Zendt and Bergersen 2000, Hiner and Moffitt 2001, Downing et al. 2002, DuBey and Caldwell 2004,

Krueger et al. 2006,) and among drainages (McGinnis 2007, Anlauf and Moffitt 2008), and why parasite success differs among locations is not well understood.

The success of *M. cerebralis* can be measured by quantifying the number of viable spores in hosts or the environment. Infection and the production of myxospores by infected fish are proportional to TAM exposure (Markiw 1992, Hedrick et al. 1999), which can be determined by measuring TAM abundance (Lukins et al. 2007) or by examining infection severity in sentinel fish (Kelley et al. 2004, Krueger et al. 2006). Infection in *T. tubifex* is also proportional to myxospore exposure (Elwell et al. 2009), but myxospores are difficult to quantify in the environment (Gates 2007) or in the oligochaete host except by molecular assays.

The success of *M. cerebralis* may be influenced by genetic variation within hosts. Differences in susceptibility among genetically variable *T. tubifex* are hypothesized to influence *M. cerebralis* success within and among drainages (Stevens et al. 2001, Beauchamp et al. 2005). Susceptibility to *M. cerebralis* appears to be correlated with variation at the 16SrDNA locus in *T. tubifex* (mitochondrial DNA lineages I, III-VI found within North America, Beauchamp et al. 2001, Arsan et al. 2007). *Tubifex tubifex* from lineages V and VI appear to be resistant to *M. cerebralis* (Beauchamp et al. 2006, Elwell et al. 2006). In general, lineage I *T. tubifex* also appear to be resistant (Beauchamp et al. 2002, Arsan et al. 2007), but at least one strain exhibited low susceptibility when infected in the laboratory (Kerans et al. 2005). Most lineage III *T. tubifex* that have been tested are susceptible, but TAM production and parasite amplification vary among strains (e.g., Stevens et al. 2001, Kerans et al. 2004, Arsan et al. 2007, Baxa et al. 2008, Rasmussen et al. 2008).

Recent surveys have shown that whirling disease risk to fish is high in at least four native Yellowstone cutthroat trout spawning tributaries in Yellowstone National Park (Koel et al. 2006, Murcia et al. 2006, Alexander et al. submitted, Chapter 4). Previous collections of *T. tubifex* from these tributaries indicated that *T. tubifex* belonging to lineages I, III, and, VI were present, but lineage III *T. tubifex* were more abundant than *T. tubifex* belonging to other lineages (Chapter 4). This result may partially explain why whirling disease risk is high in tributaries in the Yellowstone system, but it suggests it may be dissimilar to other systems in the intermountain west, where *T. tubifex* belonging to several lineages co-exist (e.g., DuBey and Calwell 2004, Beauchamp et al. 2005, Lodh et al. in press).

Characterizing genetic variation and phylogeography of *T. tubifex* from tributaries in Yellowstone National Park may provide information to help us better understand how *M. cerebralis* may spread through this system. In addition, this information may provide a template for assessing the spread of invasive parasites into other remote locations where lineages and susceptibility of *T. tubifex* have previously been characterized. Susceptibility has not been directly examined for any *T. tubifex* from Yellowstone National Park. In addition, the effects of *M. cerebralis* on *T. tubifex* populations have not been assessed, which may have important implications for shifts in community structure, particularly for tributaries in which *T. tubifex* comprise a high proportion of the oligochaete community (Chapter 4).

In this study, genetic analyses of *T. tubifex* from tributaries in Yellowstone National Park were combined with a dose challenge experiment. First, we examined genetic variation and phylogenetic relationships among *T. tubifex* from tributaries in Yellowstone National Park and previously described genetic variants of *T. tubifex* from other geographic regions.

Then, we examined susceptibility to *M. cerebralis* and effects of *M. cerebralis* infection on several cultured strains of *T. tubifex*. The aims of this study were 1) to place *T. tubifex* from Yellowstone National Park into context with *T. tubifex* from other geographic areas, and 2) to determine susceptibility of, and effects of *M. cerebralis* on, *T. tubifex* from Yellowstone National Park.

Methods

We determined the mitochondrial 16S rDNA lineage of *T. tubifex* and examined susceptibility to *M. cerebralis* and effects of parasitism using laboratory-cultured strains of *T. tubifex*. We used laboratory-cultured strains instead of field-collected *T. tubifex* because many field-collected tubificids are indistinguishable from *T. tubifex*, and the accidental inclusion of non-*T. tubifex* tubificids in the dose challenge experiment could influence the experimental outcome (e.g., Kerans et al. 2004) by affecting infection of susceptible *T. tubifex* (Elwell et al. 2009). In addition, field-collected tubificids may already be infected by *M. cerebralis* (e.g., Hallett et al. 2009) or other myxozoan parasites (Hallett et al. 2005, 2006, Koel et al. 2006) that could confound experimental results.

Strain Establishment

Tubificids were collected by kick net from one to two reaches on 18 tributaries in Yellowstone National Park in 2006-2007, and sorted under a dissecting microscope. Selected tributaries were located in either the Yellowstone Lake drainage (12 tributaries; Pelican, Astringent, Footbridge, Chittenden, Raven, Unnamed, Pelican Cone, Clear, Beaverdam, Arnica, and Beaver Creeks, and the Upper Yellowstone River) or the

Yellowstone River drainage (six tributaries; Otter, Alum, Thistle, Slough, Trout, and Elk Antler Creeks, Figure 6.1). Selected reaches were characterized by variable habitat features including geothermal influence (Chapter 3) and confinement (Chapter 4), and variable abundances of *T. tubifex* and *M. cerebralis* infected *T. tubifex* (Chapters 3 and 4, Table 6.1).

Forty-eight tubificids morphologically similar to *T. tubifex* (characterized by hair and pectinate chaete, Kathman and Brinkhurst 1998) from each reach were placed in individual wells in 4 x 12 well cell culture plates (1 mL volume) and held in an incubator at 12°C. Every 48 hrs, water was changed by removing and replacing ~90% of the water in each well. A small piece of previously frozen organic spinach was added to each well once a week as a food source.

Wells were briefly examined with a dissecting microscope (20-50x) once a week to determine if progeny were present. We used this method (termed “well plate method”) because the adult could be removed and slide-mounted for morphological identification immediately following the detection of progeny. Timely processing is important for morphological identification because reproductive structures are required for morphological identification (Kathman and Brinkhurst 1998), and these structures may be reabsorbed following reproduction (Kaster 1982). Another impetus for immediately removing and identifying the adult was to determine whether the cultured tubificids were *T. tubifex*.

However, after 60 days we switched to another method (termed “substrate container method”) because individuals were not reproducing and mortality was high using the first method. In this second method, 12 individuals from each reach (three from each well plate, if available) were placed into 12 individual containers (0.15 m x 0.15 m x 0.10 m) with 100

mL masonry sand and maintained as in Stevens et al. (2001). Containers were examined bi-monthly for progeny. Once progeny were detected, randomly selected individuals were removed and preserved for genetic analyses. When possible, adults were removed and preserved for morphological identification (Kathman and Brinkhurst 1998) and genetic analyses.

Genetic and Phylogenetic Analyses

To examine genetic variation, we compared PCR-amplified portions of mitochondrial 16S rDNA among *T. tubifex* from Yellowstone National Park, *T. tubifex* from other geographic regions, and other closely related tubificids to determine mitochondrial lineage and assess phylogenetic relationships of *T. tubifex* from Yellowstone National Park. The 16S rDNA region was selected because sequence data are available for a large number of *T. tubifex*. In addition, susceptibility to *M. cerebralis* appears to be correlated with genotype (lineages I, III-VI) of *T. tubifex* (Beauchamp et al. 2002, 2005, Elwell et al. 2006, Arsan et al. 2007). Initially, we attempted to determine *T. tubifex* genotype using lineage-specific primers (Beauchamp et al. 2001), but we found this method unreliable and switched to examining sequence data.

DNA was extracted from up to three *T. tubifex* from each culture for sequencing. When *T. tubifex* were small (<3 mm long) we used three individuals combined into a pool to ensure enough DNA and only one individual when tubificids were large (>3 mm long). DNA was extracted using Nucleospin Tissue Kits (Clontech Inc., Mountain View, CA) and amplified using standard PCR protocols (Beauchamp et al. 2001). PCR products were

purified and sequenced directly. Sequences were aligned and edited in Bioedit (version 7.0.9.0, Hall 1999).

Sequences were analyzed by simple parsimony analysis (criterion=parsimony, taxlabels=full torder=right, maxtrees=5000, increase=no, root=outgroup, outroot=monophyl storebrlens=yes, warnreset=no, warntree=no, warntsave=no, warnroot=no, warnredef=no, autoclose=yes, hsearch start=stepwise, hold=2, addseq=random, nreps=20, swap=tbr steepest=yes multrees=yes) in PAUP (Version 4.0, Sinauer Associates Inc., MA). Branch support was evaluated by non-parametric bootstrap analysis based on 10,000 pseudoreplicates with tree bisection reconnection branch swapping. *Tubifex tubifex* were assigned to specific mitochondrial lineages based on *T. tubifex* sequences from Genbank (Figure 6.2, accession numbers follow labels). We also obtained sequences for *Limnodrilus hoffmeisteri* and *T. ignotus* for comparison (Figure 6.2, accession numbers follow labels). Trees generated using other alignments (gaps included or excluded) as well as other phylogenetic methods (maximum likelihood and neighbor joining) had the same topology.

Susceptibility and Effects of Infection on Laboratory Populations

Experimental *T. Tubifex* populations. We examined susceptibility and effects of infection using laboratory-reared *T. tubifex* (see Strain Establishment, above) in a dose challenge experiment (Elwell et al. 2006, 2009). All reproducing cultures (>40 individuals/culture by May 2007, Appendix E) were used in the experiment. This included two cultures from Pelican Creek (Pel_e7; lineage III, Pel_d14; lineage III) and one culture from Clear Creek (ClearCk; new lineage) in the Yellowstone Lake drainage, and two cultures from Elk Antler Creek (ElkAntlerCk13; lineage III, ElkAntlerCK16; lineage III) in the

Yellowstone River drainage (Figure 6.1). We also included *T. tubifex* from the Mt. Whitney, CA (lineage III) strain as a standard (susceptibility to *M. cerebralis* has been previously established and this culture is maintained in our laboratory; Stevens et al. 2001, Kerans et al. 2004, Rasmussen et al. 2008). We hypothesized that lineage III *T. tubifex* would be susceptible to *M. cerebralis* and that susceptibility of Yellowstone strains of lineage III *T. tubifex* would be similar to that of Mt. Whitney *T. tubifex* (lineage III). We hypothesized that *T. tubifex* from other lineages, including the previously undescribed lineage (ClearCk; new lineage), would exhibit low to no susceptibility to *M. cerebralis*.

Experimental Design and Statistical Analysis. *Myxobolus cerebralis* myxospores were extracted from infected rainbow trout (*Onchorhynchus mykiss*) using a continuous plankton centrifuge (O'Grodnick 1975, Elwell et al. 2009). Trout were obtained from the Aquatic Sciences Laboratory, Montana State University, Bozeman, MT and were infected at the laboratory using TAMs from our *T. tubifex* cultures. Myxospores were extracted from infected fish by the plankton centrifuge method (Lemmon and Kerans 2001) and enumerated in 3x 1 μ L suspensions using on a hemacytometer and a compound microscope (400x). Total number of myxospores in suspension was determined by extrapolating from the mean number of myxospores obtained from hemacytometer counts. Spore-free emulsion was extracted from disease-free laboratory-reared rainbow trout by the same method.

Prior to the experiment, 35 *T. tubifex* were removed from cultures and held without substrate for 24 hrs to equalize hunger levels. Thirty *T. tubifex* were wet weighed to the nearest mg in groups of five and randomly assigned to containers (9 x 9 x 5 cm) with 25 mL of substrate (masonry sand) and 150 mL dechlorinated water. Containers were assigned to

one of two doses (0 or 500 myxospores/worm) and a suspension containing approximately 2500 myxospores (500 myxospores/worm) or an equivalent volume of spore-free suspension was added to each container. Doses of 10-100 myxospores/worm caused 100% infection in groups of *T. tubifex* held in the same containers (Elwell et al. 2009). We used a dose of 500 myxospores/worm because we used only five *T. tubifex* individuals per container and hoped to achieve 100% infection. During myxospore administration, the suspensions were mixed with a magnetic stirrer to ensure even distribution of myxospores, or spore-free suspension. Following dosing, containers were held in incubators at 15°C on a 12:12 light-dark cycle without air for 24h to allow myxospores to settle. An air source was connected to each container for the remainder of the experiment. Once a week, containers were maintained by changing approximately 90% of the water and then rotating containers between incubators. A suspension of *Spirulina* sp. (1 mL of 0.25 g/100 mL) was added to containers bi-monthly as a food source.

We examined susceptibility to *M. cerebralis* by comparing total number of TAMs released by *T. tubifex* strains against the Mt. Whitney standard. Beginning 60 days post-exposure, water removed from each container during water changes was filtered through a 20 µm sieve and examined for the presence or absence of TAMs. Triactinomyxons were enumerated as in Kerans et al. (2004), except TAMs were enumerated in three 100 µL aliquots on slides that were air-dried prior to enumeration. The total number of TAMs produced was calculated as [sum of TAMs produced from day 60 to day 150]. When mortality was 100%, replicates were excluded from total TAM production calculations.

We determined effects of *M. cerebralis* infection on *T. Tubifex* in three ways; by examining relationships between TAM production and *T. tubifex* population growth, by examining relationships between TAM production and relative biomass change, a measure of individual growth, and finally, by comparing population growth and relative biomass change among *T. tubifex* strains by myxospore dose. Population growth rate is influenced by adult mortality and progeny production, both of which have previously been shown to be negatively affected by *M. cerebralis* infection (e.g., Elwell et al. 2006, 2009). Biomass change is related to individual *T. tubifex* size (Chapter 3, Appendix A), and has also been shown to be influenced by *M. cerebralis* infection (e.g., Elwell et al. 2006, Shirakashi and El-Matbouli 2009).

On day 150, surviving *T. tubifex* were counted and wet-weighed to the nearest mg. Population growth rate was calculated as $[\ln(\text{number of } T. \text{ tubifex post-experiment}+1) - \ln(\text{number of } T. \text{ tubifex pre-experiment}+1)]/\text{total days of experiment}$. Biomass change was calculated as relative biomass change $[(\text{weight of } T. \text{ tubifex post-experiment}) - (\text{weight of } T. \text{ tubifex pre-experiment})]/(\text{weight of } T. \text{ tubifex pre-experiment})$, because pre-experiment weights of *T. tubifex* differed among groups (Table 6.2).

To examine differences in susceptibility among *T. tubifex* strains, we tested for differences in total TAM production using one-way analysis of variance (ANOVA). To test for effects of *M. cerebralis* infection on *T. tubifex*, we examined relationships between total TAM production and a) population growth rate and b) relative biomass change in *T. tubifex* exposed to the 500 myxospore dose using multiple linear regression (PROC REG, SAS Inc., NC, USA). We tested for differences in a) pre-experiment weight, b) population growth rate,

and c) relative biomass change among *T. tubifex* strains and between myxospore doses using two way multiple analysis of variance (MANOVA, PROC GLM, SAS Institute Inc., NC, USA). Significant effects were examined with individual ANOVAs and Tukey's honestly significant difference (HSD) tests.

Results

Strain Establishment

We successfully established reproducing laboratory cultures of *T. tubifex* from Pelican (five cultures), Astringent (two cultures), Footbridge (one culture), Chittenden (one culture), Raven (one culture), the unnamed tributary to Pelican Creek (four cultures), Pelican Cone (one culture), Clear (six cultures), Beaverdam (three cultures), Bridge (one culture), Thistle (three cultures), Slough (one culture), Trout (two cultures), and Elk Antler Creeks (three cultures), and the Upper Yellowstone River (three cultures) using the substrate container method. We did not obtain reproducing laboratory cultures from Arnica, Otter, or Alum Creeks (see Figure 6.1, Appendix E).

Genetic and Phylogenetic Analyses

Sequences were obtained for 83 *T. tubifex* from 15 tributaries (Table 6.1, Figure 6.1), which were combined into 30 consensus sequences. We did not detect length variation ($p = 0.330$). Of 487 total characters, 209 base pairs were variable and 150 base pairs were phylogenetically informative. Relationships were best explained by a single most parsimonious tree (consistency index excluding uninformative characters: 0.61, Figure 6.2). *Tubifex tubifex* and *T. ignotus* formed subgroups, and relationships among *T. tubifex* lineages

were consistent with other studies (Sturmbauer et al. 1999, Beauchamp et al. 2001, Crottini et al. 2008). Yellowstone strains of *T. tubifex* formed three clades supported by high bootstrap values (see Figure 6.2). The largest clade included *T. tubifex* from the majority of tributaries in Yellowstone National Park and grouped with lineage III *T. tubifex*. *Tubifex tubifex* from Slough Creek differed slightly but grouped with lineage I *T. tubifex*. The other clade included *T. tubifex* from cultures originating from Astringent Creek, Thistle Creek, Clear Creek, Bridge Creek and the Upper Yellowstone River, and did not group with any previously described *T. tubifex* lineages. We did not identify any strains of *T. tubifex* that belonged to lineages V or VI in laboratory strains.

Susceptibility and Effects of Infection on Laboratory Populations

During the dose challenge, all but two replicates included in the 500 myxospore dose produced TAMs. The two exceptions included one replicate each of the Clear Creek strain (ClearCk; post-experiment number of *T. tubifex* = 6) and the Elk Antler Creek strain (Elk_k16; post-experiment number of *T. tubifex* = 1). We did not detect differences in total TAM production among *T. tubifex* strains ($F_{5,11} = 1.45$, $p = 0.282$, Figure 6.3).

Total TAM production was correlated with population growth rate (positive correlation) and relative biomass change (negative correlation) in *T. tubifex* exposed to the 500 myxospore dose ($r^2 = 0.458$, $p = 0.014$, Table 6.2, Figure 6.4). This result suggested that among infected cultures, parasite proliferation was associated increased population growth and decreased individual growth (but see Figure 6.5 b and c for contrasting relationship between infection and population growth, and biomass change among infected and uninfected cultures).

Pre-experiment weights of Mt. Whitney (0 dose only), Pelican Creek (Pel_e7; 0 and 500 doses), and Clear Creek (ClearCk; 0 and 500 doses) *T. tubifex* cultures were greater than pre-experiment weights of Elk Antler Creek (Elk_k16; 0 and 500 doses) and Unnamed tributary to Pelican Creek (Pel_d14; 0 and 500 doses) *T. tubifex* (Wilks' $\lambda = 0.070$, $F_{15, 61.13} = 6.59$, $p < 0.0001$, Table 6.3, Figure 6.5a). However, we did not detect differences in effects of culture on population growth rate ($F_{5,24} = 0.98$, $p = 0.452$, Figure 6.5b) or relative biomass change ($F_{5,24} = 1.93$, $p = 0.126$, Figure 6.5c). We did not detect effects of dose (Wilks' $\lambda = 0.807$, $F_{3,22} = 1.76$, $p = 0.185$) or interactions between dose and *T. tubifex* culture (Wilks' $\lambda = 0.849$, $F_{15, 61.13} = 0.25$, $p = 0.998$) on pre-experiment weight, population growth, or relative biomass change.

Discussion

We characterized genetic variation of *T. tubifex* populations from Yellowstone National Park using mitochondrial 16s rDNA sequence data in order to compare them with *T. tubifex* from other geographic regions. Previous research suggested that different lineages of *T. tubifex* that are characterized by variable susceptibilities to *M. cerebralis* (e.g., Beauchamp et al. 2002, Kerans et al. 2004, 2005, Elwell et al. 2006, Rasmussen et al. 2008, but see Baxa et al. 2008) co-exist in other systems, and that interactions among them may drive differential *M. cerebralis* success. We also assessed susceptibility of and effects of parasitism on *T. tubifex* from Yellowstone National Park in a dose challenge experiment.

We found little evidence to suggest genetic variation in *T. tubifex* explains variation in parasite success in Yellowstone National Park because the majority of cultured *T. tubifex*

belonged to lineage III, and those tested appeared to exhibit similar susceptibilities to *M. cerebralis* and experience similar costs of *M. cerebralis* infections to one another as well as other lineage III *T. tubifex* from other geographic regions.

Tubifex tubifex from tributaries in Yellowstone National Park belonged to three lineages: Lineage III (11 tributaries), which is considered to have moderate to high susceptibility to *M. cerebralis*, lineage I (one tributary), which is considered to have low susceptibility to *M. cerebralis*, and a lineage that had not previously been described (five tributaries), for which susceptibility to *M. cerebralis* was unknown.

The clade that corresponded to lineage III *T. tubifex* (Figure 6.2) contained *T. tubifex* from tributaries spanning the Yellowstone River and Yellowstone Lake in Yellowstone National Park. The environments of these tributaries, which were previously described (Chapter 4), are characterized by a wide range of features, including geothermal influence and variable geomorphologies (confinement). In addition, *M. cerebralis* has been detected in six of the tributaries (Murcia et al. 2006, Koel et al. 2006, Alexander et al. submitted, Chapter 4). This suggests that lineage III *T. tubifex* from tributaries in Yellowstone National Park may be able to tolerate a wide range of environmental conditions. Others have described lineage III *T. tubifex* from a variety of habitats in other systems worldwide (Sturmbauer et al. 1999, Beauchamp et al. 2001, Crottini et al. 2008), so it was not surprising that the majority of *T. tubifex* cultured from Yellowstone belonged to this lineage.

The clade that corresponded to lineage I *T. tubifex* contained *T. tubifex* from Slough Creek. Unlike the lineage III *T. tubifex*, Slough Creek *T. tubifex* differed from lineage I *T. tubifex* sequences in Genbank by three nucleotide changes. Arsan et al. (2007) also observed

differences between lineage I *T. tubifex* from Alaska and sequences from Genbank. If this region is linked to susceptibility or is indicative of DNA regions that confer susceptibility, this result may explain why some strains of lineage I *T. tubifex* exhibit lower susceptibility to *M. cerebralis* (Beauchamp et al. 2002, Kerans et al. 2005, Arsan et al. 2007). However, it is more likely that the various lineages of *T. tubifex* represent cryptic species of *Tubifex* with differing compatibilities to this parasite. If variability (or lack thereof) in the 16S rDNA region is indicative of regions of DNA that may confer susceptibility to parasitism, this result may also explain the discrepancies others have observed when examining the susceptibility of lineage I *T. tubifex* (Beauchamp et al. 2002, Kerans et al. 2005, Arsan et al. 2007).

Tubifex tubifex sequences that did not correspond with previously described lineages included those obtained from *T. tubifex* from cultures originating from Astringent Creek, Thistle Creek, Clear Creek, Bridge Creek and the Upper Yellowstone River. Examination of adults from Astringent Creek (n = 1), Clear Creek (n = 3), and the Upper Yellowstone River (n = 1) indicated they were morphologically identifiable *T. tubifex* (Kathman and Brinkhurst 1998). The sequence data suggests these individuals may belong to undescribed lineages or different (potentially new) species of *Tubifex*. Astringent and Clear Creeks, and the Upper Yellowstone River, are characterized by almost opposite, extreme environments (Chapter 4). For example, the Astringent Creek tributary on Pelican Creek is geothermally influenced and has low pH (mean pH is 3.28) and high specific conductivity (mean specific conductivity is 797 μ S). Although *T. tubifex* is tolerant of low pH (Degn and Kristensen 1981, Bonacina et al. 1999), populations inhabiting Astringent Creek are likely stressed (e.g., Chapman and

Brinkhurst 1987, Reynoldson 1987, Reynoldson et al. 1991), which may drive speciation through reproductive isolation.

In contrast, the Clear Creek and Upper Yellowstone River tributaries are not geothermally influenced, and are characterized by more neutral pH (7.6-8.6) and conductivity (49.5-65.0 μ S) values. These tributaries may represent extreme (and thus stressful) conditions for *T. tubifex* via competition with other benthic invertebrates. *Tubifex tubifex* is considered a poor competitor: Many species that tolerate extreme conditions, e.g., anoxia or low pH (Reynoldson 1987, Reynoldson et al. 1991) are poor competitors (Dunson and Travis 1991) and these tributaries are characterized by environmental features that are ideal for many aquatic invertebrates.

All *T. tubifex* cultures exposed to *M. cerebralis* produced TAMs, demonstrating these cultures were susceptible to infection by *M. cerebralis*. This was not surprising because all but one of the cultures were identified as lineage III. When combined with the genetic data, this result suggests that the majority of *T. tubifex* from tributaries in Yellowstone are susceptible to infection by *M. cerebralis*. The results from this experiment also suggest *T. tubifex* from Yellowstone National Park may be moderately susceptible to *M. cerebralis* because all strains of *T. tubifex* produced fewer TAMs than the Mt. Whitney standard, although this result was not significant due to high variability in TAM production. The high variability was likely related to unequal dosing or to low densities of *T. tubifex* used in the experiment. Lemmon and Kerans (2001) suggested that freshly extracted myxospores may adhere to one another, which could explain differences in TAM production. Others have also

observed variability in TAM production (Blazer et al. 2003, Elwell et al. 2006, Rasmussen et al. 2008) even when using higher densities of *T. tubifex*.

Tubifex tubifex from the Clear Creek culture belonging to the new lineage were susceptible to infection by *M. cerebralis*. Membership within some lineages (V, VI) appears to be useful for assessing susceptibility. However, this result represents one of few (Kerans et al. 2004) observations of non-lineage III *T. tubifex* releasing TAMs, and further complicates the perceived relationship between susceptibility and mtDNA lineage. The inclusion of additional non-lineage III *T. tubifex* cultures in the experiment was critical for further examining the effect of lineage. Unfortunately, a problem with myxospore viability prevented the inclusion of additional cultures in a second experimental block (Appendix E). Interestingly, Clear Creek previously tested positive for *M. cerebralis* (Koel et al. 2006), but the parasite has not subsequently been detected. One possible explanation is that this lineage may be less susceptible to *M. cerebralis*: it produced fewer (although not significant) TAMs than strains from Pelican Creek, additionally *T. tubifex* abundance in Clear Creek is low (Chapter 4).

We observed an interesting relationship between TAM production and measures of *T. tubifex* fitness among infected *T. tubifex*: TAM production was negatively correlated with relative biomass change and marginally positively correlated with population growth rate in *T. tubifex* exposed to *M. cerebralis*. The negative correlation between biomass and TAM production indicates that infected *T. tubifex* may experience costs at the individual level (in terms of individual growth) and gains at the population level (in terms of progeny production). Among infected *T. tubifex*, those producing the greatest number of TAMs are

also probably the most severely infected. Thus, severe infection may trigger progeny production in *T. tubifex*, perhaps as a last resort. I observed the same phenomenon in infected *T. tubifex* (Chapter 5); interestingly, the progeny produced were much more numerous and characterized by low biomass compared to progeny of uninfected *T. tubifex*. This may provide evidence of host manipulation for parasite gain: If as a parasite, you can trigger your host to produce genetically similar progeny that you can subsequently infect, you essentially guarantee availability of future hosts. This should be examined in future investigations because it would be really interesting to know if the parasite somehow slows down TAM production to allow the host to produce offspring or whether the production of offspring is simply a last ditch effort by the host to reproduce before it dies from infection. If the former were the case, it would fit well with the description of a bet hedging strategy; some expected fitness (TAM production) is sacrificed for an increase in availability of suitable hosts for future generations (e.g., Seger and Brockman 1987, Philippi and Seger 1989).

Initially, the relationship between TAM production and measures of host fitness appear to be inconsistent with others' findings (e.g., Elwell et al. 2006, Shirasheki and El-Matbouli 2009, but see Rasmussen et al. 2008). In general, biomass increases in infected *T. tubifex* when compared to uninfected *T. tubifex*. The discrepancy is probably because previous studies did not examine the relationship between TAM production, which likely indicates severity of infection, and biomass: they only examined differences in biomass production among infected versus uninfected *T. tubifex*.

Another possibility is that the cost of *M. cerebralis* infection in *T. tubifex* (fitness) was low under the experimental conditions. Others have shown that *M. cerebralis* infected *T. tubifex* experience a cost of fitness (e.g., reduction in production of *T. tubifex* progeny, Elwell et al. 2009), but densities used were higher than those maintained in this experiment and intraspecific competition at higher densities actually appears to be more costly than *M. cerebralis* infection (Elwell et al. 2006). *Tubifex tubifex* is able to proliferate when conditions are not limiting (Poddubnaya 1980, Brinkhurst and Kathman 1998), so abundant food or space resources may have caused this result in our experiment. However, this result underscores the importance of considering densities and food concentrations when assessing susceptibility among different strains of *T. tubifex* from experiments, and may partially explain the high variability observed among other studies of susceptibility of *T. tubifex* to *M. cerebralis*.

Prior to beginning the dose challenge, weights of *T. tubifex* differed (Figure 6.5a), which may indicate cultured *T. tubifex* have different environmental optima. We maintained cultures under the same temperature, light, and resource conditions for approximately one year prior to the experiment. *Tubifex tubifex* are strongly influenced by temperature, photoperiod, and resource availability (Brinkhurst 1971, Kaster and Bushnell 1980, Poddubnaya 1980, DuBey et al. 2005) and *T. tubifex* strains are characterized by different environmental optima (Anlauf 1994, Kerans 2005). Thus, cultures that were characterized by higher pre-experiment weights may be better adapted to the temperature, light, and resource regime provided in culture and may not be indicative of other types, which may thrive under conditions naturally found in Yellowstone tributaries.

Characterizing genetic variability at the 16SrDNA locus may help us to better understand the processes that drive the geographic distribution of *T. tubifex* lineages and whirling disease in this system. *Tubifex tubifex* from the majority of native Yellowstone cutthroat trout spawning tributaries were characterized by low genetic variability, which is probably not surprising given the isolated location of the Yellowstone environment. The results from the dose challenge demonstrated *T. tubifex* cultures derived from individuals from tributaries in Yellowstone National Park do not differ significantly in their susceptibility to *M. cerebralis*, which may explain why we have observed uniformly high whirling disease risk in *M. cerebralis*-positive tributaries. Parasites that infect the most common host genotype have the potential to effect shifts in population structure (Bell and Maynard Smith 1987, Kalz and Shykiff 1998, Cairus et al. 2001) if host genotypes exhibit variability in resistance to parasitic infections and parasites exert significant effects on host fitness (e.g., reduction in production of *T. tubifex* progeny, Elwell et al. 2009). However, it is unlikely that *M. cerebralis* will effect similar shifts in *T. tubifex* populations in Yellowstone, except when resources are limiting.

In summary, the results from this study suggest that the majority of *T. tubifex* from Yellowstone National Park are not characterized by much genetic variation and that *T. tubifex* from tributaries in Yellowstone do not differ in susceptibility. Thus, the abundance of *T. tubifex*, rather than relative abundance of genetically variable lineages of *T. tubifex*, may be an important determinant of whirling disease risk in *M. cerebralis*-positive tributaries in Yellowstone National Park. Tributaries that have not been invaded by *M. cerebralis* are likely at risk of parasite establishment if *M. cerebralis* is introduced because *T. tubifex* in

these tributaries are probably susceptible to *M. cerebralis*. In isolated catchments of Yellowstone National Park (e.g., the upper Yellowstone River and Slough Creek), it may ultimately be more important to prevent the introduction and spread of *M. cerebralis*, because susceptible *T. tubifex* belonging to different lineages are likely to be present and may be abundant.

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Table 6.1. Environmental and context data for laboratory strains of *T. tubifex* from Yellowstone National Park including drainage, tributary, Status (*M. cerebralis* detected in sentinel fish; y/n), geothermal (y/n), geomorphology (C=confined, I=intermediate confinement, U=unconfined), and abundance of *T. tubifex* (catch per unit effort; CPUE) and *M. cerebralis* infected *T. tubifex* (CPUE). *= Detected once (2001) not subsequently detected. Cultures used in the dose experiment are indicated in boldface.

Drain- age	Tributary	sample_ID	Status	Geo- thermal	Geomor - phology	<i>T. tubifex</i> Abundance	<i>M.</i> <i>cerebralis</i> + <i>T. tubifex</i> Abundance
Yellow-stone Lake	Astringent Creek	Astr1_1 Astr2_1	Y	Y	I	3.00	0.00
	Pelican Creek	Pel_e7_1	Y	Y	U	99.70	0.66
		Pel_04Sa1_1	Y	Y	U	55.78	4.10
		Pel_b_1	Y	N	U	146.23	7.24
		Pel_xing8	Y	N	U	147.21	3.70
		Pel_xing1	Y	N	U	68.09	2.18
	Chittenden Creek	Chit_1	Y	N	I	8.60	0.20
	Pelican Cone Creek	Cone5_1	N	N	C	0.75	0.00
	Footbridge Creek	Ftbridge_1	N	N	C	2.50	0.00
	Unnamed tributary to Pelican Creek	Pel_d12_1	N	N	I	153.71	57.64
		Pel_d14_1					
		Pel_d24_1					
		Pel_d25_1					
	Raven Creek	Rav4_1	Y	Y	U	43.63	2.80
	Bridge Creek	Bridge1	N	N	I	21.43	0.00
	Clear Creek	ClearCk1	Y*	N	C	0.10	0.00
	Beaverdam Creek	Beav_q3_1	N	N	C	23.36	0.00
		Beav-r1					
	Upper Yellowstone River	Uy_s4_1	N	N	U	13.89	0.00
		Uy_sg_1					
		Uy_s3_1					
Yellow-stone River	Elk Antler Creek	Elk_k13_1	N	N	U	39.58	3.57
		Elk_k16_1	Y	N	U	13.36	0.91
	Trout Creek	Trout_j4_1	Y	N	U	344.00	11.68
		Trout_j9_1	Y	N	U	9.82	1.16
	Thistle Creek	Thistle3	N	N	C	5.33	0.00
	Slough Creek	Slough_1	N	N	U	1.65	0.00

Table 6.2. Multiple linear regression results output for relationship between total TAM production and a) population growth rate and b) relative biomass change in *T. tubifex* exposed to 500 myxospores per worm.

Predictor Variable	t	P-value
Intercept	6.42	<0.0001
Population Growth Rate	-1.91	0.077
Relative Biomass Change	-3.27	0.0056

Table 6.3. Analysis of variance results on the effects of myxospore dose and *T. tubifex* strain on pre-experiment weights. Transforms, if used, are indicated in parentheses below response variables and significant results are shown in boldface.

Response Variable	Source of variation	df	Sums of Squares (III)	F	P-value
Pre-Experiment Weight	Dose	1	0.00	0.01	0.908
	<i>T. tubifex</i> strain	4	3.89×10^{-5}	44.56	<0.0001
	<i>T. tubifex</i> strain*Dose	4	2.00×10^{-8}	0.02	0.999
	Error	20	4.37×10^{-6}		

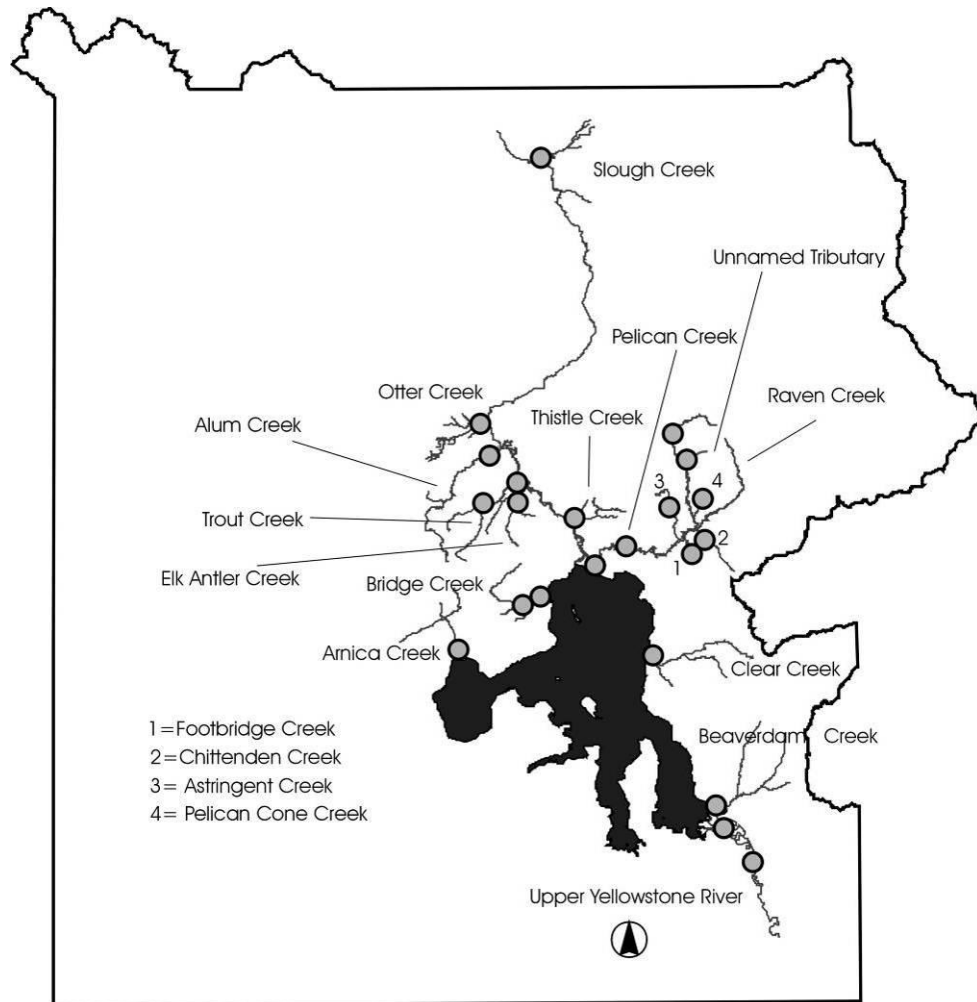


Figure 6.1. Map of locations in Yellowstone National Park that were targeted for tubificid collections for *T. tubifex* culture establishment. Tubificids were collected from one to two reaches on 12 tributaries in the Yellowstone Lake drainage and six tributaries in the Yellowstone River drainage.

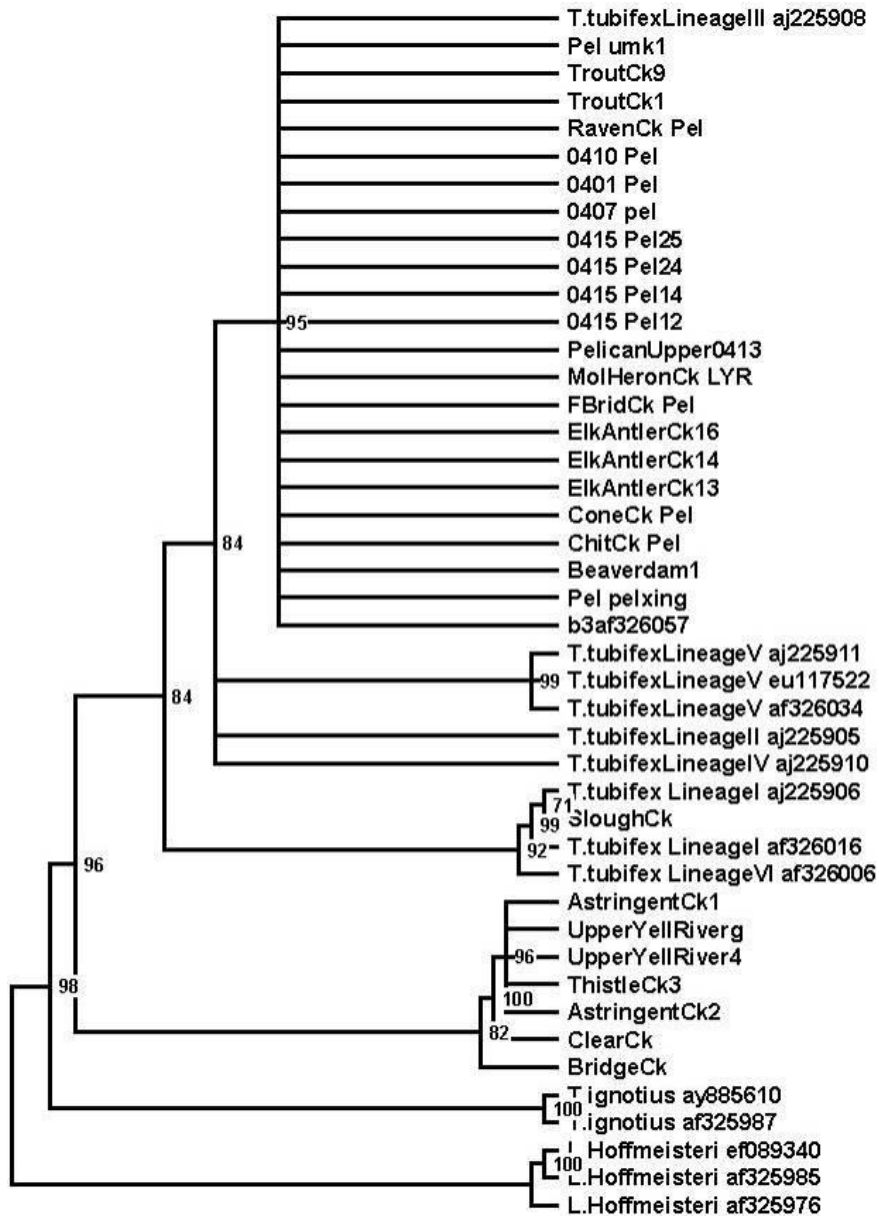


Figure 6.2. Single most parsimonious tree from analysis of a 487 base pairs in the 16SrDNA region for Yellowstone strains of *T. tubifex*, and lineages I-VI *T. tubifex*, *Limnodrilus hoffmeisteri*, and *T. ignotus*. Numbers at nodes represent bootstrap support values based on maximum parsimony. Isolate codes are given in Table 6.1.

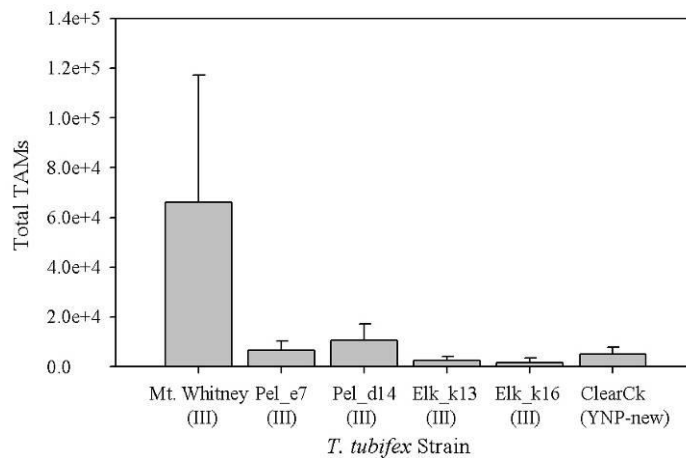


Figure 6.3. Total TAM production by *T. tubifex* strain when exposed to 500 *M. cerebralis* myxospores/worm. The Pel_e7 culture was established using *T. tubifex* collected from a geothermally influenced location on Pelican Creek, the Pel_d14 culture was established using *T. tubifex* collected from an unnamed tributary to Pelican Creek, the Elk_k13 and Elk_k16 cultures were established using *T. tubifex* from Elk Antler Creek, and the ClearCk culture was established using *T. tubifex* from Clear Creek. The Mt. Whitney culture was established using *T. tubifex* from CA and was included as a control. Lineage is indicated in parentheses below culture label.

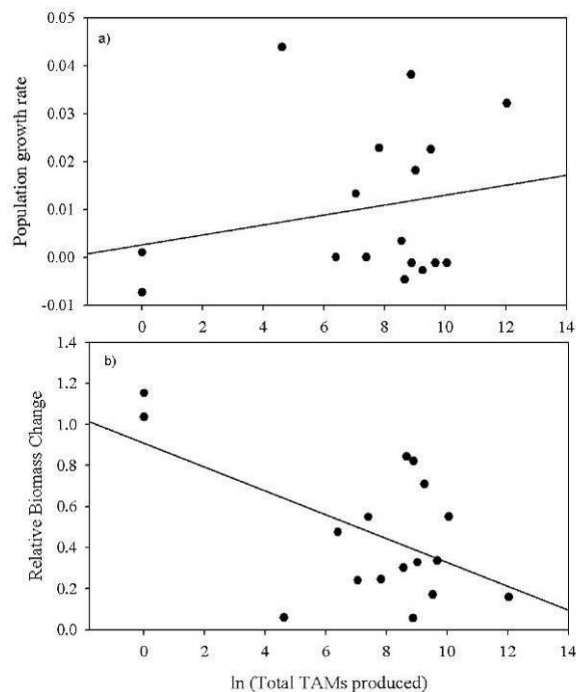


Figure 6.4. Effects of *M. cerebralis* infection. Relationship between total TAM production and a) population growth rate and b) relative biomass change of *T. tubifex*.

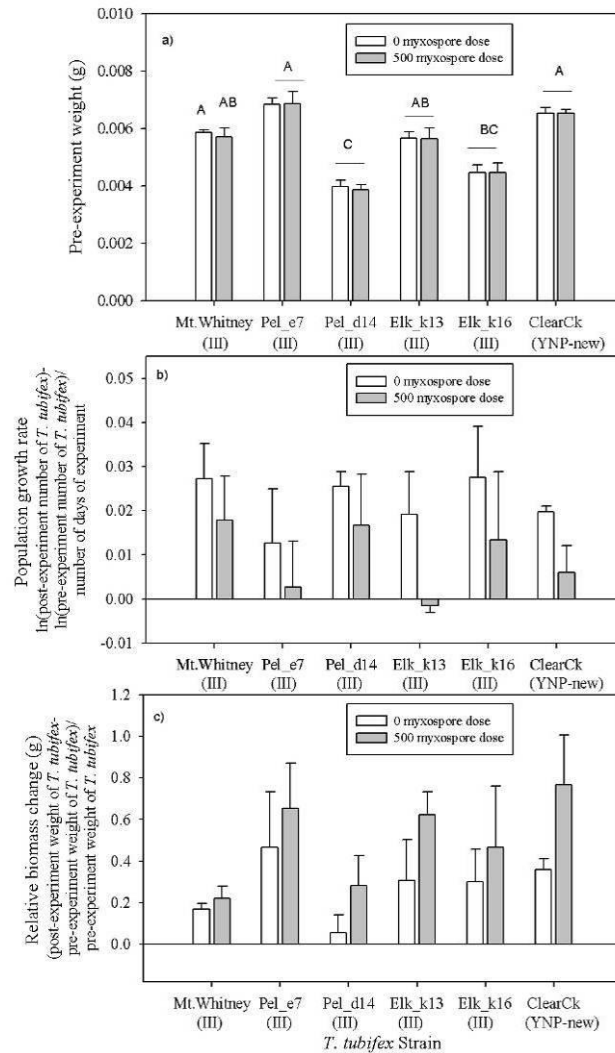


Figure 6.5. Effects of parasitism on *T. tubifex* cultures a) mean pre-experiment weight, b) population growth rate, and c) relative biomass change of *T. tubifex* shown by strain and myxospore dose. Lineage is indicated in parentheses below strain label. Letters represent Tukey's HSD results ($\alpha = 0.05$).

CHAPTER SEVEN

CONCLUSIONS

Emerging parasitic diseases constitute a significant threat to human, livestock, and wildlife health (McKenzie and Townsend 2007). Identifying factors that influence the ecology of parasitic diseases is critical for management of emerging infectious diseases. Parasite success and disease outbreaks may be influenced by aspects of parasite ecology (e. g., variation in virulence; Carius et al. 2001, Ferguson and Read 2002, Galvani 2003), host ecology (e. g., variation in susceptibility; Sorci et al. 1997, Carius et al. 2001, host community diversity; Johnson et al. 2008), the environment (e. g., factors that influence outcomes of host parasite interactions; Lafferty and Kuris 1999, Harvell et al. 2002, Vale et al. 2008), or interactions among any or all of these factors (Hedrick et al. 1998, McKenzie and Townsend 2007). However, the identification of factors most influential for parasitism and disease outbreaks is hampered by the complexity of interactions among hosts, parasites, and the environment (Hedrick 1998, Reno 1998, Agnew and Koella 1999, Rigaud et al. 2010).

Whirling disease, an emerging parasitic disease of salmon and trout in North America, may threaten the long-term survival of native Yellowstone cutthroat trout in Yellowstone National Park. The causative agent, *Myxobolus cerebralis*, appears to have become established in at least two tributaries to Yellowstone Lake (Pelican Creek and the Yellowstone River, Koel et al. 2006, Murcia et al. 2006). Factors determining parasite establishment are not understood, but may be strongly influenced by interactions between environmental features and the oligochaete host. The presence of susceptible *Tubifex tubifex*

in *M. cerebralis*-positive tributaries in Yellowstone could clearly be inferred from the detection of the parasite, but little was known about the oligochaete host in Yellowstone prior to this study. In particular, the distribution and abundance of *T. tubifex* and *M. cerebralis*-infected *T. tubifex* had not previously been examined for *T. tubifex* populations in Yellowstone cutthroat trout spawning tributaries. In addition, genetic variation and susceptibility to *M. cerebralis* had not been characterized for *T. tubifex* from Yellowstone National Park. Finally, whether (and if so, how) these factors influenced parasite success and whirling disease risk to Yellowstone cutthroat trout was unknown.

The goal of this dissertation was to characterize the ecology of the oligochaete host of *M. cerebralis* and *T. tubifex*, and identify factors that may influence *M. cerebralis* infections in *T. tubifex* and whirling disease risk to fish in Yellowstone National Park. I addressed knowledge gaps related to *T. tubifex* in four separate chapters (Chapters 3-6). In Chapter 3, I characterized *T. tubifex* and *M. cerebralis* success in reaches with variable geothermal influence in the Pelican Valley, where high whirling disease risk had previously been detected. In Chapter 4, I examined the distribution of *M. cerebralis* in *T. tubifex* populations and assessed the potential for *M. cerebralis* establishment in others by characterizing *T. tubifex* populations in environmentally variable Yellowstone cutthroat trout spawning tributaries to Yellowstone Lake and the Yellowstone River. In Chapter 5, I examined how substrate, an environmental feature influential for the abundance of *T. tubifex* in tributaries to Yellowstone Lake and the Yellowstone River (Chapter 4), influenced infection prevalence and parasite proliferation (TAM production) in *T. tubifex*. In Chapter 6, I characterized genetic variation in geographic isolates of *T. tubifex* from Yellowstone National Park and

tested the susceptibility of several cultured strains to *M. cerebralis*. Below, I provide a summary of major findings from each chapter and discuss how these results may influence whirling disease risk to Yellowstone cutthroat trout and the potential for their long-term survival in the greater Yellowstone ecosystem.

Tubifex tubifex were widely distributed and abundant in the Pelican Valley, which was characterized by high whirling disease risk (Koel et al. 2006, Murcia et al. 2006, Alexander et al. submitted). *Myxobolus cerebralis* infected *T. tubifex* were also widespread and abundant (collected from all but two of the reaches where *T. tubifex* was collected). The majority of *T. tubifex* were identified as lineage III *T. tubifex*, which included all infected *T. tubifex*. Other lineages were also detected but they were far less abundant than lineage III *T. tubifex*. Thus, myxospores released in this system clearly have a high likelihood of being encountered and ingested by lineage III *T. tubifex* (susceptible, Chapter 6), which probably explains why whirling disease risk to fish is high in Pelican Creek.

Geothermal influence appeared to constrain *M. cerebralis* success in *T. tubifex* in Pelican Creek. In reaches characterized by high geothermal influence, uninfected *T. tubifex* were abundant but *M. cerebralis*-infected *T. tubifex* were not abundant. Abundance of infected *T. tubifex* may have been low relative to abundance of uninfected *T. tubifex* in reaches with high geothermal influence for several reasons. First, survival of infected *T. tubifex* may be reduced because environmental conditions in reaches with high geothermal influence may be sub-optimal for *T. tubifex*, and potentially too stressful for *M. cerebralis*-infected *T. tubifex*. Second, myxospore availability may have been low in reaches with high geothermal influence because fish may avoid these reaches. Finally, a combination of these

factors may explain why abundance of infected *T. tubifex* was low in reaches with high geothermal influence. Regardless of the mechanism, the low abundances of infected *T. tubifex* relative to abundances of uninfected *T. tubifex* in these reaches suggest parasite distribution does not completely overlap with the distribution of the invertebrate host, and thus the success of *M. cerebralis* may be constrained in these reaches. However, whirling disease risk was high, and I did not detect differences in risk among reach types. This suggested that the decreased parasite success in the oligochaete host in reaches with high and no geothermal influence was still above the threshold required for producing high infection severity in fish.

Tubifex tubifex were widely distributed in tributaries throughout Yellowstone National Park: *Tubifex tubifex* populations were detected in at least one reach on all Yellowstone cutthroat trout spawning tributaries included in the study area, however, abundance of *T. tubifex* was highly variable. *Tubifex tubifex* were abundant in unconfined reaches and were not abundant in confined reaches, which suggested environments characterized by low slope and high proportions of fine sediments composed of sand, silt, and clay particles were optimal for *T. tubifex*, and those characterized by high proportions of coarse substrate were sub-optimal for *T. tubifex*. As in Pelican Creek *T. tubifex* populations, the majority of *T. tubifex* were identified as lineage III, which suggested most individuals would exhibit susceptibility to *M. cerebralis* (Chapter 6). *Myxobolus cerebralis* infected *T. tubifex* were only detected in Pelican Creek, tributaries to Pelican Creek, Trout Creek and Elk Antler Creek. I did not detect differences in abundance of *M. cerebralis* infected *T. tubifex* among confinement types, nor in whirling disease risk among confinement types. However,

I never detected *M. cerebralis* in *T. tubifex* from, or sentinel fish exposed in, tributaries characterized by high confinement, which clearly indicated that environments characterized by increased slopes and coarse substrates were unfavorable for *M. cerebralis*.

I examined the influence of substrate on interactions between *T. tubifex* and *M. cerebralis* because *T. tubifex* were present in confined tributaries (characterized by high proportions of coarse substrate), but I never detected infected *T. tubifex* in these environments. I hypothesized that fine substrates would be optimal for *M. cerebralis* because I assumed that rates of myxospore encounter (parasite dose) and parasite proliferation (TAM production) would be high on silt, intermediate on fine sand, and low on coarse sand. I also hypothesized that fine substrates would be optimal for *T. tubifex* fitness and success, which would affect *M. cerebralis*, albeit indirectly. During the dose period (when *T. tubifex* were exposed to 0 or 500 myxospores per worm), *T. tubifex* on silt and fine sand gained weight (positive adult growth) when not exposed to *M. cerebralis*, and lost weight (negative adult growth) when exposed to *M. cerebralis*. In contrast, individual adult growth did not differ among *T. tubifex* on coarse sand during the dose period, regardless of exposure to *M. cerebralis*. The negative adult growth suggested *T. tubifex* exposed to *M. cerebralis* on fine sand and silt were infected and that myxospore exposure (via encounter) may have been higher on fine sand and silt than coarse sand. During the second period of the experiment I did not detect differences in TAM production among *T. tubifex* from any exposure -rearing combinations. However, *T. tubifex* exposed to *M. cerebralis* and reared on coarse sand did not produce TAMs because they all died during this period, which suggested

infected *T. tubifex* could not survive on coarse substrate, and would explain why *M. cerebralis* was never detected in confined reach types.

I examined genetic variation in *T. tubifex* because results of my field studies suggested *T. tubifex* in Yellowstone National Park were relatively genetically homogeneous. Comparatively high genetic diversity has been detected in *T. tubifex* in other populations (Sturmbauer et al. 1999, Beauchamp et al. 2001, 2005, DuBey and Caldwell 2004, Crottini et al. 2008). *Tubifex tubifex* cultured from tributaries in Yellowstone National Park belonged to three lineages. Lineage III were cultured from the majority of the tributaries, and included all of the tributaries where *M. cerebralis* was detected in *T. tubifex* (Chapter 4). Although I was unable to establish susceptibility for all cultured *T. tubifex* belonging to lineage III, those that were examined were susceptible to infection. When combined with the genetic data, which indicate lineage III *T. tubifex* were genetically similar among tributaries throughout Yellowstone (susceptibility was established for lineage III *T. tubifex* cultured from tributaries to both the Yellowstone River and Yellowstone Lake), this suggests that the majority of *T. tubifex* found in Yellowstone are susceptible to infection by *M. cerebralis*.

Lineage I *T. tubifex* were cultured from Slough Creek, where *M. cerebralis* has not previously been detected. *Tubifex tubifex* belonging to lineage I are considered resistant to *M. cerebralis* (Beauchamp et al. 2002, Arsan et al. 2007, Lodh et al. in press). However, at least one strain has previously produced TAMs when exposed to high numbers of myxospores under laboratory conditions (Kerans et al. 2005), which suggests that additional strains of lineage I *T. tubifex* should be examined. I attempted to examine the susceptibility of Slough Creek *T. tubifex* in a second block exposure to complement the exposure discussed in

Chapter 6, but I had problems with myxospore viability and TAMs were never produced (Appendix E). *Myxobolus cerebralis* has been detected in tributaries located both upstream (e.g., Trout Creek, Chapter 4, Yellowstone River downstream from its confluence with Alum Creek, Murcia 2008) and downstream (e.g., Mol Heron Creek outside Yellowstone National Park, Appendix F), which suggests that Slough Creek may have been exposed to the parasite. Consequently, if Slough Creek *T. tubifex* exhibit low susceptibility to *M. cerebralis*, this may explain why *M. cerebralis* has not yet been detected in Slough Creek.

Tubifex tubifex belonging to a previously undescribed lineage were cultured from Thistle, Bridge, Clear, and Astringent Creeks, and from the Upper Yellowstone River. Although I was only able to test the susceptibility of one of these strains (from Clear Creek; the susceptibility of additional strains was to be established in a second experimental block, see Appendix E), *T. tubifex* from within this lineage from Yellowstone were susceptible to *M. cerebralis*. This was surprising because *T. tubifex* from this lineage were genetically distinct from lineage III *T. tubifex* (based on sequence data, Chapter 6).

These results suggest that environmental features, rather than oligochaete host factors, appear to strongly influence *M. cerebralis* success among tributaries in Yellowstone (except possibly in Slough Creek). Potentially susceptible *T. tubifex* were detected in all Yellowstone cutthroat trout spawning tributaries, which clearly affords *M. cerebralis* the opportunity to be transmitted to fish. However, data from Chapters 4 and 5 suggests that infected *T. tubifex* probably cannot survive in stressful environments (e.g., in highly confined tributaries). Thus, environmental factors, influenced by confinement, may preclude establishment of *M. cerebralis* in Yellowstone cutthroat trout spawning tributaries in

Yellowstone National Park. Based on these results, I have assigned tributaries to rough risk categories (Table 7.1, Figure 7.1) to indicate predicted whirling disease risk to Yellowstone cutthroat trout populations using Murcia (2008) as a model. Risk score was calculated by adding values assigned to factors found to influence *M. cerebralis* in *T. tubifex*, including confinement, abundance of susceptible lineages of *T. tubifex*, presence of other oligochaetes (including non-susceptible lineages of *T. tubifex*) and then multiplying by the presence or absence of Yellowstone cutthroat trout, which was assessed by performing limited sampling in tributaries in 2007 (Appendix G). Future research should evaluate the potential for myxospore deposition in these tributaries by examining distribution and movement of infected fish to and within these tributaries.

In addition, future research should directly assess the impact of whirling disease on Yellowstone cutthroat trout populations in Yellowstone National Park because it is difficult to separate the effects of lake trout from the effects of whirling disease, and both factors may be causing the Yellowstone cutthroat trout to decline. The combined pressure from lake trout predation in the lake and *M. cerebralis* infection in spawning tributaries influence almost every aspect of the Yellowstone cutthroat trout life cycle (Figure 7.2). Consequently, the establishment of *M. cerebralis* in additional spawning tributaries (lacustrine-adfluvial Yellowstone cutthroat trout may spawn in 68 of the 124 tributaries to Yellowstone Lake, Gresswell et al. 1994) may further decrease the chances for persistence of native Yellowstone cutthroat trout in Yellowstone National Park.

Combined with population data for Yellowstone cutthroat trout in this system, future monitoring of *M. cerebralis* among tributaries could provide an opportunity to examine

hypotheses regarding persistence and evolution of parasites. My research, combined with other research focused on the dynamics of *M. cerebralis* in this system (Koel et al. 2006, Murcia et al. 2006, Murcia 2008) indicates that Yellowstone National Park may be ideal for testing hypotheses regarding *M. cerebralis* evolution: Yellowstone cutthroat trout is the only compatible fish host species present, and variable susceptibilities to *M. cerebralis* are considered unlikely (Koel et al. 2006). In addition, the oligochaete host is widely distributed, and variable susceptibility also appears unlikely. Consequently, this unique combination may provide an ideal opportunity to examine how environmental conditions, which may effect change over a short time period (in the evolutionary sense), influence the persistence of *M. cerebralis* and perhaps even the evolution of virulence (Rigaud et al. 2010).

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Table 7.1. Risk for *M. cerebralis* establishment in tributaries to Yellowstone Lake and the Yellowstone River in Yellowstone National Park. Risk was determined based on environmental and oligochaete risk factors and the presence or absence of Yellowstone cutthroat trout. Assigned risk value (0 or 1) is indicated in parentheses under each factor. The risk of *M. cerebralis* establishment was assessed by adding factors found to influence *M. cerebralis* in *T. tubifex*, including confinement, abundance of *T. tubifex*, lineage, and multiplied by the presence or absence of Yellowstone cutthroat trout.

Drainage	Tributary	Overall Confinement	Abundance of <i>T. tubifex</i>	Dominant <i>T. tubifex</i> lineage	Yellowstone cutthroat trout detected?	Risk of <i>M. cerebralis</i> establishment if introduced
Yellowstone Lake	Pelican Creek	Unconfined (1)	High (1)	III (1)	Y (1)	3
	Clear Creek	Confined (0)	Low (0)	New lineage (1)	Y (1)	1
	Beaverdam Creek	Confined (0)	Low (0)	III (1)	Y (1)	1
	Upper Yellowstone River	Unconfined (1)	High (1)	New lineage (1)	Y (1)	3
	Bridge Creek	Intermediate (1)	High (1)	New lineage (1)	Y (1)	3
	Arnica Creek	Confined (0)	High (1)	III (1)	Y (1)	2
Yellowstone River	Elk Antler Creek	Unconfined (1)	100 High (1)	III (1)	Y (1)	3
	Trout Creek	Unconfined (1)	High (1)	III (1)	Y (1)	3
	Alum Creek	Intermediate (1)	High (1)	III (1)	N (0)	0
	Otter Creek	Confined (0)	Low (0)	III (1)	N (0)	0
	Slough Creek	Intermediate (1)	High (1)	I (0)	Y (1)	2
	Thistle Creek	Confined (0)	Low (0)	New lineage (1)	Y (1)	1

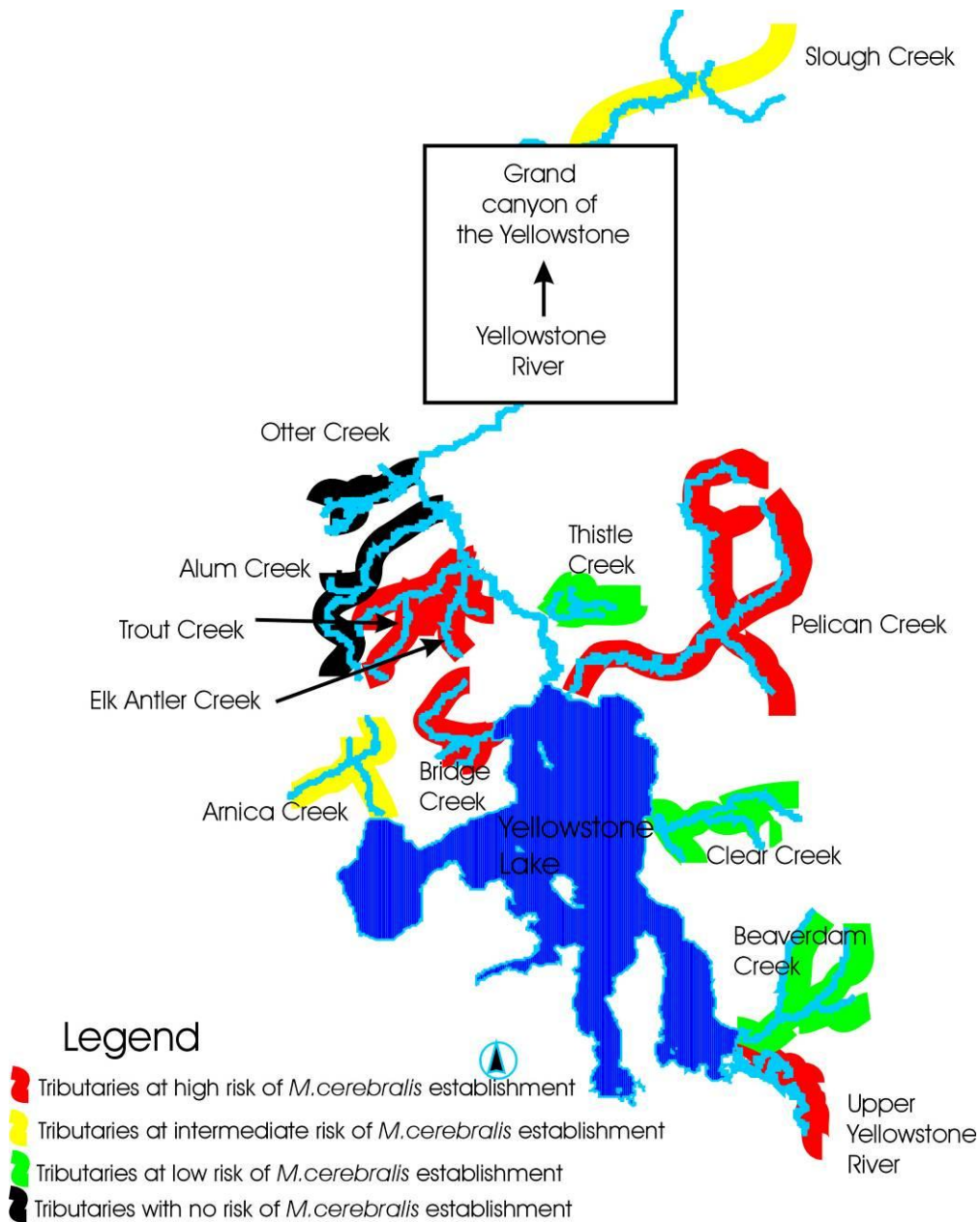


Figure 7.1. Schematic illustrating risk of *M. cerebralis* establishment in tributaries to the Yellowstone River and Yellowstone Lake in Yellowstone National Park. Risk is based on environmental and oligochaete risk factors and the presence or absence of Yellowstone cutthroat trout (see Table 7.1).

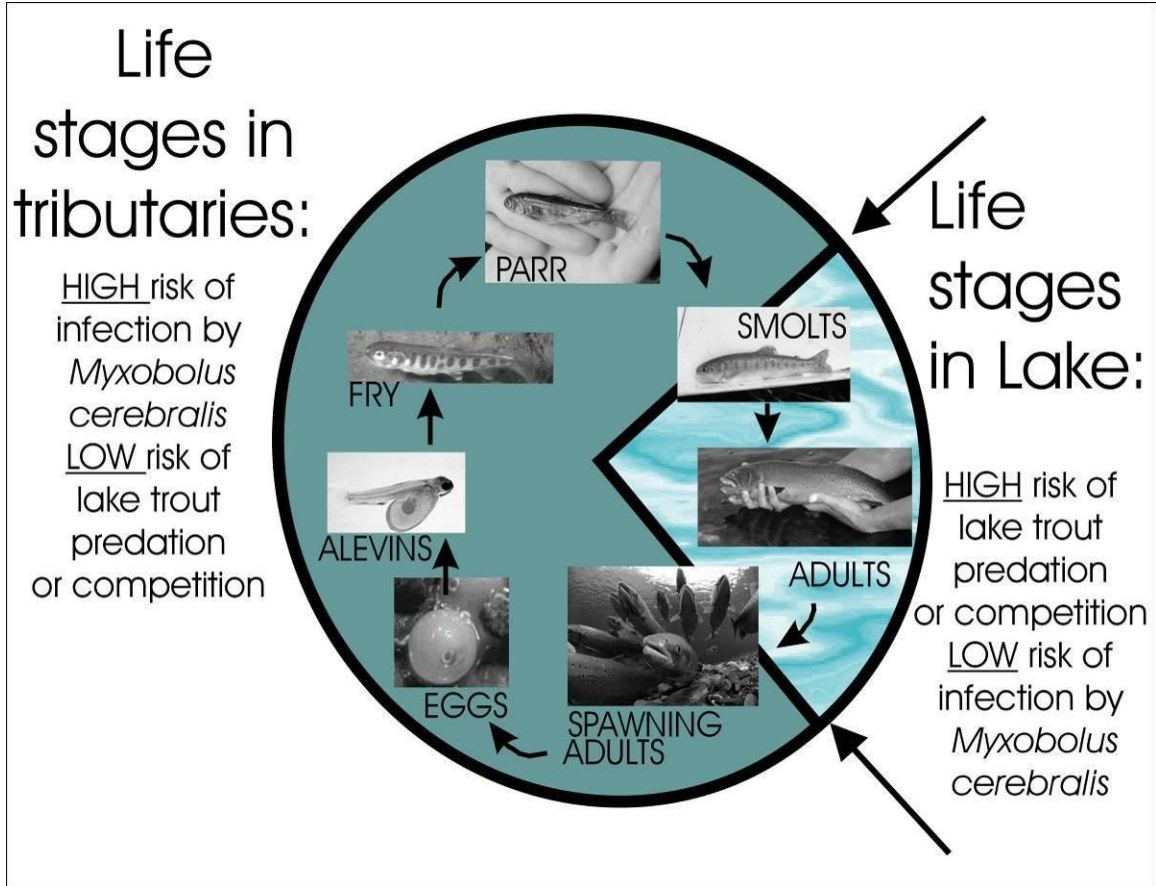


Figure 7.2. Schematic illustrating aspects of Yellowstone cutthroat trout life cycle that are likely influenced by lake trout versus *Myxobolus cerebralis*. *Myxobolus cerebralis* infects emerging fry and parr. Lake trout prey upon parr and smolts once they have moved into the lake from natal tributaries. Adults are not preyed upon by lake trout but compete with them for resources. Spawning adults moving up tributaries may be exposed to *M. cerebralis* but adults are considered resistant to infection. Eggs and alevin stages, which are restricted to gravels are probably not exposed to TAMs but upon emergence from the gravel, fry are likely infected if TAMs are released upstream.

APPENDICES

APPENDIX A:

WIDTH OF 5TH *TUBIFEX TUBIFEX* SEGMENT AND BIOMASS

The relationship between biomass and segment width was examined using laboratory cultured *T. tubifex*. The *T. tubifex* culture was established in 2006 with individual *T. tubifex* (lineage: lineage III) collected from reach 15 on Pelican Creek (chapter 2). Culture propagation methods were as described in Stevens et al. (2001). *Tubifex tubifex* were removed from culture and held without food or substrate for 48 hours. Forty individuals were randomly selected, weighed to the nearest mg, preserved in Kahle's solution and slide mounted. Segment width was measured as distance from the outer edges of body wall across the point of hair chaetae insertion on segment five. Segment five was considered a representative segment because it is located forward of the area covered by the clitellum in mature specimens and behind the narrow prostomium.

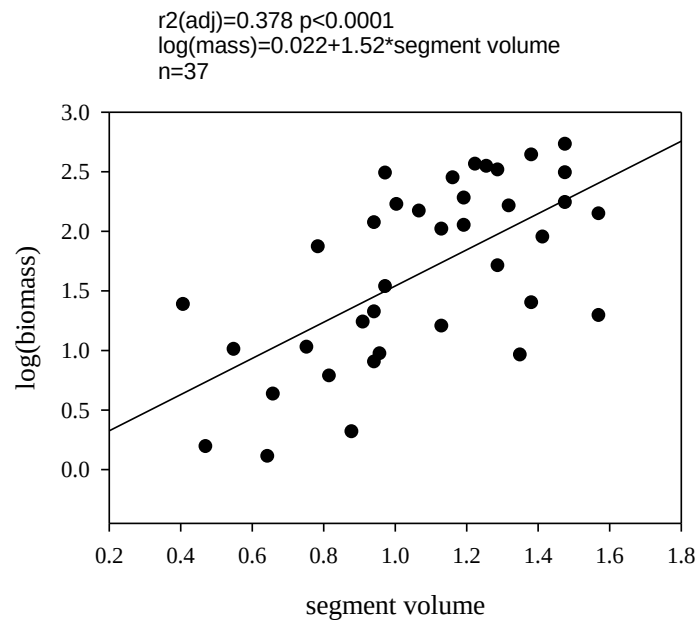


Figure A1. Relationship between the width of *Tubifex tubifex* 5th segment and biomass.

APPENDIX B:

REACH MEANS OF ENVIRONMENTAL AND OLIGOCHAETE VARIABLES IN
PELICAN CREEK AND PELICAN CREEK TRIBUTARIES

In chapter three I examined relationships among reaches classified into four categories based on distance to geothermals so reach means were not shown. The purpose of this appendix is to show environmental (Table B1), oligochaete (Table B2), and segment data (Tables B3-B5) by reach.

Table B1. Mean environmental data (+1. S.E) for Pelican Creek reaches (chapter 3) summarized by reach.

reach	Upstream elevation (ft)	downstream elevation (ft)	temperature (C)	dissolved oxygen mg/L	Specific conductivity	conductivity	pH	confinement	distance from thermal (km)	Estimated thermal size
1	7787	7771	17.420	6.610	0.298	0.255	7.820	1	9.571	3
2	7768	7760	9.100	8.540	0.203	0.142	7.800	1	7.238	3
3	7790	7775	23.150	4.670	0.285	0.281	8.640	3	0.524	3
4	7812	7809	21.980	12.660	0.292	0.273	9.090	1	17.714	1
5	7857	7856	13.900	10.100	0.255	0.199	8.300	1	7.643	1
6	7869	7862	14.600	9.700	0.239	0.193	7.600	1	3.429	1
7	7860	7859	17.400	7.240	0.481	0.413	7.100	2	0.119	1
8	7848	7847	15.200	5.960	0.299	0.266	8.000	1	19.810	3
9	7907	7876	19.360	6.310	0.276	0.246	7.390	1	13.333	3
10	7891	7889	22.030	6.050	0.286	0.266	7.900	1	9.048	3
11	7947	7884	21.400	8.400	0.343	0.319	7.800	2	4.119	3
12	7994	7932	18.100	0.000	0.267	0.232	7.800	2	0.714	3
13	8032	7964	18.100	0.000	0.244	0.211	8.400	2	7.619	2
14	8245	8245	15.600	7.250	0.086	0.070	8.200	3	no thermal	0
15	8058	8036	11.400	7.640	0.184	0.137	8.200	2	7.619	1
16	7904	7900	17.700	5.620	0.195	0.172	7.800	1	7.095	2
17	7923	7922	20.900	7.440	0.183	0.149	7.800	1	19.262	2
18	7917	7903	15.500	7.330	0.137	0.013	7.800	1	16.214	2
19	7926	7923	13.400	6.750	0.178	0.139	7.400	1	12.857	2
20	7880	7871	24.850	4.600	0.778	0.767	3.330	2	2.260	3
21	7829	7823	28.540	4.490	0.881	0.827	3.230	3	0.361	3
22	7901	7897	13.700	7.850	0.085	0.066	8.300	2	no thermal	0
23	7951	7942	12.800	7.750	0.061	0.047	8.600	3	no thermal	0
24	7930	7906	20.100	6.470	0.071	0.064	8.200	2	no thermal	0
25	7973	7969	17.700	8.230	0.061	0.053	8.200	2	no thermal	0

Table B1 continued. Mean environmental data (+1. S.E) for Pelican Creek reaches (chapter 3) summarized by reach.

reach	Width	Depth	Velocity	Organic material	Prop. fines	% sand	% clay	% silt
1	14.340 (1.00481) 16.980	0.267 (0.07396) 0.320	0.013 (0.01333) 0.289	2.180 (0.29525) 4.345	0.823 (0.09523) 0.245	80.874	8.980	10.146
2	(6.14363) 10.927	(0.04399) 0.315	(0.28919) 0.021	(0.43862) 0.897	(0.06558) 0.803	41.825	19.241	38.934
3	(0.83055) 29.137	(0.02688) 0.208	(0.02082) 0.149	(.) 1.695	(0.08275) 0.674	87.158	11.695	1.147
4	(0.71857) 20.000	(0.07984) 0.366	(0.14903) 0.024	(0.2722) 4.360	(0.14763) 0.558	84.644	7.926	7.431
5	(2.78747) 19.167	(.) 0.234	(0.02404) 0.057	(.) 1.451	(0.22735) 0.558	72.772	14.852	12.376
6	(1.67564) 19.433	(0.02688) 0.132	(0.05686) 0.160	(.) 1.886	(0.24893) 0.673	80.171	8.998	10.831
7	(0.87289) 18.800	(0.02032) 0.152	(0.16045) 0.074	(.) 4.539	(0.15408) 0.898	84.750	13.889	1.362
8	(1.60104) 5.077	(0.03048) 0.234	(0.07424) 0.027	(.) 1.231	(0.054) 0.469	83.663	8.911	7.426
9	(0.31317) 6.547	(0.0618) 0.264	(0.02667) 0.037	(0.01146) 1.302	(0.19609) 0.660	91.811	4.514	3.675
10	(0.39074) 10.200	(0.04064) 0.356	(0.03712) 0.022	(.) 1.357	(0.08843) 0.721	90.555	4.048	5.397
11	(3.60046) 6.413	(0.0618) 0.120	(0.02186) 0.528	(0.05733) 5.353	(0.14542) 0.551	88.497	6.165	5.338
12	(2.15772) 3.023	(0.01109) 0.193	(0.52843) 0.127	(0.25824) 6.624	(0.25843) 0.177	32.568	28.469	38.963
13	(0.46563) 3.803	(0.07935) 0.244	(0.12732) 0.017	(1.49198) 1.434	(0.0945) 0.027	67.088	18.885	14.027
14	(1.32269) 1.907	(0.0577) 0.170	(0.01732) 0.028	(0.00553) 32.591	(0.01799) 0.238	82.681	10.361	6.958
15	(0.03844) 8.800	(0.01107) 0.757	(0.02848) 0.006	(0.44129) 1.904	(0.05311) 0.694	49.718	22.976	27.306
16	(0.98489) 7.800	(0.10497) 0.694	(0.00577) 0.009	(0.48189) 1.610	(0.11362) 0.986	90.789	5.624	3.588
17	(0.15275) 6.107	(0.14975) 0.122	(0.00882) 0.054	(0.38896) 2.612	(0.0136) 0.483	91.000	4.909	4.091
18	(0.57185) 4.387	(0.0176) 0.345	(0.05364) 0.010	(0.04378) 2.476	(0.2316) 0.864	79.190	4.132	16.679
19	(0.6739) 2.563	(0.08857) 0.305	(0.01) 0.176	(0.44457) 2.273	(0.0981) 0.823	82.699	6.564	10.737
20	(0.26028) 3.057	(0.1099) 0.218	(0.17559) 0.055	(.) 2.032	(0.12094) 0.837	85.172	8.063	6.765
21	(0.32106) 1.450	(0.00508) 0.127	(0.05487) 0.058	(.) 0.921	(0.13384) 0.211	85.172	8.063	6.765
22	(0.16371) 2.697	(0.00508) 0.109	(0.0584) 0.020	(.) 2.077	(0.10947) 0.034	96.611	3.304	0.085
23	(0.48704) 4.780	(0.0127) 0.315	(0.02) 0.070	(.) 3.562	(0.034) 0.728	89.992	9.031	1.766
24	(0.67352) 3.277	(0.13469) 0.284	(0.06984) 0.066	(0.21404) 2.909	(0.15332) 0.231	81.124	10.248	8.628
25	(0.30278)	(0.10198)	(0.06557)	(0.30475)	(0.20148)	83.773	8.666	7.561

Table B2. Oligochaete data for Pelican Creek reaches (chapter 3) summarized by reach.

Reach number	# oligochaetes collected	man hour standard	# kick samples	# tubificids prepared as individual samples	# tubificids prepared as pool samples (number in pooled samples)	mature <i>T. tubifex</i> in pools	mature <i>T. tubifex</i> in individual samples	<i>M. cerebralis</i> + individuals	<i>M. cerebralis</i> + pools
1	346	1	3	40	7(93)	2	1	3	1
2	300	1	3	40	10(158)	11	2	1	1
3	220	1	5	40	9(128)	4	3	0	3
4	239	0.98	3	40	10(156)	2	1	0	5
5	378	0.82	3	40	10(151)	1	0	7	3
6	256	1	4	40	10(140)	0	0	0	1
7	300	1	3	40	10(150)	1	0	0	1
8	501	1	3	40	10(158)	1	0	6	6
9	550	1	3	40	10(159)	0	0	2	4
10	506	1	3	40	10(159)	0	0	1	4
11	304	1.15	3	40	9(133)	0	0	0	6
12	316	0.75	4	40	10(176)	0	0	0	3
13	310	0.52	4	40	10(142)	0	0	0	7
14	227	1	5	40	7(110)	0	0	0	0
15	351	0.83	2	40	10(157)	5	3	15	9
16	134	1	4	33	0(0)	0	0	1	0
17	258	1.5	3	40	3(46)	0	1	2	3
18	303	1.37	3	40	3(28)	0	1	4	0
19	383	0.5	3	40	10(155)	1	1	1	3
20	17	1	5	16	0(0)	.	1	0	0
21	0	1	5	0	0(0)	.	0	0	0
22	10	1	4	10	0(0)	.	1	0	0
23	3	1	4	2	0(0)	.	0	0	0
24	60	1.5	4	43	0(0)	.	4	1	0
25	3	1	4	0	0(0)	.	0	0	0

Table B3. Segment width data for mature *T. tubifex* from Pelican Creek reaches (chapter 3) summarized by reach.

reach	segment width (± 1 S.E) mm	n
1	0.580(.)	1
2	0.485 (0.105)	2
3	0.533(0.019)	3
4	0.290(.)	1
15	0.610(0.111)	3
17	0.570(.)	1
18	0.500(.)	1
19	0.420(.)	1
20	0.230(.)	1
22	0.560(.)	1
24	0.568(0.032)	4

Table B4. Segment width data for *M. cerebralis* infected *T. tubifex* from Pelican Creek reaches (chapter 3) summarized by reach.

reach	segment width (± 1 S.E) mm	n
1	0.393(0.041)	3
2	0.480(.)	1
4	0.360(.)	1
5	0.428(0.046)	6
8	0.565(0.047)	4
9	0.515(0.165)	2
10	0.480(.)	1
12	.	0
15	.680(0.038)	12
16	0.390(.)	1
17	0.490(0.060)	2
18	0.437(0.049)	3

Table B5. Segment width data for randomly selected *T. tubifex* from Pelican Creek reaches (chapter 3) summarized by reach.

reach	segment width (± 1 S.E) mm	n
1	0.428(0.029)	5
2	0.446(0.038)	5
3	0.452(0.032)	5
4	0.468(0.022)	5
5	0.448(0.055)	4
6	0.472(0.054)	5
7	0.436(0.068)	5
8	0.486(0.020)	5
9	0.415(0.034)	4
10	0.496(0.052)	5
11	0.460(0.021)	5
12	0.464(0.036)	5
13	0.510(0.079)	5
14	0.524(0.034)	5
15	0.706(0.042)	5
16	0.362(0.045)	5
17	0.464(0.039)	5
18	0.448(0.012)	5
19	0.452(0.024)	5
20	0.306(0.016)	5
21	.	0
22	0.526(0.042)	5
23	0.700(0.040)	2
24	0.536(0.074)	5
25	.	0

APPENDIX C:

COMPARISON OF *TUBIFEX TUBIFEX* ABUNDANCE ESTIMATES OBTAINED BY
SORTING KICK NET SAMPLES IN THE FIELD VERSUS LABORATORY

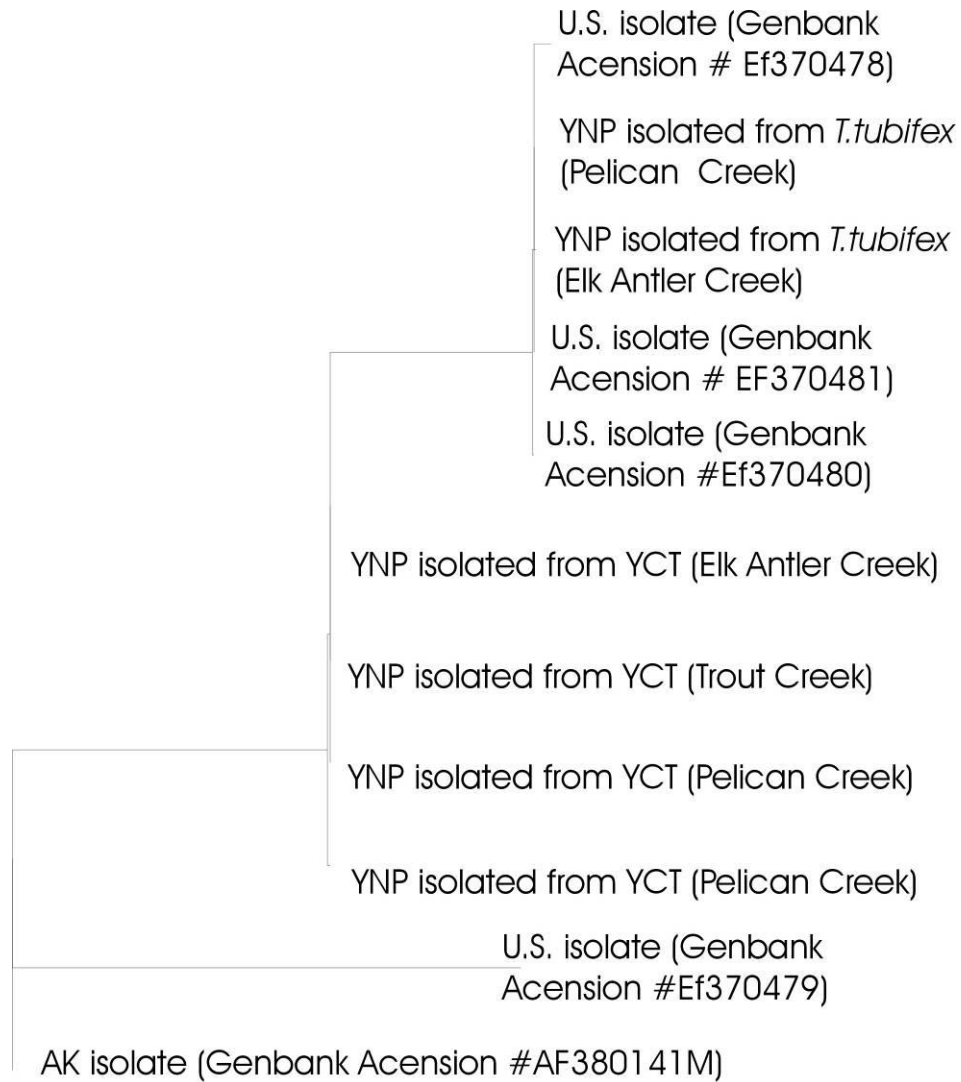
The purpose of appendix C is to demonstrate the relationship between the different measures of *T. tubifex* abundance in this dissertation. In Chapter 3, abundance of *T. tubifex* was determined from tubificids that were picked out of kick samples in the field. In this method, tubificids were removed from sorting trays in the field and subsequently sorted in the laboratory. This method was used because it ensured enough individuals (>200) were collected for molecular analyses, including *M. cerebralis* infection prevalence, in each reach. However, this method could result in biased samples because individuals may be more likely to be picked from locations where tubificids are large, and individuals may be less likely to be picked when individuals are small or conditions such as high silt make picking difficult. We calculated “field estimated *T. tubifex* abundance” as CPUE [number of *T. tubifex* (after all immatures were assigned as above)]/(time spent sorting)/(total number of kicks) per reach to compare this method with estimates of abundance (kick net samples preserved and subsequently sorted in the laboratory, see above).

The “field estimated *T. tubifex* abundance,” which was based on field sorted samples and used previously, was positively correlated with our measure of *T. tubifex* abundance ($r^2=0.627$, $p<0.0001$).

APPENDIX D:

18SRDNA SEQUENCE DATA CONFIRM *MYXOBOLUS CEREBRALIS* IS THE
MYXOZOAN AMPLIFIED IN *TUBIFEX TUBIFEX* AND SENTINEL TROUT IN
YELLOWSTONE NATIONAL PARK

The purpose of appendix D is to validate the PCR results used to assess *M. cerebralis* factors in this dissertation. PCR products isolated from sentinel fish and *T. tubifex* from Pelican, Trout, and Elk Antler and Otter Creeks were sequenced to verify the myxozoan that was amplified in PCR assays for *T. tubifex* and sentinel trout (Chapters 3 and 4) was *Myxobolus cerebralis*. Sequences were obtained from Genbank for comparison (Accession numbers follow labels on Figure D1). Parsimony analysis was conducted in Paup (set criterion=parsimony taxlabels=full torder=right maxtrees=5000 increase=no root=outgroup outroot=monophyl storebrlens=yes warnreset=no warntree=no warntsave=no warnroot=no warnredef=no autoclose=yes; bandb multrees=yes; describetrees 1/ root=outgroup plot=phylogram apolist=no; savetrees root=yes file=MC_tre.nex replace format=altnex brlens=yes; contree all/strict=yes semistrict=no majrule=no adam=no root=outgroup treefile=MC_tre.nex append=yes; bootstrap reps=10000 grpfreq=no conlevel=70 search=bandb/ multrees=no; savetrees from=1 to=1 root=yes file=MC_tre.nex append=yes format=altnex savebootp=brlens; log stop;). The samples isolated from *T. tubifex* and sentinel trout from Pelican, Trout, and Elk Antler Creeks were a 100% match with sequences available on Genbank (Figure D1).



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Figure D1. Single most parsimonious tree from analysis of a 422 base pairs in the 18S DNA region for *M. cerebralis* isolated from *T. tubifex* and sentinel Yellowstone cutthroat trout in Yellowstone National Park.

APPENDIX E:

TUBIFEX TUBIFEX CULTURES AND PRELIMINARY
RESULTS FROM TWO EXPERIMENTS

The purpose of this appendix twofold: First, to provide data about *Tubifex tubifex* cultures that were established from tributaries to the Yellowstone River and Yellowstone Lake from 2006-2008 including drainage, tributary, collection date, date reproduction was first detected, and morphological and molecular identification (Table E.1).

Culture Establishment

We successfully established reproducing laboratory cultures of *T. tubifex* from Pelican (five cultures), Astringent (two cultures), Footbridge (one culture), Chittenden (one culture), Raven (one culture), the unnamed tributary to Pelican Creek (four cultures), Pelican Cone (one culture), Clear (six cultures), Beaverdam (three cultures), Bridge (one culture), Thistle (three cultures), Slough (one culture), Trout (two cultures), and Elk Antler Creeks (three cultures), and the Upper Yellowstone River (three cultures) using the substrate container method. We did not obtain reproducing laboratory cultures from Arnica, Otter, or Alum Creeks.

Experiments

I set up two experiments in summer 2009 because I felt they would compliment my research. However, the source of myxospores (infected rainbow trout) had changed and I evidently used non-viable myxospores in the experiments, which was evidenced by a total lack of TAM production over the 130 day period each experiment ran. The aim of the first experiment was to characterize susceptibility of additional *T. tubifex* cultures from Yellowstone, by including *T. tubifex* from cultures that had not reproduced sufficiently to be included in the first block (Chapter 6). The aim of the second experiment was to establish

the relationship between infection prevalence in *T. tubifex* and TAM production because this relationship has not been clearly established.

Second Block of Susceptibility Experiment

Methods for the second block of the susceptibility experiment were as explained in Chapter 6, except “infected” rainbow trout were infected and reared at the Pony Fish Hatchery, Pont, MT (obtained from J. Hupka, MTFWP) and myxospores were extracted in August 2009. Cultures that were included in the second block are listed in Table G.1. The experiment was terminated after 130 d because no TAMs were ever detected (not even in the Mt. Whitney replicates).

Relationship Between Infection Prevalence and TAM Production Experiment

I used five treatments of infection prevalence, including 0%, 20%, 50%, 80% and 100% infected and five replicates for each container. As in Chapter 5, I conducted the experiment in two periods. During the first period, *T. tubifex* were removed from culture (culture d14, susceptibility was previously established, see Chapter 6) and held without substrate for 24 hrs to equalize hunger levels. Groups of 50 *T. tubifex* were wet weighed to the nearest mg and randomly assigned to an exposure container. A suspension of 500 myxospores per *T. tubifex* (13 containers of 50 *T. tubifex*) or an equivalent volume of spore-free emulsion (0 myxospores per *T. tubifex*, 13 containers of 50 *T. tubifex*) was added to each exposure container. Myxospores were extracted as above (see Second Block of Susceptibility Experiment). After five days, *T. tubifex* were removed from their containers, wet weighed to the nearest mg, placed into a master container with all other *T. tubifex* from that dose (0 or 1000 myxospores), and assigned to new containers as follows: Containers

assigned to the 0 infection prevalence treatment received 50 *T. tubifex* from the 0 dose master container, and 0 *T. tubifex* from the 1000 dose master container, containers assigned to the 20 infection prevalence treatment received 40 *T. tubifex* from the 0 dose master container, and 10 *T. tubifex* from the 1000 dose master container, and so on. Experimental containers were maintained as in chapter 5. The experiment was terminated after 130 d because only a single TAM was detected (from one replicate in the 20% infected treatment of all places!), which suggested myxospores may not have been mature. I calculated individual adult growth as in chapter 5 (Figure G.1), and detected differences among infection prevalence treatments (which were suggestive of infection) but I did not carry out the analyses since TAMs were not produced.

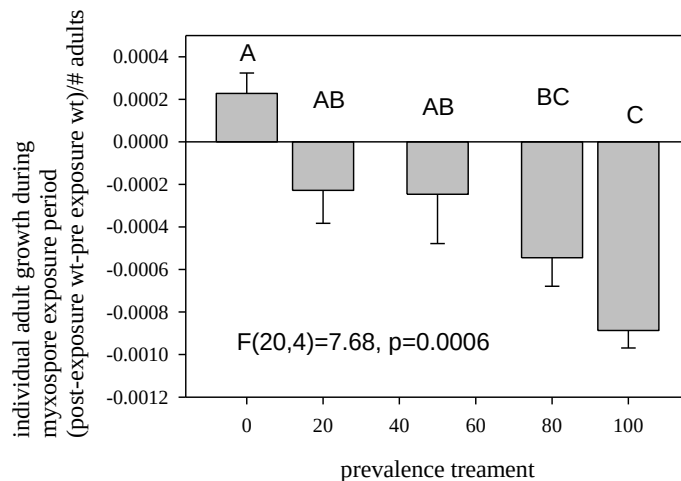


Figure E.1. Adult growth in five days following exposure to 1000 myxospores (or spore free emulsion). Pre-exposure weights did not differ.

Table E.1. Yellowstone *T. tubifex* culture data.

Drainage	Creek	Site.id	Collection date	Date reproduction first detected	Morphological ID	Molecular ID
Yellowstone Lake	Upper Yellowstone River	05.11 s	July.2007	15.jan.2008	<i>T. tubifex</i>	?
			July.2007	15.jan.2008	<i>T. tubifex</i>	?
			July.2007	15.jan.2008	<i>T. tubifex</i>	?
	unnamed tributary to Pelican Creek	04.15-05.05	Aug.2006	18.december.2006	<i>T. tubifex</i>	III
			Sept.2007	6.sept.2007	<i>T. tubifex</i>	III
			Sept.2007	6.sept.2007	<i>T. tubifex</i>	III
			Sept.2007	6.sept.2007	<i>T. tubifex</i>	III
	Raven Creek tributary to Pelican Creek	04.17	October.2006	6.sept.2007	<i>T. tubifex</i>	III
	Pelican cone tributary to Pelican Creek	4.22	October.2006	18.december.2006	<i>T. tubifex</i>	III
	Pelican Creek	4.07	Aug.2006	15.jan.2008	<i>T. tubifex</i>	III
		4.10.xing	October.2006	6.sept.2007	<i>T. tubifex</i>	III
		4.10.xing	October.2006	6.sept.2007	<i>T. tubifex</i>	III
		04.1 (a)	Aug.2006	18.december.2006	<i>T. tubifex</i>	III
		04.13 (b)	Aug.2006	6.sept.2007	<i>T. tubifex</i>	III
	Footbridge tributary of Pelican Creek	4.23	October.2006	12.sept.2006	<i>T. tubifex</i>	III
	Clear creek	05.7 (1)	October.2007	15.jan.2008	<i>T. tubifex</i>	?
			October.2007	15.jan.2008	<i>T. tubifex</i>	?
			October.2007	15.jan.2008	<i>T. tubifex</i>	?
			October.2007	15.jan.2008	<i>T. tubifex</i>	?
			October.2007	15.jan.2008	<i>T. tubifex</i>	?
			October.2007	11.oct.2007	<i>T. tubifex</i>	?
	Chittenden tributary of Pelican Creek	04.24 site	Sept.2007	18.december.2006	<i>T. tubifex</i>	III
	Bridge creek	05.15 site	Aug.2007	15.jan.2008	<i>T. tubifex</i>	III
	Beaverdam creek	05.09q	July.2007	11.oct.2007	<i>T. tubifex</i>	III
		05.09r	July.2007	11.oct.2007	<i>T. tubifex</i>	III
		05.09r	July.2007	11.oct.2007	<i>T. tubifex</i>	III
	Astringent creek	04.20 site	Sept.2007	18.december.2006	<i>T. tubifex</i>	III
			Sept.2007	18.december.2006	<i>T. tubifex</i>	III
Yellowstone River	trout creek	05.19 site	July.2007	15.july.2007	<i>T. tubifex</i>	III
			July.2007	15.july.2007	<i>T. tubifex</i>	III
	thistle creek	05.26 site	Jun.2007	15.july.2007	<i>T. tubifex</i>	?
			Jun.2007	11.oct.2007	<i>T. tubifex</i>	?
			Jun.2007	15.jan.2008	<i>T. tubifex</i>	?
	slough creek mol heron creek	05.28	Jun.2007	15.jan.2008	<i>T. tubifex</i>	I
		2008	Nov.2008	15.jan.2008	<i>T. tubifex</i>	III
	Elk antler creek	05.17 site	July.2007	15.july.2007	<i>T. tubifex</i>	III
			July.2007	11.oct.2007	<i>T. tubifex</i>	III
			July.2007	15.july.2007	<i>T. tubifex</i>	III

APPENDIX F:

WHIRLING DISEASE RISK IN THE YELLOWSTONE RIVER BELOW
YELLOWSTONE NATIONAL PARK

Data from tributaries on the Yellowstone River downstream of Yellowstone National Park. Data were collected during the winter 2007-2008 to validate relationships between confinement and oligochaete risk factors, which were examined in chapter 4. We examined five tributaries including Molheron, Mill, Reese, Emigrant, Cedar and Big Creeks in the Paradise Valley. A total of 10 study sites were allocated to the selected tributaries based on accessibility (Figure F.1). Whirling disease risk was assessed using data from sentinel fish exposures (personal communication, R. Vincent, Montana Fish Wildlife and Parks). Oligochaetes, tubificids and environmental data were sampled as in chapter 3. Abundances of oligochaetes and *T. tubifex* and infection prevalence and abundance of infected *T. tubifex* were determined as in chapter 3, except we did not calculate relative abundance of *T. tubifex* (the proportion) because in 2005, we found that more intensive sampling (e.g., sorting kick net contents under a dissecting microscope rather than in the field) was necessary to achieve reliable values for this variable.

Environmental data were summarized using PCA. Relationships among habitat types and risk factors (e.g., risk to fish, oligochaete abundance) were examined using simple correlations. Correlations among environmental features including PCA axes 1-4 and individual environmental features and WD risk factors were analyzed with simple correlations. Correlations among WD risk (including infection prevalence and severity in sentinel fish, the abundance of oligochaetes and *T. tubifex*, the abundance *M. cerebralis* infected *T. tubifex*, and infection prevalence in *T. tubifex*) factors were also analyzed with simple correlations.

Four major PCA axes described ~86.5% of the variation in environmental features among sampling sites (Table F.1). PC1 discriminated sites characterized by high conductivity and high proportions clay and silt, and low proportions of sand from sites with the opposite characteristics. PC2 discriminated sites characterized by higher dissolved oxygen, high proportions of organic material and non-fine sediments, and low velocity and pH from sites with the opposite characteristics. PC3 discriminated sites characterized by high temperatures and dissolved oxygen, and low proportions of organic material and low slopes from sites with the opposite characteristics. PC4 discriminated deeper, wider sites characterized by high temperatures from sites with the opposite characteristics.

Environmental variability, as described by principal components analysis, discriminated sites by confinement type: On average, unconfined sites plotted lower on PC2 than sites characterized by intermediate or high confinement, which indicated that unconfined sites were characterized by increased velocities and pH values, and low non-fine sediments, dissolved oxygen values and proportions of organic material. In contrast, confined sites plotted high on PC2, which indicated they were characterized by relatively lower velocities, conductivity and pH, and relatively higher dissolved oxygen, proportions of non-fine sediments and organic material.

Whirling disease risk to fish: Whirling disease risk, measured as infection severity in sentinel fish, was high in Emigrant Creek, intermediate in Mulheron Creek, and low in Big Creek (Table F.2). *Myxobolus cerebralis* was not detected in sentinel fish in Cedar and Mill Creeks, and sentinel fish were not deployed in Reese Creek (Table F.2). Infection prevalence in sentinel fish ranged from 100% (Emigrant Creek) to 5.9% (Big Creek) and infection

severity ranged from 4.48 (Emigrant Creek) to 0.09 (Big Creek; Table F2). The presence or absence of TAMs in water samples corroborated our WD risk data assessed by sentinel fish with one exception: We detected TAMs in water samples collected from the lower Reese Creek site, but sentinel fish were not sampled at this site (Table F.2). Infection prevalence in sentinel fish was highest in unconfined sites (89.80 ± 10.20 , $n=2$), lower in intermediate sites (5.90 , $n=1$), and non-existent in confined sites (0.0 ± 0.0 , $n=2$). Infection severity was also highest in unconfined habitats, lower in intermediate habitats, and non-existent in confined habitats (Table F4). Infection prevalence and severity were both negatively correlated with PC4 (Table F3) indicating that they were highest in narrow, shallow sites with low temperatures. However, neither variable was significantly correlated with individual environmental variables. Infection prevalence and severity in fish were also highly correlated, making it impossible to separate these variables at this scale (Table F3). Infection prevalence in fish was positively correlated with prevalence of infection in *T. tubifex* (Table F5). Infection severity in fish was also positively correlated with prevalence of infection in *T. tubifex* (Table F5). Surprisingly, neither variable was correlated with the abundance of infected *T. tubifex* (Table F5).

Abundance of oligochaetes and *T. tubifex*: Oligochaetes were collected at 11/12 sites (Table F2). We were unable to collect oligochaetes from Lower Emigrant Creek because the creek bed was dry during our sampling period, possibly diverted for agriculture. Abundance of oligochaetes was highest at the upper site on Reese Creek (CPUE: 18.8), and no oligochaetes were found at the lower sites on Mill and Big Creeks (CPUE: 0.0) during the search period. Abundance of *T. tubifex* was also highest at the upper site on Reese Creek and

no *T. tubifex* were found at the lower sites on Mill and Big Creeks during the search period (Table F.2). Abundance of oligochaetes was high in unconfined habitat types (13.58 ± 2.27 , $n=4$), and low in confined habitats (2.13 ± 1.03 , $n=5$). Abundance of *T. tubifex* was also highest in unconfined habitat types and lowest in confined habitats (Table 14). Abundance of oligochaetes was negatively correlated with PC4 (Table F3), which indicated oligochaetes were more abundant in narrower, shallower reaches with low temperatures than in sites with the opposite characteristics. Abundance of *T. tubifex* was negatively correlated with PC2, which indicated that sites characterized by increased pH and velocities and low dissolved oxygen levels, low proportions of organic material and low amounts of non-fine sediments have higher *T. tubifex* abundance in these drainages. The abundance of *T. tubifex* was also correlated with conductivity ($r=0.63$, $p=0.04$, $n=11$).

Neither the abundance of oligochaetes, nor abundance of *T. tubifex* were significantly correlated with infection prevalence or severity in sentinel fish (Table F.5). However, the abundance of oligochaetes was significantly correlated with the abundance of *T. tubifex* (Table F.5).

M. cerebralis infection in *T. tubifex*: As in sentinel fish, *M. cerebralis* infected *T. tubifex* were detected in Emigrant Creek, Mulheron Creek, and Big Creek (Table F2; Figure F1). Infected *T. tubifex* were also detected in Reese Creek, which was not sampled for sentinel fish (Table F2; Figure F1). Infected *T. tubifex* were not detected in Cedar or Mill Creeks (Table F2). The highest abundance of infected *T. tubifex* was observed at the upper Mulheron Creek site (Table F2).

Infection prevalence in *T. tubifex* and abundance of infected *T. tubifex* were highest in unconfined sites and lowest in confined sites (Table F4), which was consistent with our previous findings at the among catchment scale. The infection prevalence in *T. tubifex* and the abundance of infected *T. tubifex* were not significantly correlated with PC1-4 (Table F3).

The prevalence of infection in *T. tubifex* was negatively correlated with temperature ($r=-0.64$, $p=0.03$, $n=11$) and positively correlated with conductivity ($r=0.61$, $p=0.05$, $n=11$). The abundance of infected *T. tubifex* was also positively correlated with conductivity ($r=0.80$, $p=0.003$, $n=11$). Neither variable was significantly correlated with additional individual environmental variables.

The prevalence of infection in *T. tubifex* was significantly correlated with infection prevalence and severity in sentinel fish, whereas abundance of infected *T. tubifex* was not correlated with either infection prevalence or severity in sentinel fish (Table F5). However, both the prevalence of infection in *T. tubifex* and the abundance of infected *T. tubifex* were correlated with the abundance of oligochaetes and the abundance of *T. tubifex* (Table F5). In addition, the prevalence of infection in *T. tubifex* was significantly correlated with the abundance of infected *T. tubifex* (Table F5).

Summary: Catchment scale variables including confinement type (confined versus unconfined) and substrate composition were useful predictors of oligochaete and *T. tubifex* abundance, as well as infection prevalence in *T. tubifex* and abundance of infected *T. tubifex* for tributaries to the Yellowstone River, downstream of Yellowstone National Park. While further research is needed to identify specific mechanisms, our results suggest that a very basic risk assessment using environmental data that are easily collected may be useful for

assessing WD risk at broad (among catchment) scales when the oligochaete host is characterized by low genetic variability.

Table F1. Values of eigenvectors on principal components axes 1-4 (%= variation explained) from test tributaries. Significant values (>0.3) are shown in bold text. PC1-PC4 explained a total of 86.46% of the variability in the dataset.

Variables	PC1 (33.33%)	PC2 (23.21%)	PC3 (15.75%)	PC4 (14.17%)
Width	0.298	0.181	-0.045	0.504
Depth	0.272	-0.080	-0.115	0.541
Velocity	0.252	-0.427	0.110	-0.045
Slope	-0.212	0.149	-0.413	0.198
% organic material in sediments	-0.003	0.411	-0.374	-0.101
proportion non-fine sediments	0.293	0.372	0.223	0.033
proportion sand	-0.417	0.053	0.239	0.169
proportion clay	0.410	0.068	-0.061	0.188
proportion silt	0.385	-0.081	-0.240	-0.271
Temperature	-0.021	-0.106	0.553	0.309
Conductivity	0.339	-0.266	0.034	-0.278
pH	-0.130	-0.499	-0.184	0.132
Dissolved oxygen	0.145	0.322	0.391	-0.269

Table F2. Confinement types, WD risk to Yellowstone cutthroat trout (as assessed by infection prevalence and severity in sentinel fish), and oligochaete risk factors at among test sites in YNP. Drainage= Lower Yellowstone River (below YNP). (.)=not tested

Site Name	confinement	Cage Year	Infection prevalence in sentinel fish %	Mean infection severity score	TAMs detected during water filtration (y/n)?	Abundance of <i>T. tubifex</i> CPUE	Prevalence of Infection in <i>T. tubifex</i> (%)
Reese Creek lower	Unconfined	.	NT	.	Y	14.8	1.50
Reese Creek Upper	Unconfined	.	.	.	.	18.6	1.80
Cedar Creek lower	Confined	2007	0	0	N	2.8	0.00
Cedar Creek Upper	Confined	.	.	.	.	1.3	0.00
Mulheron Creek lower	Unconfined	2007	79.6	2.43	Y	12.2	0.91
Mulheron Creek Upper	Unconfined	.	.	.	.	7.6	4.69
Big Creek lower	Intermediate	2007	5.9	0.09	N	0	0.00
Big Creek upper	Intermediate	.	.	.	.	7	3.17
Mill Creek lower	Confined	2006	0	0	N	0	0.00
Mill Creek upper	Confined	.	.	.	.	0.4	0.00
Emigrant Creek lower	Unconfined	2007	100	4.48	.	.	.
Emigrant Creek Upper	Confined	.	.	.	Y	2.7	3.13

Table F3. Spearman correlations among WD risk and oligochaete variables and PCA axes describing environmental data for test sites. Significant correlations ($p < 0.05$) are shown in bold text.

PC		Infection prevalence in fish	Infection severity in sentinel fish	Oligochaete abundance	Abundance of <i>T. tubifex</i>	Prevalence of infection in <i>T. tubifex</i>	Abundance of infected <i>T. tubifex</i>
PC1	r value	0.2052	0.2052	0	-0.06378	0.31464	0.32418
	p value	0.7406	0.7406	1	0.8522	0.346	0.3308
	n	5	5	11	11	11	11
PC2	r value	0.2052	0.2052	-0.4328	-0.61504	0.0286	-0.29557
	p value	0.7406	0.7406	0.1836	0.044	0.9335	0.3775
	n	5	5	11	11	11	11
PC3	r value	-0.35909	-0.35909	0.00911	-0.06378	-0.20023	-0.11442
	p value	0.5528	0.5528	0.9788	0.8522	0.555	0.7376
	n	5	5	11	11	11	11
PC4	r value	-0.87208	-0.87208	-0.5877	-0.51481	-0.53394	-0.4958
	p value	0.0539	0.0539	0.0573	0.1051	0.0907	0.1209
	n	5	5	11	11	11	11

Table F4. Mean $\pm$ SE WD risk factor values including WD risk (infection severity in sentinel fish), *T. tubifex* abundance and *M. cerebralis* infection prevalence in *T. tubifex* shown by confinement type for test sites in 2008.

Confinement Type	Mean infection severity in fish (on 0-5 scale $\pm$ 1 S.E.)	Mean <i>T. tubifex</i> abundance (CPUE $\pm$ 1 S.E.)	Mean infection prevalence in <i>T. tubifex</i> (% $\pm$ 1 S.E.)	Mean abundance of infected <i>T. tubifex</i> ($\pm$ 1 S.E.)
unconfined	3.46 $\pm$ 1.03 (2)	13.28 $\pm$ 2.31 (4)	2.22 $\pm$ 0.84 (4)	25.52 $\pm$ 5.67 (5)
intermediate	0.09 (1)	3.50 $\pm$ 3.50 (2)	1.59 $\pm$ 1.59 (2)	11.11 $\pm$ 11.11 (2)
confined	0.0 $\pm$ 0.0 (2)	1.43 $\pm$ 0.56 (5)	0.63 $\pm$ 0.63 (5)	1.67 $\pm$ 1.67 (5)

Table F5. Spearman correlations among WD risk and oligochaete variables for test sites. Significant values ($p < 0.05$) are shown in bold text, and correlation type (Pearson/Spearman) is indicated in brackets. Correlations between non-normal and normal variables were always expressed as Spearman's rank correlations.

Variable		Infection prevalence in sentinel fish	Infection severity in sentinel fish	Oligochaete abundance	Abundance of <i>T. tubifex</i>	Prevalence of infection in <i>T. tubifex</i>
Infection prevalence in sentinel fish	r value					
	p value					
	n	5	-	-	-	-
Infection severity in sentinel fish	r value	1	1			
	p value	<0.0001		-	-	-
	n	5	5			
Oligochaete abundance	r value	0.65789	0.65789	1		
	p value	0.2275	0.2275		-	-
	n	5	5	11		
Abundance of <i>T. tubifex</i>	r value	0.28947	0.28947	0.96347	1	
	p value	0.6366	0.6366	<0.0001		-
	n	5	5	11	11	
Prevalence of infection in <i>T. tubifex</i>	r value	0.91766	0.91766	0.72628	0.64028	1
	p value	0.028	0.028	0.0114	0.0338	
	n	5	5	11	11	12
Abundance of infected <i>T. tubifex</i>	r value	0.80296	0.80296	0.85052	0.82185	0.92
	p value	0.1018	0.1018	0.0009	0.0019	<.0001
	n	5	5	11	11	11

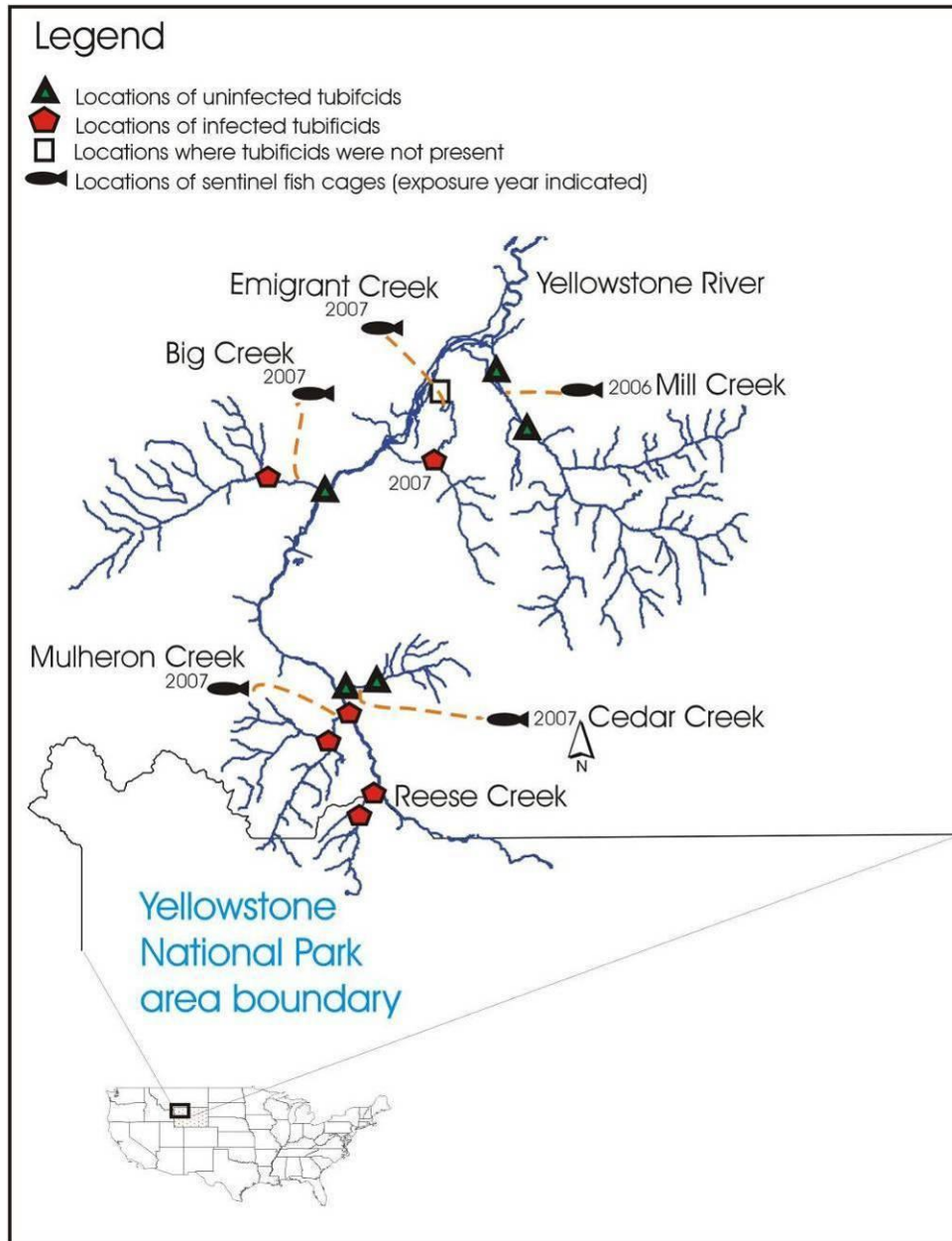


Figure F1: Map of tubificid collection and sentinel fish exposure sites on the Yellowstone River (below the Park in 2007-2008). Pentagon (red) plots indicate sites of infected tubificids, triangle (green) plots indicate sites where tubificids were not infected, and square (white) plots indicate sites where tubificids were not found.

APPENDIX G:

YELLOWSTONE CUTTHROAT TROUT
PRESENCE/ABSENCE AND DEMOGRAPHY DATA

Table G.1. Presence/absence of Yellowstone cutthroat trout in tributaries to Yellowstone Lake and the Yellowstone River in June and September 2007. NS= not sampled with backpack electroshocker, *=Yellowstone cutthroat trout observed but not collected

Drainage	Tributary	Invertebrate Sampling Reach (2005)	Confinement	Month(s) Sampled	Yellowstone cutthroat trout
Yellowstone Lake	Pelican Creek	1	Unconfined	June/September	N
	Mainstem	2	Unconfined	September	N
	Chittenden Creek	3	Intermediate	June/September	Y
		4	Intermediate	NS*	Y
	Upper Pelican Creek	5	Intermediate	June/September	Y
		6	Confined	June/September	Y
	Clear Creek	7	Confined	June/September	Y
		8	Intermediate	NS *	Y
	Beaverdam Creek	9	Unconfined	NS	
		10	Confined	NS *	Y
	Upper Yellowstone River	11	Unconfined	NS*	Y
		12	Unconfined	NS*	Y
	Arnica Creek	13	Confined	June/September	Y
		14	Confined	NS	
	Bridge Creek	15	Intermediate	NS*	Y
		16	Intermediate	NS	
Yellowstone River	Elk Antler Creek	17	Unconfined	June/September	Y
		18	Unconfined	June/September	Y
	Trout Creek	19	Unconfined	June/September	
		20	Unconfined	June/September	Y
	Alum Creek	21	Intermediate	June	N
		22	Intermediate	June	N
	Otter Creek	23	Confined	June	N
		24	Intermediate	June	N
	Slough Creek	25	Confined	NS*	Y
		26	Intermediate	NS	
	Thistle Creek	27	Confined	June/September	Y
		28	Intermediate	NS	

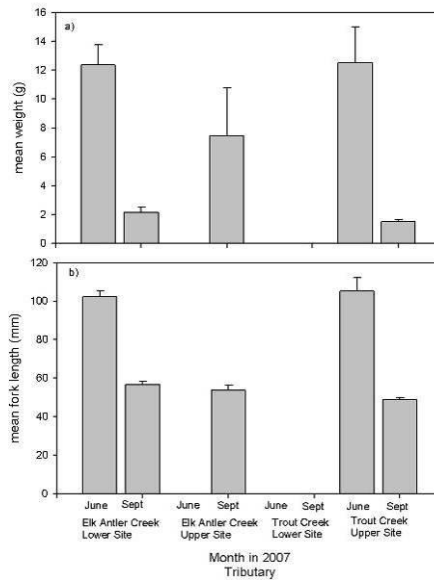


Figure G.1. Length and size data for all Yellowstone cutthroat trout collected during single pass electrofishing in Trout and Elk Antler Creeks in June and September 2007. The dissimilar sizes and weights of Yellowstone cutthroat trout in June and September suggest young of year may only be present in September.

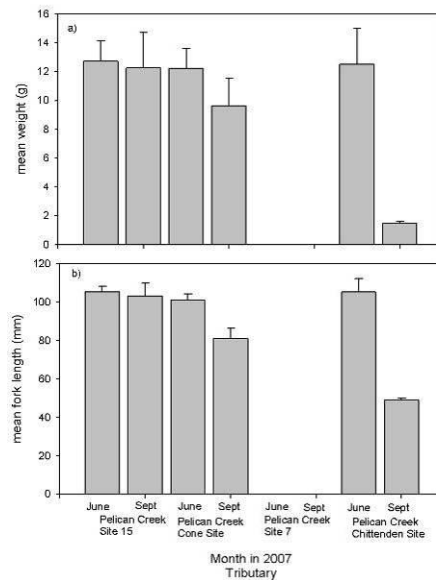


Figure G.2. Length and size data for all Yellowstone cutthroat trout collected during single pass electrofishing in Trout and Elk Antler Creeks in June and September 2007. The similar sizes and weights of Yellowstone cutthroat trout in June and September suggest resident adults may be present.