ECOLOGY OF WHIRLING DISEASE IN ARID LANDS WITH AN EMPHASIS ON *TUBIFEX TUBIFEX*

BY

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ABSTRACT

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The novel pathogen hypothesis describes host parasite relationships where a pathogen spreads into new geographical areas or into areas of previously unexposed `virgin' hosts. Often, measures of parasite virulence and host resistance are elucidated through pathogenic impacts on the 'virgin' hosts. The myxosporean *Myxobolus cerebralis,* the causative agent of whirling disease in salmonid fish, qualifies as a novel pathogen with its recent introduction into North America from Europe in the 1950s. This introduction of a novel pathogen provides opportunity for insight into the etiology of host-parasite life cycles, parasite virulence, and host resistance.

The devastating effect of whirling disease on wild salmonid populations was not fully realized until its discovery in the inter-mountain west. The presence of the

whirling disease parasite in rainbow trout was confirmed in New Mexico the spring of 1999. The most devastating potential of the parasite in New Mexico lies in the threat it poses to native salmonid populations that rely on natural reproduction.

In this dissertation, I investigated the distribution *T. tubifex* lineages within waters that support salmonids within the State of New Mexico, ecological relationships and physiological responses to *T. tubifex* infection with *M. cerebralis*, and analyzed the genetic divergence between *T. tubifex* lineages from varied habitats in a system that harbors the parasite. The goal of my research was to establish which *T. tubifex* lineages are present in arid lands habitat and whether they are differentiated by ecological factors. I clarified the taxonomic status of *T. tubifex* lineages found in New Mexico through examination of genetic divergence between lineages III and VI. Furthermore, I have investigated the relative susceptibility of the lineages to *M. cerebralis* to aid in the assessment of risk of parasite establishment in sensitive waters.

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CHAPTER 1: INTRODUCTION

The novel pathogen hypothesis describes host parasite relationships where a pathogen spreads into new geographical areas or into areas of previously unexposed `virgin' host populations (Grenfell and Gulland 1995). Often measures of parasite virulence and host resistance are elucidated through the pathogenic impacts on the 'virgin' hosts. The myxosporean *Myxobolus cerebralis,* the causative agent of whirling disease in salmonid fish, qualifies as a novel pathogen with its recent introduction into North America from Europe in the 1950's. This introduction of a novel pathogen provides opportunity for insight into the etiology of host-parasite life cycles, parasite virulence, and host resistance.

Background

The *M. cerebralis* parasite has a two-host life cycle involving separate stages of sporogony in each host (a salmonid fish and the aquatic oligochaete *Tubifex tubifex*) (Wolf and Markiw 1984; El-Matbouli and Hoffman 1989). The importation of rainbow trout (*Oncorhynchus mykiss*) to Germany from the United States led to the discovery of the parasite. *Myxobolus cerebralis* isolated from farm raised rainbow trout was first described in Germany by Höfer (1903). *Myxobolus cerebralis* is believed to have co- evolved with brown trout (*Salmo trutta*) which show resistance to the parasite (Hoffmann 1970). Hoffmann (1970) also suggested that dissemination of the parasite throughout Europe occurred in the first half of the twentieth century with shipments of live rainbow trout within the continent. The

parasite was first detected in North America in 1958 when it was found in hatchery stocks of rainbow trout in Pennsylvania (Hoffmann et al. 1962). To date, whirling disease has been reported in a total of 22 states and 26 different countries (Bartholomew and Reno 2002).

Evidence of recent introduction into the United States is supported by sequence analysis of 18S and ITS-1 ribosomal deoxyribonucleic acids (rDNA) of *M. cerebralis* isolates from Europe and the United States (Andree et al. 1999; Whipps et al. 2004). Initially the disease was perceived to be only a problem in hatcheries and would have minimal impact on natural salmonid populations (Wolf 1986). The devastating effect of whirling disease on wild salmonid populations was not fully realized until its discovery in the inter-mountain west. Whirling disease was first detected in Montana in 1994 with the sudden collapse of trout populations in the Madison River (Vincent 1996). Wild rainbow trout populations decreased by 90% between 1991 and 1995 in a 93 km reach of the Madison River (Rognlie and Knapp 1998). The presence of the whirling disease parasite in rainbow trout was confirmed in New Mexico the spring of 1999 (Hansen 2002). Since this confirmation, three of the six state hatcheries, several private ponds, and salmonid populations from the San Juan River tailwater and other riverine systems in New Mexico have tested positive for the parasite. As a result, routine testing and remediation procedures have been instituted in state-run hatcheries and a testing program has been initiated for New Mexico's 173 coldwater streams and reservoirs which may have been inadvertently stocked with rainbow trout carrying the parasite, or infected through transmission by

other natural or manmade vectors. The most devastating potential of the parasite in New Mexico lies in the threat it poses to native salmonid populations that rely on natural reproduction.

Myxobolus cerebralis

The phylum Myxozoa is represented in more than 1300 parasitic species of fish, reptiles and amphibians; the genus *Myxobolus* is the largest, consisting of over 450 species (Lom and Dykova 1992). Myxozoans were once classified as parasitic protozoa but now are placed with the metazoa as bilateria or cnidarians (Smothers et al 1994; Sidall et al. 1995). In addition to molecular phylogenetic and dimorphic sessile and pelagic life stage similarities, the myxozoan polar capsules and filaments have functional similarities to cnidiarian nemocysts providing strong evidence for association with the cnidarians (Sidall et al. 1995). Myxozoans are characterized by one or more sporoplasms and polar filaments contained within protective shells or valves. The size, shape, and number of these three elements are often used to differentiate species as well as tissue trophism, developmental cycles, and host species (Lom et al. 1997). The myxosporean spore stage of *M. cerebralis* are characterized by an elliptical shaped shell (approximately 10 μm diameter) consisting of two hardened valves which contain two polar capsules with coiled polar filaments and a binucleate sporoplasm cell (Hedrick et al. 1998) (Figure 1.1). The discovery of the actinosporean form of myxozoan spore morphology occurring in aquatic oligochaetes (Wolf and Markiw 1984) and polychaetes (Bartholomew et al. 1997) provided additional criteria for taxonomic assignment (Figure 1.2.).

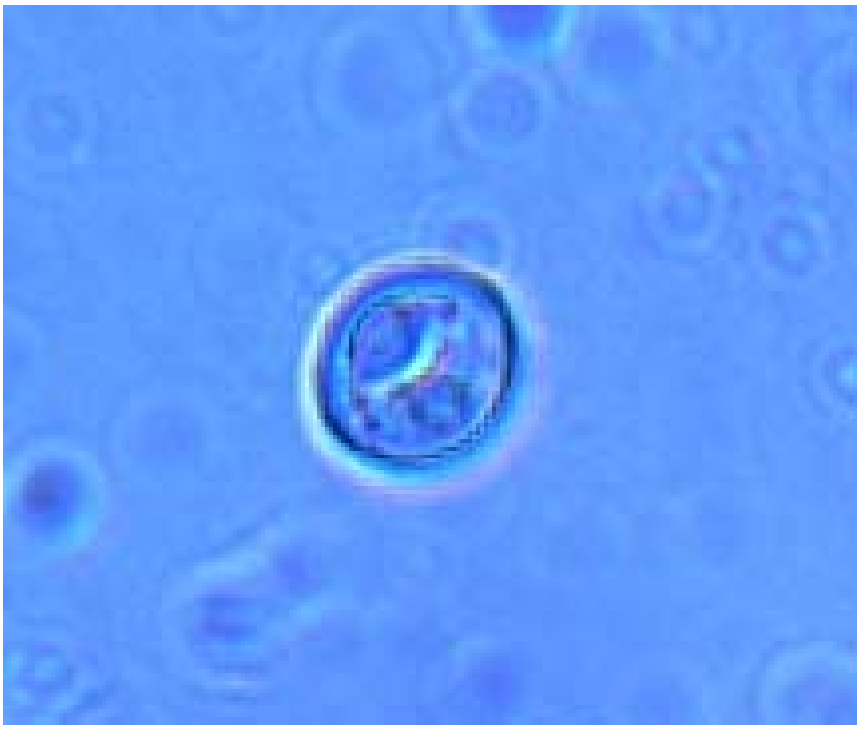

Figure 1.1. Phase contrast microscopy image of *Myxobolus cerebralis* myxospore approximately 10 microns in width isolated from infected rainbow trout (400x) (Image by R. DuBey 2004).

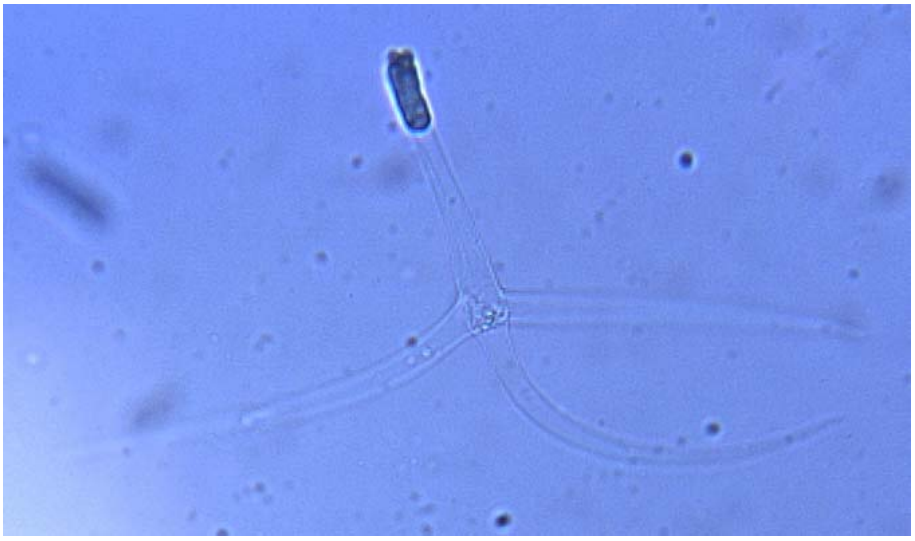

Figure 1.2. Phase contrast microscopy image of *Myxobolus cerebralis* triactinomyxon (TAM) approximately 70 microns stylus length and 160 microns process width, note sporoplasm in packet at end of stylus (400x) (Image by R. DuBey 2004).

The triactinomyxon stages of *M. cerebralis* are characterized by an anchor shape. The sporoplasts are contained at one end of a stylus (approximately 90 µm length) and by three fluke-like processes at the other (Wolf and Markiw 1984). To date, few myxozoans have been linked to their actinosporean stages (Kent et al. 1996).

Myxobolus cerebralis myxospores are released into aquatic sediments when infected fish die and decompose, or, are consumed by predators or scavengers. Myxospores resist rigorous environmental conditions which include retaining infectivity after desiccation, freezing, low pH, and enzymatic degradation in the alimentary tract of predators (El-Matbouli et al. 1992). The myxospores are released into the sediments from decomposing salmonids or feces from predators. The myxospores are then ingested by *T. tubifex* in whose gut epithelium the next phase of transformation into the actinosporean triactinomyxon (TAM) occurs. Mature TAMs are released into the aquatic environment in fecal pellets released from infected *T. tubifex* (Gilbert and Granath 2001). In contrast to *M. cerebralis* myxospores, TAM's are relatively fragile and short-lived (2-5 d, Markiw 1992; 15 d, El-Matbouli et al. 1999b). Salmonids contract *M. cerebralis* by brief epidermal contact with waterborne TAMs (Markiw 1989; El-Matbouli et al. 1995). The TAMs attach their polar filaments to the secretory openings of epidermal mucous cells and release their sporoplasm germ cells into the fish.

The *Myxobolus cerebralis* life cycle within *T. tubifex* was described by El-Matbouli and Hoffmann (1998) who used light and electron microscopy to delineate

developmental stages. When *T. tubifex* ingest *M. cerebralis* myxospores, they enter the intestinal lumen and extrude polar filaments attaching themselves to intestinal muscosa. After attachment, the two outer valves open to release the infective germ cell (binucleate sporoplasm) contained within the myxospore. The germ cell migrates from the myxospore into the intestinal mucosa intercellular space. The germ cell then undergoes an asexual stage (schizogenic phase) where both diploid nuclei undergo multiple divisions followed by plasmotomy producing uninucleate cells which disperse throughout the gut epithelia. Some of the uninucleate cells undergo schizogamy and others plasmogamy to produce binucleate stages. The binucleate stages then undergo a sexual stage (gametogony) starting with the formation of pansporocysts with four cells from different origins (two forming enveloping cells and two representing dipliod cells). A gametogenesis stage follows which involves three mitotic divisions and a meiotic stage where 8 - and 8 + gametes expulse polar bodies forming 16 haploid gametocytes. Copulation occurs between positive and negative gametocytes with one cell enveloping the other. The enveloping cells undergo two mitotic divisions resulting in 8 zygotes (each surrounded by a somatic cell). Each zygote then undergoes a series of mitotic divisions resulting in a mature TAM spore with 64 germ cells in the sporoplasm. The mature TAM spores are then shed by the infected *T. tubifex*.

The TAMs are released into the aquatic environment in fecal packets (Gilbert and Granath 2001), egested from the anus directly into the water (El-Matbouli and Hoffmann (1998), from damaged segments, or after the death of infected *T. tubifex*

(El-Matbouli et al. 1992). When TAMs are released into the water column their stylus and processes inflate with water and they become planktonic. Triactinomyxon development in experimentally exposed *T. tubifex* ranges from 74 to 120 days post-exposure and is dependent on water temperature (Markiw 1986; El-Matbouli and Hoffmann 1998; Gilbert and Granath 2001; Stevens et al. 2001).

Myxobolus cerebralis' life cycle within the salmonid host was described by El-Matbouli et al. (1995) who used light and electron microscopy to delineate *M. cerebralis* developmental stages in rainbow trout. Briefly stated, the fish become infected with *M. cerebralis* when TAMs attach themselves to the fish's skin using their polar filaments. The triactinomyxon spores attach to the secretory openings of mucous cells of the epidermis, the respiratory epithelium and the buccal cavity of trout and use them as portals of entry. Complete penetration of sporoplasm germ cells occur as early as 1 min from the attachment of the triactinomyxon spores (El-Matbouli et al. 1999a).

After the sporoplasm germ cells enter the mucous cell the sporoplasm enveloping cell disintegrates and the sporoplasm penetrates an epithelial cell. The germ cell then undergoes an endogenous cleavage producing a cell doublet consisting of a primary enveloping cell and a secondary inner cell. The secondary inner cell then undergoes schizogony with a series of mitotic divisions increasing the number of secondary cells. The secondary cells then undergo another endogenous cleavage stage producing numerous cell doublets. The cell doublets rupture the plasmodium aggregate membrane and the host cell and enter the extracellular space to migrate to

another cell. The cell doublets continue a series of intercellular endogenous cleavages and ruptures to proliferate and migrate through the epithelial cells and central nervous system to body areas containing cartilage. When the cell doublets reach cartilaginous areas, they either continue the schizogony proliferation cycle or enter an asexual sporogony stage with the formation of panosporoblasts. Panosporoblasts are formed by one cell doublet enveloping another (i.e., one becoming the cyst envelope and the inner one forming the sporogenic cell with two spores). The panosporoblasts mature into myxospores which are infective to the definitive host *T. tubifex* (El-Matbouli et al. 1995).

Salmonid Host Pathology

Myxobolus cerebralis is one of the most pathogenic myxoans known in fish (Hedrick et al. 1998). The main pathogenic effect of the parasite is damage to cartilage of the axial skeleton. This damage includes parasitism of the cartilaginous capsule of the auditory apparatus in fish resulting in an impaired ability to maintain an upright position which causes the fish to swim with a corkscrew motion (Platt 1983). The whirling behavior is likely due to constriction of the spinal cord and brain stem caused by an inflammatory response (Rose et al. 2000) giving the appearance of whirling behavior. Infection of the spinal column interferes with the posterior sympathetic nerves controlling the melanocytes. The caudal region of the fish becomes dark producing the clinical symptom "black-tail" (Halliday 1976). If the

fish survives, infection will often result in permanent deformities (e.g., misshapen cranium, twisted lower jaw, severe spinal curvature).

Salmonid Susceptibility

Myxobolus cerebralis parasitizes a number of salmonid species, however, not all fish that are infected exhibit clinical symptoms of whirling disease (Hoffmann 1990; Hedrick et al. 1999a). The known fish hosts for *M. cerebralis* have been derived from observations of epizootics in captive and wild salmonid populations and controlled laboratory and sentinel experiments (O'Grodnick 1979; reviewed by MacConnell and Vincent 2002). The severity of disease depends on the age at exposure (Markiw 1991), rearing temperature (Halliday 1973), dose of TAMs the fish receives, and the species (Markiw 1991; Hedrick et al. 1999b).

Among the species exhibiting clinical symptoms of infection, Rio Grande cutthroat trout (*Oncorhynchus clarki virginialis*) (DuBey et al. In Prep), rainbow trout (O'Grodnick 1979; Vincent 2002), brook trout (*Salvenlinus fontinalis*) (Vincent 2002) and chinook salmon (*Oncorhynchus tshawytscha*) (Hedrick et al. 2001) may suffer the worst pathology (Table 1.1). These species exhibit high cartilage lesion scores and spore counts compared to other salmonid species. In recent susceptibility experiments, Rio Grande cutthroat trout exhibited increased disease severity and higher mortality when compared to RBT (DuBey et al. In Prep). In contrast, arctic grayling (*Thymallus articus*) showed no cartilage lesions and *M. cerebralis* spores were not recovered from either low or high TAM dose exposure groups up to 5 months post-exposure (Hedrick et al. 1999b).

Table 1.1. Salmonid species susceptibility to *Myxobolus cerebralis* infection by recent laboratory challenges using pepsin-trypsin digest method to enumerate spores and/or histology scores to rate susceptibility (susceptibility scores, 0 = resistant; 1= low; 2 = high; 3 = very high).

Genus Species	Common Name	Susceptibility	Reference
Oncorhynchus clarki bouveri	Yellowstone cutthroat	2	Hedrick et al. (1999b), Vincent (2002)
O. c. lewisi	Westslope cutthroat	2	Hedrick et al. (1999b), Vincent (2002)
O. c. behnkei	Snake River cutthroat	2	Vincent (2002)
O. c. virginialis	Rio Grande cutthroat	3	DuBey et al. (In Prep)
O. kisutch	Coho salmon	1	Hedrick et al. (2001b)
O. mykiss	Rainbow trout	3	Bartholomew et al.(2003), Densmore et al. (2001), Hedrick et al. (2001), Hedrick (1999b), Vincent (2002)
O. mykiss	Steelhead trout	3	Hedrick et al. (2001)
O. tshawytscha	Chinook salmon	2	Hedrick et al. (2001), Sollid et al. (2003)
Salmo trutta	Brown trout	1	Hedrick et al. (1999a), Vincent (2002)
Salvelinus confluentus	Bull trout	1	Bartholomew et al. (2003), Vincent (2002)
S. fontinalis	Brook trout	3	Vincent (2002)
Thymallus arcticus	Arctic grayling	0	Hedrick et al. (1999b), Vincent (2002)
Hucho hucho	Danube salmon	3	El-Matbouli et al. (1992)

In exposure challenges from 10 to 10,000 TAMs/fish, brown trout exhibited resistance to infection when compared to rainbow trout (Hedrick et al. 1999a). In the same experiment, rainbow trout exhibited a 10-fold higher lesion score and cranial spore concentration than brown trout. Black-tailing was not observed in brown trout at exposures from 10 to 100 TAMs/fish, however, in the same series of laboratory exposures of more than 1000 TAMs/fish resulted in clinical signs of infection (Hedrick et al. 1999a). In more recent studies, Rio Grande cutthroat trout challenged with *M. cerebralis* at a range of 0, 50, 100, 250, 500 and 1000 TAMs/fish exhibited higher cumulative mortality and histology scores reflecting very high severity of infection when compared to Erwin strain rainbow trout (known to exhibit high susceptibility to the disease) (DuBey et al. In Prep). The higher mortality and histological response indicated that Rio Grande cutthroat trout are extremely susceptible to *M. cerebralis* and suffer higher morbidity and mortality than most trout species. In contrast, brown trout resistance to the parasite has been offered as evidence of co-evolution with the parasite in Europe (Hoffmann 1970). However, brown trout co-occur with danube salmon (*Hucho hucho*), which exhibit high susceptibility (El-Matbouli et al. 1992).

The current knowledge of the immune response for salmonid hosts to *M. cerebralis* is limited. The wide range of susceptibility among the different species, however, suggests the immune response of salmonid hosts can be effective in eliminating the parasite. An initial humoral response in the host's skin after attachment of the TAM is suggested as sporoplasm numbers are reduced or

eliminated in some species before the sporoplasm migrates to nerve ganglia (Hedrick et al.1998). Resistant species, such as coho salmon (*O. kisutch*), prevent most of the sporoplasm somatic cells from entering the epithelium after contact by the TAM. Brown trout inhibit *M. cerebralis* somatic cells in the nerve ganglia or roots between the epithelium and cartilage. Leukocytes were found in cranial nerve ganglia of infected brown trout but not rainbow trout suggesting differing immune response and giving some insight into brown trout resistance to infection (Hedrick et al. 1999a). However, varied antibody response and no antigen recognition pattern were observed among rainbow trout (Adkison 2003). Rainbow trout cellular immune response is elicited by TAMs during their developmental stages and active immunity is thought to be acquired after the development of cartilage lesions (Halliday 1973). Rainbow trout exhibit epithelial cell inflammation with initial infection, but nerve cells do not exhibit inflamation suggesting that the parasite is sheltered by the central nervous system (El-Matbouli et al. 1995). Rainbow trout exhibited a significant immune response to the pathogen 12 weeks post-exposure (Ryce et al. 2002). The response, however, provides little protection against parasite development at this late time in exposure.

Oligochaete Host

In contrast to our extensive knowledge of the interaction between *M. cerebralis* and salmonid hosts, the knowledge of the interactions within *T. tubifex* is limited. *Tubifex tubifex* is the only known oligochaete host for *M. cerebralis* (Gilbert

and Granath 2003). Unsuitable oligochaete hosts for *M. cerebralis* include *Aeolomsoma* spp., *Dero* spp., *Stylaria* spp. (Markiw and Wolf 1983), *Limnodrilus hoffmeisteri*, *Ilyodrilus templetoni*, Qus*tadrilus multisetosus* (Wolf et al. 1986), and *Tubifex ignotus* (El-Matbouli and Hoffman 1989).

Tubifex tubifex is a cosmopolitan freshwater species that is taxonomically identified using morphological characteristics of sexually mature adults (e.g., chaetae and reproductive structures) (Kathman and Brinkhurst 1999) (Figure 1.3). These characteristics have been proven inadequate to effectively distinguish *T. tubifex* as the species exhibits phenotypic plasticity of its chaetal morphology (Chapman and Brinkhurst 1987) and absorption of sexual organs (Poddubnaya 1984). It has been suggested that some forms of *T. tubifex* may represent distinct species (Paoletti 1989). Crossbreeding and temperature threshold tests between sympatric 'tubifex' and 'blanchardi' forms indicated the forms did not interbreed and with sufficient genetic differences between the forms to be considered distinct species. Crossbreeding studies of *T. tubifex* are complicated by the fact that the species are hermaphroditic and can reproduce through parthogenesis (Poddubnaya 1984). Paoletti's (1989) experiments relied on observing incomplete spermatogenesis in parental worms and peculiarities of the sexual structure to infer parthogenic reproduction in place of sexual reproduction or self-fertilization.

Recent molecular studies (Anlauf 1990, 1994, 1997; Anlauf and Neumann 1997; Sturmbauer et al. 1999; Beauchamp et al. 2001, 2002) also suggest the existence of cryptic species of *T. tubifex* exhibiting varied physiological

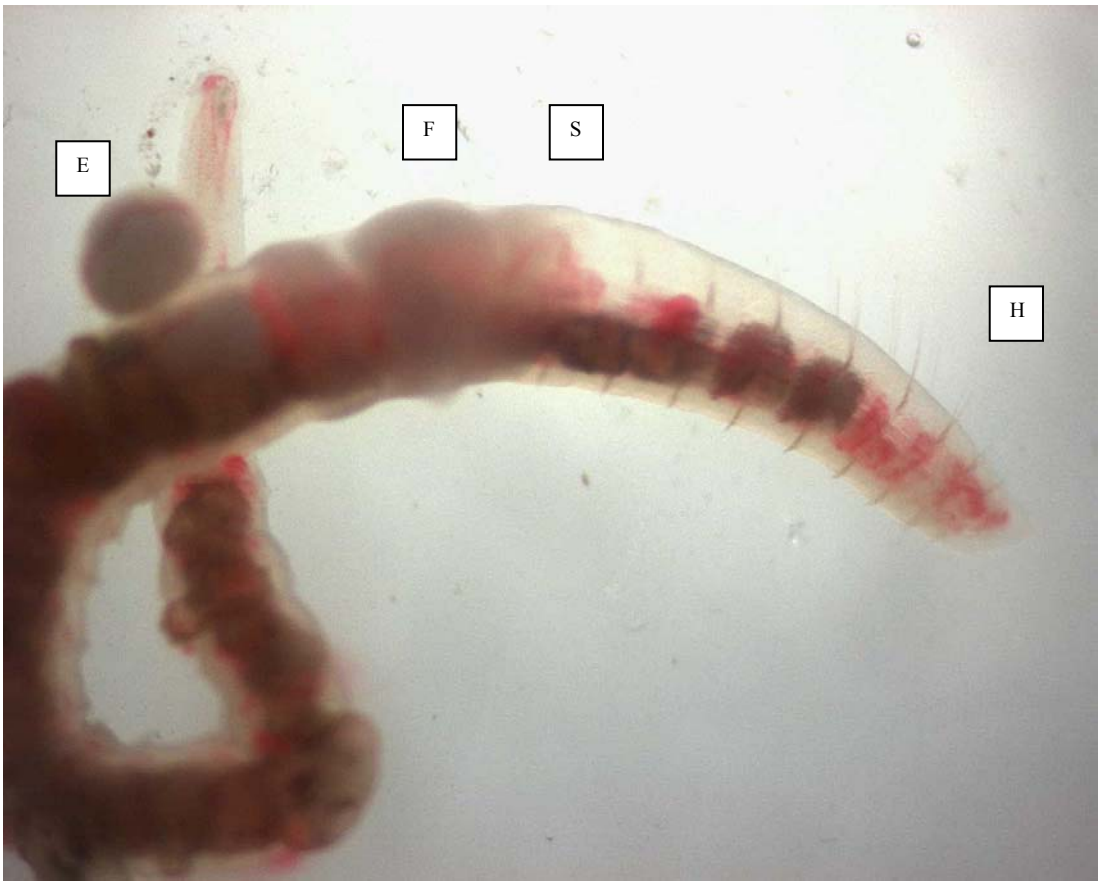

Figure 1.3. Microscopy image of sexually mature *Tubifex tubifex* with hair cheatae (H) on dorsal side of anterior segments, sexual organs (S), egg packet (E), and egg packet formation (F) in segments 11 - 13 (approximately 30 mm total length) (100x) (Image by DuBey 2002).

characteristics. Anlauf (1990, 1994, 1997) and Anlauf and Neumann (1997) described ecological races of *T. tubifex* were differentiated by tropic conditions through allozyme screening. Phylogenetic analysis of mitochondrial 16S DNA from geographically distinct populations of *T. tubifex* provided evidence that similar cryptic lineages exist within both North American and European populations (Sturmbauer et al. 1999; Beauchamp et al. 2001, 2002).

Tubifex tubifex distribution is influenced by sediment composition and organic content (Robbins et al. 1989). Anlauf's (1994) allozyme study of *T. tubifex* differentiated several ecological lineages taken from habitat characterized by their trophic condition. He described ecological lineages from oligotrophic coldwater systems and from ephemeral eutrophic waters. Tubificid abundance has often been positively correlated with sedimentation of fines and organic matter (Robbins et al. 1989). Anlauf (1997) further speculated that habitat temperature may influence growth and reproduction within these lineages.

Tubifex tubifex are often found in mutualistic association with *Limnodrilus hofmeisteri*, in which one species feeds on the bacteria associated with the fecal pellets of the other species and vice versa (Brinkhurst 1971, 1974). *Tubifex tubifex* also absorb small organic molecules through the epithelium, sometimes obtaining up to 40% of their nutritional requirements in this manner (Hoffmann et al. 1987). *Tubifex tubifex* also exhibit anaerobic respiration and can survive under anoxic conditions (Reynoldson 1987). In cases of eutrophication, *T. tubifex* and *L. hofmeinsteri* may be the only invertebrates present in sediments (Brinkhust 1996).

Tubifex tubifex are thought to survive drought, freezing, and food shortages by secreting a protective cyst (Anlauf 1990). Increased cyst formation was observed in Rocky Mountain *T. tubifex* during winter and may serve as a protective mechanism from predation (Kaster et al. 1981). Cysting of *T. tubifex* was also only observed in winter collections from the San Juan River, New Mexico (DuBey and Caldwell 2004; see Chapter 3). Winter occurrence of cysting among *T. tubifex* suggest that decreases in temperature or photoperiod may induce cysting. Some researchers have also speculated that cysting facilitates dispersal within and between watersheds (Anlauf 1990).

Tubifex tubifex lineages also vary in susceptibility to *M. cerebralis*. Krueger et al. (2000) reported variability in tubificid assemblages and infection rates of *T. tubifex* within side channels of the Madison River. Furthermore, Stevens et al. (2001) observed that within geographically differentiated populations of *T. tubifex*, doses of 50, 500, and 1000 myxospores/worm did not affect TAM production. Thus, different TAM production levels may, in part, be explained by sympatric cryptic populations of *Tubifex* species. Some lineages may be resistant while other are susceptible to *M. cerebralis* infection. For example, Beauchamp et al. (2002) reported that several *T. tubifex* lineages from the upper Colorado River exhibited resistance while others were infected with *M. cerebralis*. Furthermore, *T. tubifex* lineage V from Ontario Bay exhibited complete resistance. This lineage ingests and inactivates *M. cerebralis* spores, thus, effectively removing spores from habitat by acting as biological filters and preventing contact with susceptible lineages (El-Matbouli et al. 1999b).

Direct correlation between water temperature and *M. cerebralis* infection was observed in trout using sentinel cage studies with the most severe infection occurring at 10-12 ^{o}C (Baldwin et al. 2000). The same authors also observed seasonal periodicity in infection. These findings are congruent with observations of optimal TAM production and survival from cultured populations of *T. tubifex* at 10 and 15 ^{o}C while minimal TAM releases occurred at 5, 20, 25 and 30 ^{o}C (El-Matbouli et al. 1999b).

Persistent infection and varied TAM release is also supported by Gilbert and Granath's (2001) laboratory observations. These authors observed *T. tubifex* releasing TAMs 12 times throughout 58 days and intermittent TAM releases up to 606 days post-exposure. The variation of TAM production with temperature suggests an optima. Temperature optima for TAM development *T. tubifex* lineages is supported by Anlauf (1994, 1997). Anlauf and Neumann (1997) observed ecological lineages of *T. tubifex* having different environmental requirements including temperature. However, an optima for TAM production of *T. tubifex* lineages is unknown.

Pathology of *M. cerebralis* infection on *T. tubifex* has been described by several authors. El-Matbouli and Hoffmann (1998) observed discoloration of intestines and distortion due to large clusters of *M. cerebralis* cells and speculated that the clusters may decrease the absorptive surface of the intestine. They also reported that there was no evidence of parasitic castration as the parasite was not observed in histology slides of gonads. They did suggest, however, the parasite may have an indirect effect on reproduction. Infected *T. tubifex* from different

geographical populations exhibited decreased biomass, abundance, and individual weights (Stevens et al. 2001). Furthermore, Stevens et al. (2001) observed a dramatic decline in abundance within populations of *T. tubifex* suggesting that *M. cerebralis* infection may cause significant mortality to distinct lineages.

Whirling Disease in New Mexico

The San Juan River is the largest river in arid western New Mexico. The river is controlled by Navajo Dam and supports a world renowned "blue-ribbon" rainbow trout fishery in the "Quality Waters" section of the tailwater containing a high density of trophy size trout. Pilot studies focusing on the ecology and distribution of *T. tubifex* were initiated in response to reports of high *M. cerebralis* myxospore loads in rainbow trout in the tailwater. Previous research of benthic responses to changes in flow regime in the San Juan River tailwater observed an altered benthic community structure (due to a modified thermal regime) compared to a non-regulated system (DuBey 1996). Specifically, annelida density and biomass were higher in tailwater sections with a uniform thermal differential when compared to sites with a greater temperature differential. The stable water temperatures and high organic load supported a large *T. tubifex* population. It was believed the high *M. cerebralis* spore loads in rainbow trout that inhabit the tailwater were directly related to the uniform tailwater temperatures and high primary production. The pilot study also revealed distinct habitats in the San Juan River tailwater. *Tubifex tubifex* were found in both

organically enriched side channels and river bank areas and in deeper (>1m) main-channels dominated with sand substrate.

Goals and Objectives

In this dissertation, I have investigated the distribution *T. tubifex* lineages within waters that support salmonids within the State of New Mexico, ecological relationships and physiological responses to *T. tubifex* infection with *M. cerebralis*, and analyze the genetic divergence between *T. tubifex* lineages from varied habitats in a system that harbors the parasite. The goal of my research was to establish what *T. tubifex* lineages are present in arid lands habitat and whether they are differentiated by ecological factors. Furthermore, I investigated the relative susceptibility of the lineages to *M. cerebralis* to aid in the assessment of risk for introduction of the parasite into sensitive waters. I also clairified the taxonomic status of *T. tubifex* lineages found in New Mexico through examination of genetic divergence between lineages III and VI.

Varied *M. cerebralis* infection levels in *T. tubifex* have been observed in populations from different geographical areas (Stevens et al. 2001; Beauchamp et al. 2002) and among different lineage assemblages from habitats exhibiting varying sediment loads (Beauchamp et al. 2002). The presence of resistant *T. tubifex* and elevated water temperatures in an infected system may reduce the availability of spores and therefore severity of the disease. The variation of TAM production with temperature suggests an optima. I suggest that habitat and lineage of *T. tubifex* are important factors in characterizing high prevalence of disease within the San Juan

River tailwater. Thus, the objectives of the research in Chapter Two were to characterize the effects that environmental variables (i.e., water velocity, depth, and organic matter) have on the distribution and infection level within populations of *T. tubifex* and to determine whether these populations can be distinguished by genetic lineage.

Little is known regarding the interactive effects of *M. cerebralis* infection, temperature, and photoperiod on the demography and fitness of *T. tubifex* lineages. *Tubifex tubifex* from allopatric geographic populations differed in biomass, reproduction and growth in *M. cerebralis* infection experiments (Stevens et al. 2001). Earlier research demonstrated that growth, reproduction, and development of TAMs were influenced by habitat temperature (El-Matbouli et al. 1999b). *Tubifex tubifex* are thought to survive drought, freezing, and food shortages by secreting a protective cyst (Anlauf 1990). Furthermore, the parasite may have an indirect effect on reproduction of the tubificid host. Within sympatric populations of *T. tubifex* in the Colorado and the San Juan rivers, several lineages were thought to be resistant to infection resulting in a reduction in the prevalence of infection. Thus, the objectives of the research in Chapter Three were to characterize the response of two lineages of *T. tubifex* (III, VI) under a range of photoperiod and thermal regimes and determine if infection by *M. cerebralis* would negatively affect growth and survival of the lineages.

It has been suggested that some lineages of *T. tubifex* may represent distinct species (Paoletti 1989; Anlauf 1994; Sturmbauer et al. 1999; Beauchamp et al. 2001,

2002). These authors suggested that genetic distances between some of these lineages provided evidence of the existence of cryptic species of *Tubifex*. Furthermore, Beauchamp et al. (2002) hypothesized the presence of sympatric cryptic populations of *Tubifex* species (one species resistant and others susceptible to *M. cerebralis* infection). The experimental evidence to date suggests resistant lineages may have significant genetic divergence to indicate that speciation may be occurring. Thus, determining the genetic variance of *T. tubifex* lineages may provide a key component to understanding the etiology of whirling disease. Chapter Four investigated the genetic diversity within *T. tubifex* lineages from different habitats within the San Juan River, New Mexico tailwater.

The most devastating potential of the disease in New Mexico lies in the threat it poses to native salmonid populations that rely on natural reproduction. These native populations are isolated to headwater systems in New Mexico. Little is known, however, about *T. tubifex* distribution in these headwater systems and the associated ecological factors that result in greater risk to salmonids. Thus, the objectives of Chapter Five were to characterize the *T. tubifex* lineage distribution, and the relationship to habitat variables and salmonids in headwater systems in New Mexico.

CHAPTER 2: DISTRIBUTION OF *TUBIFEX TUBIFEX* LINEAGES AND *MYXOBOLUS CEREBRALIS* INFECTION IN THE TAILWATER OF THE SAN JUAN RIVER, NEW MEXICO

Introduction

Whirling disease is a parasitic infestation of cartilage in salmonids by the myxosporean *Myxobolus cerebralis*. Salmonids infested with *M. cerebralis* exhibit skeletal and cranial deformities, erratic whirling behavior, and young-of-year may suffer a high rate of mortality (Gilbert and Granath 2003). The parasite has a two-host life cycle involving separate stages of sporogony in salmonid fishes and the aquatic oligochaete *Tubifex tubifex* (Wolf and Markiw 1984; El-Matbouli and Hoffman 1989). Typically, myxosporean-type spores are released from infected fish when they die and are ingested by *T. tubifex* in whose gut epithelium the next phase of transformation into the actinosporean Triactinomyxon (TAM) occurs (El-Matbouli et al. 1995; El-Matbouli and Hoffman 1998). Salmonids contract whirling disease by brief contact with waterborne TAMs released from infected *T. tubifex*. The devastating effects of whirling disease on wild salmonid populations was not fully realized until its discovery in the inter-mountain west with the sudden collapse of many wild rainbow trout populations (Nehring and Walker 1996; Vincent 1996).

Tubifex tubifex is a cosmopolitan freshwater species with morphological characteristics that exhibit phenotypic plasticity resulting in difficulty distinguishing it from closely related species (Chapman and Brinkhurst 1987; Kathman and Brinkhurst 1999). It has been suggested that *T. tubifex* can be differentiated into ecological lineages inhabiting specific habitats with distinct environmental parameters (Poddubnaya 1979; Anlauf 1994). Poddubnaya (1979) described populations of rheophilic and limnophilic forms of *T. tubifex* were differentiated by

habitat types that exhibited different tropic conditions. Furthermore, Anlauf and Neumann (1997) utilized allozymes to differentiate ecological races of *T. tubifex* from lakes, streams, and ponds each exhibiting different tropic conditions. Phylogenetic analysis of mitochondrial 16S ribosomal DNA from geographically distinct populations of *T. tubifex* provided evidence that similar cryptic lineages existed within both North American and European populations (Sturmbauer et al. 1999; Beauchamp et al. 2001).

Varied *M. cerebralis* infection levels in *T. tubifex* have been observed in populations from not only different geographical areas (Stevens et al. 2001; Beauchamp et al. 2002) but among different lineage assemblages as well (Beauchamp et al. 2002). For example, Beauchamp et al. (2002) observed varying infection levels within waters that supported different lineage compositions exhibiting varying sediment loads. The varying infection levels, in part, may have been due to the presence of resistant *T. tubifex* lineage V (Beauchamp et al. 2002). Resistant worms may ingest and inactivate *M. cerebralis* spores**,** thus, effectively removing spores from the habitat by acting as biological filters and preventing contact with susceptible lineages (El-Matbouli et al. 1999b).

The majority of the major rivers in the United States are regulated by reservoirs having a hypolimnion release which provide habitat for salmonid species. Regulated flows within these tailwaters create artificial environments that often exhibit diurnally and seasonally constant temperatures and increased total dissolved solids and nutrients (Ward 1974). Flow regime directly affects benthic community structure and density by altering the availability of organic matter as well as altering suitable habitat through changes in water velocity (Nowell and Jumars 1984). Thus, the environment below deep-release reservoirs may provide a unique set of conditions

where invertebrate standing crop may be enhanced with increased oligochaetan populations (Lehmkuhl 1972; Ward 1974, 1978; Rader and Ward 1988; Munn and Brusven 1991).

The San Juan River is the largest river in arid western New Mexico and is regulated at Navajo Dam where water is released from a coldwater hypolimnion directly into the river. In 1999, rainbow trout, *Oncorhynchus mykiss,* inhabiting the tailwater tested positive for whirling disease harboring extremely high *M. cerebralis* spore loads (Hansen 2002). Previous research demonstrated high organic loading of sediments in the tailwater below Navajo Dam supported high tubificid densities (DuBey and Jacobi 1996). Thus, the objectives of the study were to characterize the effects that environmental variables (i.e., water velocity, depth, and organic matter) have on the distribution and infection level within populations of *T. tubifex* and to determine whether these populations can be distinguished by genetic lineage.

Methods and Materials

The San Juan River flows out of Colorado headwaters through northwestern New Mexico and southeastern Utah to the Colorado River at Lake Powell. The study area was defined as the San Juan River tailwater starting 2 km from the dam face and ending 2 km downstream and was chosen because it contained diverse instream habitats with the presence of both riffle-run and pool-glide reaches (Figure 2.1). Within this same reach, DuBey and Jacobi (1996) reported water temperatures that ranged from 4.5 to 12.5^{o}C throughout the year and elevated *T. tubifex* populations (2130 individuals/m^{2}) in riffle habitat. Samples were collected August and December 2001 and June 2002 to characterize temporal and spatial changes in habitat resulting

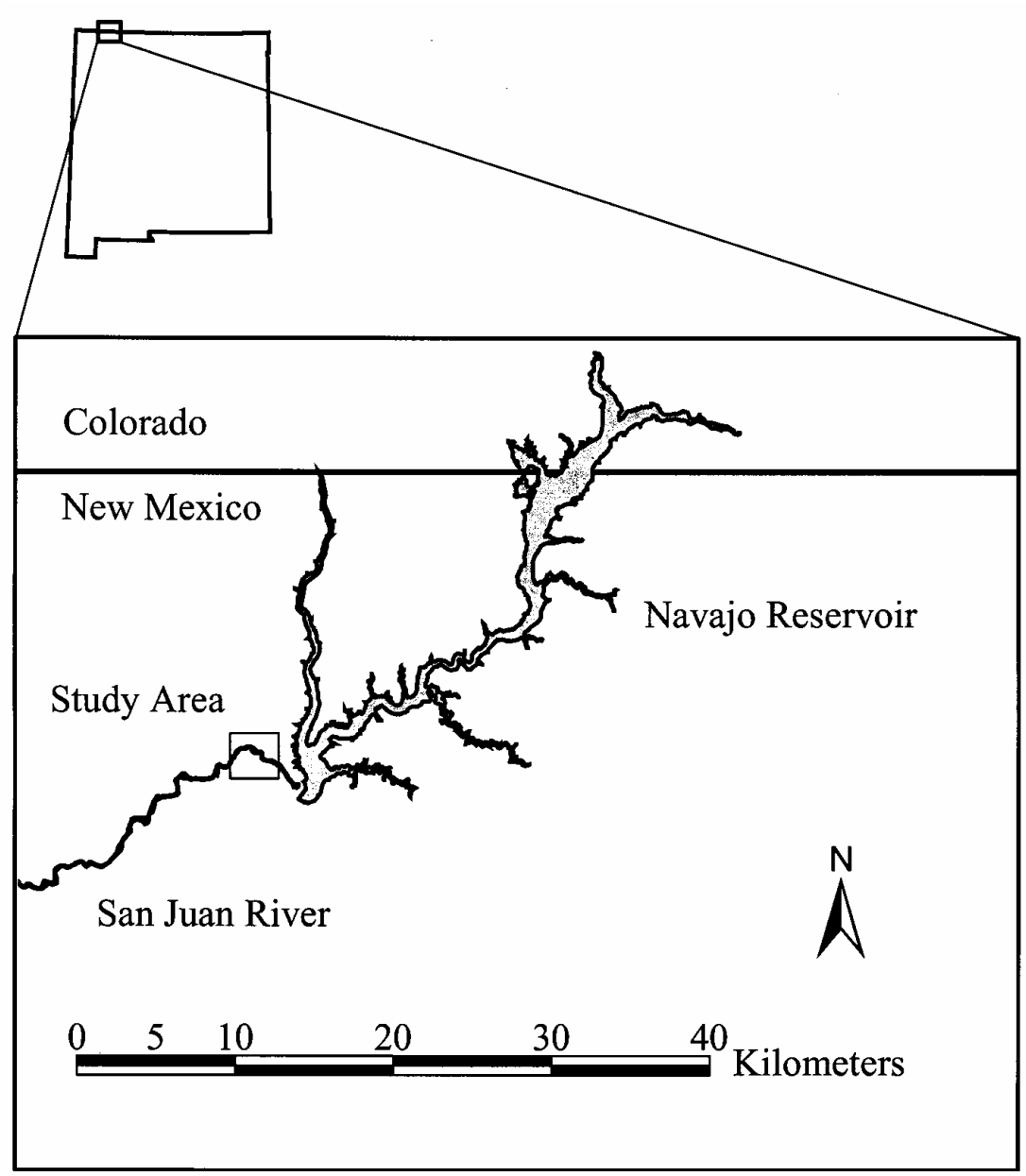

Figure 2.1. Location of study area on the San Juan River, New Mexico.

from a scheduled scouring flow that peaked on May 29, 2001at 142.0 m^3/s. The August 2001 sample collection was completed 38 days after the scouring flow at a reduced flow rate of 23.6 m^3/s. The second sample collection was conducted December 2001 at a low flow of 13.9 m^3/s and the third collection was completed June 2002 at a flow of 24.5 m^3/s (USGS 2002).

A multistage sampling design was developed to effectively characterize tubificid habitat and their distribution within the study area. Thirty transects were randomly selected with replacement from 200 transects located at 10 m intervals perpendicular to the main channel. Each transect was classified for habitat type as either riffle or pool habitat by width and depth. The selected transects were then post-stratified into deep (> 1 m) and shallow habitats (< 1 m). One deep and one shallow habitat were randomly selected for sampling along each transect.

At each sample location, a petite ponar dredge was used to collect *T. tubifex* and sediments for determining organic material (ASTM 2000). In addition, physical and chemical parameters were collected at the substrate level to reflect environmental conditions for *T. tubifex*. Depth (m) was measured with a sounding line, velocity (m/s) was measured with a Marsh-McBirney direct-reading flow meter, temperature and dissolved oxygen (mg/L) were measured with a dissolved oxygen meter (Yellow Springs Instruments, Ohio), and turbidity (NTU) was measured with a turbidity meter (H F Instruments). All meters were calibrated daily. Water samples were collected with a Kemmerer vertical water sampler to assess biochemical oxygen demand (BOD), and stored in 300 ml glass BOD bottles and analyzed in accordance to *Standard Methods for the Examination of Water and Wastewater* (APHA 1999).

Benthic and experimental methodology incorporated the *Standard Protocols for Whirling Disease Research* (Bartholomew 2001). Briefly stated, benthic samples

were processed to establish oligochaete density, community structure, and percentage of *T. tubifex* within that community. Oligochaetes from each sample were microscopically sorted and identified using a dissecting microscope into morphological groups. Oligochaetes, Naididae, Tubificidae without hair chaetae, and Tubificidae with hair chaetae (morphology consistent with *T. tubifex)* were sorted and enumerated. The tubificid group which includes *T. tubifex* can be morphologically identified by hair and pectinate chaetae starting at the second dorsal segment and definitive identification of *T. tubifex* can be made microscopically by the penal sheath of slide-mounted mature specimens (Kathman and Brinkhurst 1999). Species composition within each sample was determined from a randomly selected sub-sample of tubificids. Polymerase chain reaction (PCR) for molecular marker screening (Beauchamp et al. 2002) was utilized to characterize *T. tubifex* lineages from the randomized sub-sample of mature *T. tubifex.*

Triactinomyxon screening was performed on sub-samples of *T. tubifex* for *M. cerebralis* infection and definitive diagnosis of infection through genetic analysis (Andree et al. 1998). Sample size was determined utilizing a hyper-geometric distribution with 1% disease incidence level (Thompson 1992). Thus, a sub-sample of 4 to 16 individuals from each sample site containing a *T. tubifex* population were screened resulting in a total of 840 individuals from the pool habitat and 584 individuals from riffle habitat. These *T. tubifex* were then evenly distributed in a sorting tray containing a grid and individuals randomly selected and placed in multi-well plates filled with water. The *T. tubifex* were cultured at 15^{o}C for 7 d. Water from each well was viewed daily by phase-contrast microscopy for the presence of TAMs (Markiw 1989). *Tubifex tubifex* found producing TAMs were genetically tested for confirmation of *M. cerebralis* infection using molecular PCR markers

developed by Andre et al. (1998) and were genotyped for lineages I, III, V, and VI utilizing the primers and protocol developed by Beauchamp et al. (2002).

Statistical analysis were performed using the software Statistical Analysis System (SAS 2001). Parametric comparisons of tubificid density and organic matter between collection periods were not performed because the distribution were skewed. Thus, tubificid density (i.e., number of individual tubificids with hair chaetae/m^2) at each site were log transformed and the non-parametric Kruskal-Wallace test performed for both the density and organic matter among collection periods. Regression analysis was used to examine the relationship between tubificid densities and habitat (deep versus shallow), water quality parameters, and organic matter. One-way analysis of variance was used to compare water quality parameters between collection periods. Infection rates and lineage composition were grouped by habitat type (riffle versus pool) and examined using *t*-tests. Differences were considered significant at an α level of 0.05.

Results

Tubificid densities ($P = 0.0030$) and percent organic matter in sediments ($P = 0.0227$) increased through time in deep habitats (Table 2.1). Thus, the scouring flow initially reduced sediments in deep habitats resulting in an increase in tubificid density and organic matter through time. In contrast, tubificid densities in shallow habitat ($P = 0.1614$) and percent organic matter in shallow habitat ($P = 0.9646$) did not differ among the sample dates. Although tubificid densities within the shallow habitat in August 2001 were twice the densities of December 2001 and June 2002, the difference was not detectable due to the high variability of densities between sites. Shortly after the scouring flow, lower organic matter was observed in deep habitats

than in shallow habitats (P = 0.0157). Organic matter did not differ between deep and shallow habitats for either December 2001 or June 2002 collections, reflecting the initial effect of scouring flow on deep habitats.

Determination of genetic lineage of *T. tubifex* from randomly selected sub-samples revealed the study reach was inhabited primarily by genetic lineages III and VI. One pool area with two individuals was represented by genetic lineage I. The resistant lineage V was not identified from collections in the study area. Lineage VI exhibited greater dominance in riffle habitats compared to lineage III (P = 0 .0438); a total of 42 lineage VI individuals and 17 lineage III individuals were sampled from riffles. The lineage proportions in pool habitats did not differ; 42 individuals from lineage VI and 36 individuals from lineage III were found in pools. Regression analysis showed no detectable differences between lineages in relation to water quality parameters, organic matter, or velocity.

Water quality parameters did not differ between deep and shallow habitats. However, there were detectable differences among sample periods. Temperatures were lower in December 2001 (6.9^{o}C) than in August 2001 (8.3^{o}C) or June 2002 (7.8^{o}C) (P<0.0001). Dissolved oxygen (9.7 mg/L) was lower in June 2002 than in August 2001 (12.7 mg/L) or December 2001(12.1 mg/L) (P< 0.0001). The mean water quality parameters (7.67^{o}C, SE = 0.081; dissolved oxygen 11.69 mg/L, SE = 0.134; BOD 1.48 mg/L, SE = 0.147; and turbidity 2.11 NTU, SE = 0.119) reflected relatively homogeneous habitat conditions within the study reach and consistent conditions among sample dates. In addition, water quality was within acceptable limits established for high quality coldwater fisheries (NMWQCC 2001).

Triactinomyxons were identified in 29 of the 1424 *T. tubifex* screened and all TAM producing *T. tubifex* tested positive for *M. cerebralis* (Table 2.2). *Myxobolus*

Table 2.1. Tubificid density (individuals/m^2) and ash free dry weight organic matter (%) in sediments collected during the three sample dates in the San Juan River tailwater from shallow and deep habitats. Standard error in parentheses. Significant differences among sample dates have different letters.

	Shallow Habitat		Deep Habitat	
	Density	Organic Matter	Density	Organic Matter
August 2001	16,696 (± 4599.6)z	2.90 (± 0.39)y	6,922 (± 1467.9)u	1.75 (± 0.242)r
December 2001	8,799 (± 2238.9)z	2.81 (± 0.45)y	7,636 (± 2160)v	3.54 (± 0.62)s
June 2002	8,571 (± 802.8)z	4.28 (± 1.77)y	10,397 (± 1078.2)w	4.78 (± 0.93)t

Table 2.2. Total *Tubifex tubifex* reflect sub-samples that were combined among collection dates and screened for triactinomyxons. Infected *Tubifex tubifex* (percentage in parentheses) exhibiting infection with *Myxobolus cerebralis* from pool and riffle habitats in the San Juan River tailwater. Significant differences between habitats have different letters.

	Pool Habitat	Riffle Habitat	Combined Habitats
Total *Tubifex tubifex*	840	584	1424
Infected *Tubifex tubifex* (%)	26 z (3.01%)	3 y (0.51%)	29 (2.04%)

cerebralis infection rates were detectably higher in pool habitats (3.01%) than in riffle habitats (0.51%)($P < 0.001$); however, there were no differences in infection levels among sample dates. Only *T. tubifex* with molecular markers corresponding to lineage III were found to produce TAMs.

Discussion

Hydraulic stress and the associated substrate disturbance is a likely determinant of the abundance and diversity of benthic communities (Sousa 1984). We observed an increase in tubificid densities and organic matter within deep habitats reflecting the effect of a scouring flow on a tailwater system as it stabilized through time. It follows that tubificid densities would increase through time. Tubificid abundance has often been positively correlated with sedimentation and organic matter (Robbins et al. 1989). Whether the lower tubificid densities observed in deep habitat after the scouring flow were due to reduced organic matter in sediments or to removal of tubificids from deep habitat by physical displacement was not determined. However, it is probable that both reduced organic matter (Peralta et al. 2002) and physical disturbance (Sousa 1984) were factors of lower tubificid density in deep habitats shortly after the scouring flow. Tubificid densities measured shortly after the scouring flow were higher in shallow habitats than in deep habitats, which may have been due to tubificid displacement between the two habitats. However, tubificid densities and organic matter from shallow habitats were not different among the sampling periods.

Tubificid densities throughout the 2-km study reach ranged from 57 to 123,029 individuals/m^2. While these densities were considered high when compared to those in intermountain headwater systems (Allen and Bergersen 2002), they are

similar to those in tailwaters in well developed riverine systems and lakes (Robbins et al. 1989; Dumnica 2002). Allen and Bergersen (2002) reported the Cache la Poudre River in Colorado exhibited a disparate oligochaete community with a peak abundance of 50 oligochaetes/m^2 (including *T. tubifex*) in side alcoves and eddies. Oligochaete densities, which included high tubificid densities, ranged from 10,000 to 79,000 individuals/m^2 in the Vistula River in Poland (Dumnica 2002) and from 6,600 to 55,300 individuals/m^2 in depositional basins in Lake Erie (Robbins et al. 1989).

Genetic analysis for *T. tubifex* lineage revealed three of the five North American lineages in the tailwater of the San Juan River. *Tubifex tubifex* lineages III and VI were found throughout the study area while lineage I was found within a pool habitat at only one site. The resistant lineage V was not found in the study area. When compared to habitat parameters, lineage VI predominated in riffle habitats, while both lineages III and VI were observed in pool habitats. These findings were similar to lineage distributions reported by Beauchamp et al. (2002) in which lineages I and III were dominant in reservoir collections and lineages III and VI were dominant in riverine collections. However, Beauchamp et al. (2002) found the resistant lineage V only in riverine collections, not in tailwater collections. The dominance of lineage VI in riffle habitats combined with the absence of lineage I suggests that depth of habitat may influence the distribution of *T. tubifex* lineages.

Of tubificids screened for *M. cerebralis* infection, only *T. tubifex* was found to be carrying the parasite. This is in agreement with previous studies that report *T. tubifex* as the sole oligochaete host for *M. cerebralis* (Markiw and Wolf 1983; Wolf and Markiw 1984; Markiw 1986; Wolf et al. 1986; El-Matbouli and Hoffman 1989; Beauchamp et al. 2002). Even though three *T. tubifex* lineages were identified in the tailwater, *M. cerebralis* infection was observed solely in lineage III. Lineages I and

VI did not exhibit presence of the parasite, and lineage V was not detected in collections from the study area. Previous studies have also reported susceptible and resistant *T. tubifex* genotypes and variance in TAM production among *T. tubifex* populations from different geographical locations (Beauchamp et al. 2001, 2002; Stevens et al. 2001). The *M. cerebralis* infection rate of *T. tubifex* in this study ranged from 0.51 to 3.01%. Similarly, field infection rates in populations of *T. tubifex* from other riverine habitats were 2.6% (Rognlie and Knapp 1998) and from 1.2 to 6.8% (Zendt and Bergerson 2000).

Anlauf and Neumann (1997) suggested environmental factors could act as selective pressures for various *T. tubifex* genotypes; however, very little is known about this. Lower temperatures increased susceptibility rates of *T. tubifex* to infection by *M. cerebralis* (El-Matbouli et al. 1999b). Water temperature in this study was within the tolerance limits for both *T. tubifex* and *M. cerebralis* and infection rates were similar to reported rates in riverine systems (Rognlie and Knapp 1998; Zendt and Bergerson 2000). In addition, size, age, and genotype of *T. tubifex* were presumed to affect susceptibilities (Gilbert and Granath 2001; Beauchamp et al. 2002). This study illustrates that the habitat and genotype of *T. tubifex* are important in characterizing disease prevalence within the San Juan River tailwater. Elevated populations of susceptible *T. tubifex* lineage III were observed in pools while lower infection occurred in riffles dominated by the resistant *T. tubifex* lineage VI. This study also suggests that scouring flows may affect the incidence of the disease by reducing *T. tubifex* abundance and organic loading typical of regulated tailwaters.

CHAPTER 3: EFFECTS OF TEMPERATURE, PHOTOPERIOD AND *MYXOBOLUS CEREBRALIS* INFECTION ON GROWTH, REPRODUCTION AND SURVIVAL OF *TUBIFEX TUBIFEX* LINEAGES

Introduction

Whirling disease is a parasitic infestation of cartilage in salmonids by the myxosporean *Myxobolus cerebralis*. Evidence of recent introduction in North America is supported by genetic sequence data from *M. cerebralis* isolates from Europe and the United States (Andree et al. 1999; Whipps et al. 2004). The devastating effects of whirling disease on wild salmonid populations was not fully realized until its discovery in the inter-mountain west with the rapid decline of salmonid populations in Montana (Vincent 1996) and Colorado (Nehring and Walker 1996).

The aquatic oligochaete, *Tubifex tubifex*, is the definitive host for *M. cerebralis* (Wolf and Markiw 1984; El-Matbouli and Hoffmann 1989). The myxosporean-type spores released by infected salmonids are ingested by *T. tubifex* and are transformed into the actinosporean triactinomyxon (TAM) in the gut epithelium of the oligochaete hosts (El-Matbouli and Hoffmann 1989). Salmonids contract whirling disease by brief contact with waterborne TAMs (El-Matbouli et al. 1995).

Tubifex tubifex is a cosmopolitan freshwater species with morphological characteristics that exhibit phenotypic plasticity such as pectinate chaetae that make it

difficult to distinguish from closely related species (Chapman and Brinkhurst 1987; Kathman and Brinkhurst 1999). Crossbreeding studies between sympatric morphological forms of *T. tubifex* (*tubifex* and *blanchardi*) indicated that they did not interbreed with sufficient genetic differences to be considered distinct species (Paoletti 1989). Using allozymes, Anlauf (1994, 1997) described lineages of *T. tubifex* that differed between pond and lake habitats. Phylogenetic analysis of mitochondrial 16S ribosomal DNA from geographically distinct populations of *T. tubifex* provided evidence that cryptic lineages within both North American and European populations were similar (Sturmbauer et al. 1999; Beauchamp et al. 2001). Sturmbauer et al. (1999) described European lineages that exhibited differential tolerance to cadmium exposure. Beauchamp et al. (2002) reported four lineages of *T. tubifex* (I, III, V, and VI) at two sites on the Colorado River, Colorado. The authors also observed varied susceptibility of the lineages to experimental infection of *M. cerebralis*. Recently, three lineages (I, III, and VI) were identified in sympatric populations from the tailwater of the San Juan River, New Mexico (DuBey and Caldwell 2004). No infection was detected in lineages I and VI, in contrast to lineage III. Furthermore, lineage III exhibited higher infection levels in pools (3.0%) than in riffle habitats (0.5%), demonstrating varied infection presumably due to different environmental conditions.

Anlauf (1994) observed differences in growth, reproduction, and survival among cultures of three *T. tubifex* lineages originating from different habitats at 5^{o}C, 15^{o}C, and 20^{o}C. A positive linear relationship between *T. tubifex* fecundity,

temperature and percentage of organic matter in sediments was observed in Montana headwater streams (Kaster 1980). *Myxobolus cerebralis* development in infected *T. tubifex* was also shown to be temperature dependent, the spore loads being highest at 15°C, wheras non-viable degenerating *M. cerebralis* spores were detected at 30°C (El-Matbouli et al. 1999b). Furthermore, allopatric geographic populations of *T. tubifex* differed in biomass, reproduction, growth, and TAM production when subjected to controlled infection of *M. cerebralis* (Stevens et al. 2001). However, little is known regarding lineage specific responses of *M. cerebralis* infection to combinations of temperature and photoperiod. Thus, the objective of this research was to characterize the responses of two lineages of *T. tubifex* to a range of photoperiod and thermal regimes and determine if infection by *M. cerebralis* would affect growth and survival of lineages.

Methods and Materials

Tubifex tubifex are parthogenic (Poddubnaya 1984). Thus, progeny from this form of reproduction were used to establish laboratory monocultures of lineages III and VI. Original stocks of *T. tubifex* lineages were obtained from the San Juan River, New Mexico. The lineage of each monoculture was verified by screening individuals using a PCR amplification of 16S mRNA developed by Beauchamp et al. (2002). The PCR methodology for lineage determination used the DNA template from DNeasy (Qiagen, Valencia, California) mouse-tail protocol extractions and three rounds of amplification using primers. In this protocol, four North American lineages

were distinguished by size-specific PCR product. Of the two lineages tested the PCR product is 147 base pairs (bp) for lineage III and 125 bp for lineage VI. *Myxobolus cerebralis* spores were isolated from infected rainbow trout (*Oncorhynchus mykiss*). The fish heads were de-fleshed by heating at 45^{o}C, and flesh and non-cartilaginous material manually removed. The cartilaginous material was pulverized and centrifuged to concentrate spores in a pellet. The pellet was resuspended and spores were quantified by microscopic examination and enumerated within a known volume.

Tubifex tubifex lineages III and VI were randomly assigned to each of 18 treatment combinations within nine 38 L glass aquaria using a split-split plot experimental design. Each aquarium was divided in half with clear Plexiglass. Eight 350 ml plastic tubs were placed within each aquarium half (four tubs containing lineage III and four tubs containing lineage VI). Before placement within the aquaria, 25 immature tubificids were randomly assigned to each tub with a mixture of sterilized silt, sand, and water. Subsequently, *T. tubifex* in eight tubs were challenged individually with an infection of 500 spores per worm, a rate similar to Stevens et al. (2001) and Blazer et al. (2003). Eight plastic tubs received no spores. The challenged *T. tubifex* tubs were then assigned to an aquarium half with the non-challenged *T. tubifex* tubs in the other half to ensure independence of the treatment and control groups.

A range of temperatures (5, 17, 27^{o}C) were selected to represent the optimum range at which *T. tubifex* is commonly found. Each temperature treatment was

applied to three aquaria at three separate locations. Treatment at $5 \pm 1^{o}C$ was conducted in a Living Stream (Frigid Units, Toledo, Ohio) in a laboratory using a temperature controlled refrigeration unit (model DI-500, Aquarium Systems, Mentor, Ohio). Treatment at $17 \pm 1^{o}C$ was conducted within the same laboratory on a bench top. Treatment at $27 \pm 1^{o}C$ was conducted in a walk-in Sheerer environmental chamber having a UP 780 digital control system to maintain the desired temperature (Yokogama Corporation of America, Newton, Georgia). Chamber temperature was measured by a National Institute of Standards and Testing certified sensor (Model MRHT3-2-1-D, General Instruments, Woburn, Massachusetts).

For each photoperiod treatment, fluorescent lamps were used to provide approximately 50 lumens/cm2 at the water surface of each aquarium. Photoperiod treatment for two of three aquaria (at each location) was manipulated using darkened plexiglass covers that were placed on top of each aquarium to achieve desired light exposure (14:10, 16:8; dark:light). The aquaria exposed to 12 hours of light (12:12) remained uncovered but light exposure was regulated by an Electronic Time Switch controller (Model TS110S, BRK Electronics, Aurora, Illinois) in the laboratory and the UP 780 digital control system regulated photoperiod in the environmental chamber. Water temperature (^{o}C) and light intensity (lumens) were measured continuously by Hobo^{TM} recorders (Model H8, Onset Computer Corp., Bourne, Massachusetts).

All tubificids were fed an aqueous solution of ground spirulina (0.5 mg dry weight spirulina per tubificid) twice weekly. Temperature (^{o}C) and dissolved oxygen

(mg/L; Yellow Springs Instrument, Yellow Springs, Ohio) were monitored daily and pH (pHPlus, LaMotte, Chestertown, Maryland), nitrite (mg/l NO_2^--N, diazotization method) and ammonia (mg/l NH_3-N, salicylate method) (Model DR/2010 spectrophotometer, Hach Company, Loveland, Colorado) were monitored in tubs from all aquaria each week throughout all treatments for the experiment duration. Water in tubs was changed weekly with aerated well water. The experiment was run for 70 d.

At 70-d post-exposure, surviving *T. tubifex* were separated into adult, immature, and cysted individuals, counted and weighed to the nearest 0.1 mg. *Tubifex tubifex* from infected treatments were randomly selected for determining infection from each container using a gridded sorting tray. The tubificids were placed in centrifuge tubes, frozen with liquid nitrogen, and stored at -70^{o}C until analyzed for *M. cerebralis* infection with diagnostic PCR markers (Andree et al. 1998). *Myxobolus cerebralis* infection was determined for individual worms in the infected treatment and for worms composited from non-infected treatments. Infection level for each treatment combination was computed by dividing the total number of infected worms by total number of worms examined (lineage- specific) from each aquarium half.

The presence or absence of *M. cerebralis* infection was determined using a nested PCR amplification of 18S rRNA. The PCR methodology for *M. cerebralis* used DNA template from phenol extraction, two rounds of amplification according to protocol, and primers developed by Andree et al. (1998). The diagnostic

amplification marker for *M. cerebralis* round two was a 410 bp product. Amplification was performed with a GeneAmp PCR System (Model 9700, Applied Biosystems, Foster City, California). After amplification, 10 µl of the DNA sample, and 6 µl 1x loading buffer (Sigma Chemical Co., St. Louis, Missouri) were loaded into an ethidium bromide-containing agarose gel (2%) with tris-citrate borate ETDA buffer for electrophoresis and diagnostic DNA identification (Andree et al. 1998).

The experiment was analyzed as a split-split plot with three levels of experimental units. Treating the nine glass aquaria as the experimental units at the first level, whole plot treatments consisted of temperature and photoperiod, using the interaction between the two as the error term. The infected and non-infected halves within each aquarium were the experimental units at the second level. The experimental units at the third level consisted of the individual plastic tubs. Bonferroni's multiple comparison procedure was used to control the experiment-wise error rate ($\alpha = 0.10$) using a comparison-wise rate of $\alpha = 0.026$. Response variables were infection level, adult mortality, average adult net weight change, number of young produced per surviving adult, and average weight per young worm. All statistical analyses were performed with SAS software (SAS Institute 2001). Interactive effects were not detectable unless otherwise reported.

Results

Survival differed among individual tubs. Thus, infection levels for treatment combinations are based on the weighted average of worms. There was no

photoperiod effect; therefore, results are presented for each lineage-temperature combination. Lineage III exhibited infection levels of 4.3% at 5°C, 3.3% at 17°C and 0% at 27°C, while no infection was detected in lineage VI (Table 3.1). Polymerase chain reaction diagnostics detected no infection in *T. tubifex* of either lineage from the non-challenged group.

Under experimental conditions in this study, lineage VI exhibited detectably higher survival ($F_{1,120} = 100.7$; $P < 0.0001$) than lineage III (Table 3.2). Of the 72 tubs containing lineage III, 9 tubs exhibited complete mortality at 27°C and 4 at 5°C. Of the 72 tubs containing lineage VI, only 1 exhibited complete mortality at 27°C and no complete mortality was observed at either 5 °C or 17°C. Although effects of temperature on survival were not statistically detectable at the $\alpha = 0.026$ level ($F_{2,4} = 7.83$; $P = 0.041$), survival was consistently higher at 17°C. No effect of photoperiod ($F_{2,4} = 0.69$; $P = 0.55$) or infection ($F_{1,6} = 1.45$; $P = 0.27$) was identified.

The individual mean starting weight of lineage VI (1.35 mg, SE = 0.021) was greater than the individual starting weight of lineage III (1.10 mg, SE = 0.022); thus, we based our analysis on the average net weight change per adult worm. Lineage VI individuals gained more weight (3.17 mg, SE = 0.25) than did lineage III individuals (2.34 mg, SE = 0.20), ($F_{1, 106} = 25.47$; $P < 0.0001$). Interaction between infection and lineage was detectably different ($F_{1,106} = 5.69$; $P = 0.019$). Infected individuals of lineage III gained more weight than the uninfected individuals. Lineage VI worms, however, had the same weight gain regardless of parasite challenge. Although

Table 3.1. Infection prevalence of *Tubifex tubifex* lineages III and VI experimentally challenged with 0 (non-exposed) and 500 (exposed) *Myxobolus cerebralis* myxospores/worm at 5°C, 17°C, and 27°C.

	III		VI	
	Exposed	Non-exposed	Exposed	Non-exposed
5°C	4.3%	0%	0%	0%
17°C	3.3%	0%	0%	0%
27°C	0%	0%	0%	0%

Table 3.2. Average survival (%; ± standard error) of *Tubifex tubifex* lineages III and VI subjected to exposure (exposed) or no exposure (non-exposed) by *Myxobolus cerebralis* at 5°C, 17°C, and 27°C. Different letter subscripts within treatment groups indicate detectable differences (n=12 for all combinations).

	III $_z$		VI $_y$	
	Exposed $_x$	Non-exposed $_x$	Exposed $_w$	Non-exposed $_w$
5°C $_v$	27.0 ± 6.05	11.7 ± 3.32	53.3 ± 7.11	35.7 ± 5.96
17°C $_v$	51.3 ± 6.07	44.7 ± 3.74	68.0 ± 3.88	79.3 ± 2.45
27°C $_v$	21.0 ± 5.59	10.0 ± 5.01	57.0 ± 6.68	68.3 ± 10.21

temperature effects were not detectable in adult tubificids ($F_{2,4} = 8.46$; $P = 0.037$), individuals held at 17°C had higher net weight gains.

Natality (number of young produced per surviving adult) was not affected by exposure to *M. cerebralis* in either lineage (Table 3; $F_{1,6} = 0.03$; $P = 0.87$). The lack of a detectable effect on natality was due to negative population growth rates for both the 5°C and 27°C treatment groups, whereas positive growth rates were exhibited within the 17°C treatment groups. Natality was greater in lineage VI than in lineage III ($F_{1,106} = 19.32$; $P < 0.0001$). The degrees of freedom in this error term were lower due to 14 tubs having complete mortality and therefore were excluded from analysis. Although there was no detectable effect of photoperiod ($F_{2,4} = 0.43$; $P = 0.68$), greater mean natality was observed in both lineages at 17°C than at either 5°C or 27°C (Table 3.3; $F_{2,4} = 26.15$; $P = 0.005$).

No environmental effects (i.e., temperature and photoperiod) were detected for weights of immature tubificids ($P > 0.25$), nor was there a difference attributable to infection ($F_{1,1} = 1.24$; $P = 0.4662$). However, there was a detectable effect of lineage ($F_{1,43} = 21.35$; $P < 0.0001$) on the mean weight of immature tubificids. Lineage VI exhibited higher mean weight (1.28 mg/worm, SE = 0.15) than lineage III (0.82 mg/worm, SE = 0.06). Cysting was observed at only 5°C in both lineages and was similar throughout all photoperiods (20 - 22 cysted worms). The overall cysting rate for all surviving adult tubificids was 3.9% and none of the cysted individuals were infected.

Table 3.3. Average number of young tubificids produced in 70 days per adult *Tubifex tubifex* of lineages (III and VI) subjected to exposure (exposed) or no exposure (non-exposed) by *Myxobolus cerebralis* at 5°C, 17°C, and 27°C. Different letter subscripts within treatment groups indicate detectable differences and standard error and sample size are in parenthesis.

	III $_z$		VI $_y$	
	Exposed $_x$	Non-exposed $_x$	Exposed $_w$	Non-exposed $_w$
5°C $_v$	0.5 (0.48, 12)	0.2 (0.17, 8)	0.4 (0.28, 12)	0.03 (0.03, 12)
17°C $_u$	3.8 (1.25, 12)	5.4 (1.46, 12)	13.2 (2.12, 12)	12.3 (1.61, 12)
27°C $_v$	0 (n=9)	1.0 (0.68, 6)	0.4 (0.30, 12)	0.9 (0.59, 11)

Discussion

Lineage VI exhibited wider tolerance to experimental conditions than lineage III, corresponding to earlier field observations of DuBey and Caldwell (2004). Lineage VI resisted *M. cerebralis* infection while those in lineage III exhibited infection. Previous experimental exposure studies reported *T. tubifex* exhibiting variations in TAM production and infection levels at several substrate and temperature combinations (Blazer et al. 2003) and in susceptible and resistant lineages of *T. tubifex* (Beauchamp et al. 2002). The infection levels of lineage III (4.3% at 5^{o}C, 3.3% at 17^{o}C, and 0% at 27^{o}C) were generally low compared to the wide range of infection levels in *T. tubifex* from West Virginia reported by Blazer et al. (2003). These authors reported infection levels from an experimental challenge of 350 myxospore dose per worm that ranged from 8.3 to 16.7% at 9^{o}C , from 6.3 to 22.9% at 13^{o}C, from 12.5 to 33.3% at 17^{o}C, and 0% at 20^{o}C. A wide range of infection levels were also exhibited by *T. tubifex* collected from Windy Gap Reservoir and Breeze Bridge on the upper Colorado River, Colorado, when exposed to spore doses of 6,000 and 11,000 spores per worm (Beauchamp et al. 2002). *Tubifex tubifex* from these collections were screened for TAM production; those not producing TAMs after one month were experimentally challenged with *M. cerebralis* spores. Beauchamp et al. (2002) observed infection levels ranging from 0 to 76.7% for lineage I and from 2.3 to 30.0% for lineage III; no infection was exhibited in lineages V or VI. Thus, the results of this research and those of others suggest a wide

range of susceptibility of *T. tubifex* to *M. cerebralis* within lineages susceptible to infection.

Lineage III exhibited detectably lower adult survival than lineage VI regardless of temperature, photoperiod, or infection. These observations were similar to those of Beauchamp et al. (2002) who demonstrated survival ranging from 15.3 to 91.0% and from 67.4 to 75.4% in two *T. tubifex* populations experimentally exposed to *M. cerebralis*. The authors, however, did not distinguish lineage differences before exposure. Negative effects of the parasite on survival among different populations of *T. tubifex* were inferred by dramatic decrease in abundance in one of two populations originating from different geographical origins when subjected to *M. cerebralis* challenges of 50, 500, and 1000 myxospores per worm (Stevens et al. 2001). These authors did not distinguish mitochondrial lineage differences among populations, however, analysis of rRNA ITS-1 locus determined that the two geographic populations were genetically distinct. Furthermore, differences in survival were exhibited among three ecological lineages of *T. tubifex* subjected to experimental temperature increases of 11 and 12^{o}C (type A: from 71.4 to 73.1%; type B: from 89.6 to 95.0%; type C: from 30.8 to 37.5%) (Anlauf 1994). Effects of temperature on survival were not detected in this study; however, survival was consistently higher at 17^{o}C suggesting that further work on this topic is warranted. *Tubifex tubifex* of lineage VI exhibited greater weight gains and greater fecundity than those of lineage III. This is the first report of differences in growth and reproduction between these two lineages. Similar observations were observed among ecological lineages of *T.*

tubifex (Anlauf 1994), *T. tubifex* forms (Poddubnaya 1984), between two geographic populations (Stevens et al. 2001), and different population growth rates between exposed and unexposed *T. tubifex* from three geographical populations (Kearns et al. 2004).

Infection had no detectable effect on growth, reproduction, or survival of lineage III worms. However, infection had a negative effect on population growth rate (fecundancy) in two of three geographical populations of *T. tubifex* (Kearns et al. 2004). These authors reported fecundancy rates that ranged from 0.001 to 0.009 individuals/d for the exposed population and from 0.002 to 0.013 individuals/d for the unexposed population. In addition, this study's exposed and unexposed treatment groups exhibited higher population growth rates at 17^{o}C. The lack of a detectable exposure effect on natality in this study reflected negative growth rates for both the 5 and 27^{o}C treatment groups.

Bonomi and DiCola (1980) observed that within same age cohorts of *T. tubifex*, the faster-growing worms matured earlier, remained fecund longer and produced more young. Thus, the observed reduction in growth, reproduction, and survival of lineage III adults (compared to lineage VI) may result from lineage differences. If time to reproductive maturity is accelerated, the greater weight gain by lineage VI may infer increased fitness over lineage III.

Of the three temperatures examined, the survival of young tubificids was highest at 17^{o}C. Bonomi and DiCola (1980) observed a positive correlation of egg production in *T. tubifex* at temperatures ranging from 5^{o}C to 20^{o}C. Density was

shown to affect natality in experimental cultures of *T. tubifex* (Bonomi and DiCola 1980). Density in this study was not manipulated; however, adults in lineage VI exhibited higher survival rates and produced more young per adult than lineage III. This suggests that the differences in natality are attributable to lineage differences and not density dependence. Lineage VI densities in tubs were as high as 21 adult worms (mean weight 7.86 mg) and 482 young (mean weight 1.88 mg). In contrast, the greatest densities exhibited in lineage III were 17 adult worms (mean weight 7.14 mg) and 177 young (mean weight 1.52 mg). These adult mean weights were higher than the means for field-collected worms which ranged from 2.37 to 6.90 mg (Bonomi and DiCola 1980), and surpassed Anlauf's (1994) experimental means, which ranged from 1.73 to 3.16 mg. Thus, natality differences between the lineages may have been the result of faster growing worms within lineage VI that matured sooner and remained fecund longer.

Cysting for all surviving adult tubificids was 3.9% and was observed in only the 5^{o}C treatment while photoperiod had no effect. None of the cysted *T. tubifex* exhibited infection as determined by PCR screening. Several authors have described *T. tubifex* cysting as a life history strategy to survive changes in environmental conditions (Kaster and Bushnell 1981; Anlauf 1990). Kaster (1980) observed cysting in *T. tubifex* in a mountain headwater stream in winter. Anluaf (1990) also observed cysted *T. tubifex* survived lower experimental temperatures than those that did not cyst. Furthermore, the number of cysted *T. tubifex* observed was higher in winter than summer or spring in the tailwater of the San Juan River, New Mexico

(DuBey and Caldwell 2004). The tailwater exhibited a relatively constant temperature (7 - 9.5^{o}C) through the seasons, thus, cysting by *T. tubifex* in the New Mexico tailwater may have been initiated by the reduction in photoperiod in winter. Anlauf (1994) found that only one of three ecological lineages cysted and speculated that cysting reduced metabolic activity when food was limited. Our mean ending weights for lineage VI (4.45 mg/adult worm) were similar to mature *T. tubifex* weights where food was not a limiting factor (i.e., 4.6 mg/worm, Bonomi and DiCola 1980). Anlauf (1994) also reported one cysted ecological lineage exhibited higher weights and greater egg production than other ecological lineages. Although lineage VI exhibited greater weight, greater weight gains, and greater egg production than lineage III in this study, both lineages of *T. tubifex* cysted. Additional work is needed to better understand environmental pressures associated with cysting of *T. tubifex*.

Triactionomyxon production by tubificids was directly related to disease severity in juvenile rainbow trout in other susceptibility experiments (Ryce et al. 2001). Thus, the presence of resistant lineages may reduce the prevalence of TAMs and lessen the impact of infection on resident salmonids. El-Matbouli et al. (1999b) observed resistant *T. tubifex* lineage V from Hamilton Bay, Ontario, Canada, ingested and inactivated *M. cerebralis* spores. Presumably, these tubificids were effectively removing spores by acting as biological filters and preventing contact with susceptible lineages. Furthermore, none of our lineage VI worms were infected and habitats dominated by resistant lineage VI exhibited lower infection rates in the San Juan River tailwater (DuBey and Caldwell 2004). It is possible that resistance of a *T.*

tubifex lineage to infection may serve to mitigate the prevalence of *M. cerebralis* in infected systems. Competition of resistant *T. tubifex* with other lineages may reduce incidence of infection by eliminating spores from the habitat that would otherwise be available to susceptible *T. tubifex*.

CHAPTER 4: GENETIC DIFFERENTIATION OF *TUBIFEX TUBIFEX* LINEAGES FROM THE SAN JUAN RIVER, NEW MEXICO

Introduction

Since Lewontin and Hubby's (1966) work showing extensive polymorphism in *Drosophila*, allozymes have been used to study genetic variation in many organisms. Most of the work on aquatic invertebrates has been on organisms that inhabit the planktonic or intertidal zones (Weider 1992). The discovery of the devastating effects of whirling disease in the Rocky Mountains in the 1990's has increased the activity level of the examination of genetic variation of *Tubifex tubifex*. *Tubifex tubifex* is a cosmopolitan freshwater species in the family Tubificidae that is taxonomically identified using morphological characteristics of sexually mature adults (Kathman and Brinkhurst 1999). These characteristics have been proven inadequate to effectively distinguish *T. tubifex* because the species exhibits phenotypic plasticity of its external morphology (Chapman and Brinkhurst 1987). In earlier work, Paoletti (1989) suggested that some 'forms' of *T. tubifex* may represent distinct species. Crossbreeding tests between sympatric *T. tubifex* forms indicated that the forms did not interbreed with sufficient genetic differences to be considered distinct species.

Anlauf (1994) differentiated cryptic ecological lineages of *T. tubifex* from oligotrophic coldwater systems and from ephemeral eutrophic waters by the allelic frequencies of glucose 6-phosphate isomerase (GPI) and isocitrate dehydrogenase

(IDH). Phylogenetic analysis of mitochondrial 16S ribosomal DNA from geographically distinct populations of *T. tubifex* provided evidence that similar cryptic lineages existed within both North American and European populations (Sturmbauer et al. 1999; Beauchamp et al. 2001). Both Sturmbauer et al. (1999) and Beauchamp et al. (2001) suggested that the genetic distances between some of these lineages provided evidence of the existence of cryptic species of *Tubifex*. Furthermore, a survey of *T. tubifex* revealed European mitochondrial lineages were differentiated by distinct electrophoretic patterns of GPI and IDH (Sturmbauer et al. 1999).

Stevens et al. (2001) demonstrated different *Myxobolus cerebralis* triactinomyxon (TAM) production levels between geographically differentiated *T. tubifex* and Krueger et al. (2000) reported variability in tubificid assemblages and infection rates of *T. tubifex* within side channels of the Madison River. Several *T. tubifex* lineages were found at sites within the Colorado River (I, III, V, and VI) (Beauchamp et al. 2002). At least one *T. tubifex* mitochondrial lineage (V) in the Colorado River was resistant to infection. The authors hypothesized the presence of sympatric cryptic populations of tubificid species (species that are resistant and others which are susceptible to *M. cerebralis* infection). Recently, lineage VI was shown to exhibit resistance to infection among sympatric populations of lineages I, III and VI from the tailwater of the San Juan River, New Mexico (DuBey and Caldwell 2004) and when subjected to experimental challenges (Dubey et al. 2005). DuBey and Caldwell (2004) observed *T. tubifex* lineage III and VI were co-dominant in pool

habitats characterized by sediments with high silt and organic matter content where lineage VI dominated riffle habitats which were characterized by coarser substrates with lower organic matter content. Lineage I was found within a few shallow main channel sites characterized by high silts and organic matter. Furthermore, *M. cerebralis* infection rates exhibited in pool habitats were higher (3.01%) than those collected in riffle habitats (0.51%). Thus, determining the genetic differentiation exhibited among sympatric populations of *T. tubifex* lineages may provide a key component to understanding the etiology of whirling disease. Based on the differences of infection rates (Beauchamp et al. 2002; DuBey and Caldwell 2004), physiological differences between lineages (DuBey et al. 2005), and genetic differences exhibited between *T. tubifex* lineages (Sturmbauer et al 1999; Beauchamp et al. 2001), this study hypothesizes that considerable genetic variation exists between *T. tubifex* lineages and that the lineages can be differentiated and considered cryptic species. The objective of this study was to examine the genetic divergence within two *T. tubifex* lineages (III and VI) from different habitats within the San Juan River, New Mexico tailwater.

Materials and Methods

Starch gel electrophoresis was used to screen allozyme and buffer combinations to delineate allelic loci. This was followed by starch gel and cellulose acetate electrophoreses to assay the genetic divergence of *T. tubifex* lineages and

between *T. tubifex* from deep and shallow habitats within the San Juan River tailwater.

Electrophoresis screening of *T. tubifex* for diagnostic allozymes was designed to replicate buffer systems and allozyme stains used in starch gel electrophoresis of *T. tubifex* (Anlauf 1990, 1994, and 1997) and cellulose acetate electrophoresis of aquatic oligochaetes (Weider 1992). Starch gel electrophoresis screening of *T. tubifex* for diagnostic allozymes used buffer systems 2, 4, 5, 6, 7, 8, 9, 10, 11 and 22 allozyme stains (Shaw and Prasad 1970) (Appendix A). Cellulose acetate electrophoresis screening of *T. tubifex* for diagnostic allozymes used two buffer systems Tris Glycine (TG, 3.0 g/l tris and 14.4g/l glycine) and Citric acid-morphaline (CAAM, 10.5 g/l citric acid and 12.5 ml/l 4-(3-aminopropyl) morpholine and 8 allozyme stains (Hebert and Beaton 1993).

The characterization of genetic variation among *T. tubifex* lineages and habitats used *T. tubifex* sub-samples from frozen (-70^{o}C) cultures collected from the San Juan River tailwater in 2001 and 2002. Prior to freezing, the cultures were placed in sorting trays and individual *T. tubifex* were microscopically identified in depression slides by morphological characteristics (hair and pectinate chaetae starting on dorsal segment 2). *Tubifex tubifex* were then placed individually in 0.5 ml 24-cell well plates with aerated well water for 3 days to purge and to screen for TAMs. Each individual was then dissected into anterior and posterior sections; the anterior section for further identification of morphological characteristics (penal sheath, pectinate

chaetae starting on dorsal segment 2) and extraction of DNA and the posterior section for allozyme sample homogenate.

To prepare for allozyme analysis, the posterior section of each tubificid was placed into a depression well of a microtiter plate on ice and 10 µl of distilled water was added and ground with a glass pestle to obtain a homogenate. Filter paper wicks were soaked in the homogenate for starch gel electrophoresis and the homogenate was pipetted into wells of a Super Z applicator™ (Helena Laboratories, Beaumont, Texas) for cellulose acetate electrophoresis.

Starch gels were prepared by combining 47 g starch and 400 ml gel buffer for the system in a Pyrex™ beaker and heated until boiling for 30 sec. The gel mixture was degassed and poured into a pexiglass mold and when set covered with saran wrap to prevent dehydration and chilled to 4^{o}C. Filter paper wicks saturated with sample homogenates were loaded into wells cut into the gel. The gels were run at 50 mA for five hours at 4^{o}C. The gels were sliced horizontally into 2 mm sections providing three diagnostic sections. Individual gel sections were placed in plastic trays for staining with the diagnostic allozyme. The gels were incubated at 40^{o}C in a darkroom incubation chamber to initiate enzymatic reaction between the tubificid homogenate and stain. The gels were then fixed in a solution of 5:5:1, methanol, distilled water, and glacial acetic acid.

Starch gel electrophoresis for electrophoretic polymorphisms revealed that several buffer systems and stains were potentially diagnostic for *T. tubifex* (Table 4.1). Thus, subsequent starch gel electrophoresis used buffer systems 4 (Continuous

Tris-citrate, pH 6.3), and 5 (Continuous Tris-citrate, pH 8.0) and allozyme stains aconitate hydratase (ACON, 4.2.1.3), carboxylesterase (αEST, 3.1.1.1), fumarate hydratase (FUM, 4.2.1.2), glucose 6-phosphate isomerase (GPI, 5.3.1.9), isocitrate dehydrogenase (IDH, 1.1.1.42), leucine (LAP, 3.4.1.1), and malate dehydrogenase (MDH, 1.1.1.37) to delineate alleles from *T. tubifex*.

Cellulose acetate electrophoresis was performed on cellulose acetate plates (Helena Laboratories, Beaumont, Texas) and utilized methods described by Hebert and Beaton (1993). The plates were pre-soaked for 30 min in the buffer solution and then sample homogenates were loaded onto the cellulose acetate gel plate with an applicator. The allozymes fumarate hydratase (FUM, 4.2.1.2), glucose 6-phosphate isomerase (GPI, 5.3.1.9), leucine (LAP, 3.4.11.1), malate dehydrogenase (MDH, 1.1.1.37), malate dehydrogenase $NADP^+$ (ME, 1.1.1.40), and phosphoglucomutase (PGM, 5.4.2.2) were used with the TG buffer system. Aconitase (ACON, 4.2.1.3), carboxylesterase (α EST, 3.1.1.1) and isocitrate dehydrogenase (IDH, 1.1.1.42) were used with the CAAPM buffer system. The gels were run at 110 volts for 30 minutes at room temperature (20-25^{o}C). The stains were mixed with melted (60^{o}C) 1% agar and poured over the cellulose acetate plates on a leveled sheet of Plexiglas and incubated for 30 min.

Diagnostic alleles were scored using relative migration distance described in Ferguson (1980) and recorded. Many of the specimens did not provide staining results for all loci resulting in small sample sizes for many of the allozymes. The number of alleles and the level of heterozygosity were calculated separately for

Table 4.1. Preliminary genetic screening of *Tubifex tubifex* using starch gel electrophoresis with a suite of buffer systems and allozyme stains (+ = distinct banding, / = not distinct banding, blank = no banding).

		Buffer System							
Allozyme	EC #	2	3	4	5	6	9	10	12
ACON	4.2.1.3			+	/	/			
ACP	3.1.3.2								
ADH	1.1.1.1							/	
ALDO	1.2.3.1								
DIA	1.6.2.2								
A EST	3.1.1.1			+		+			
FBA	4.1.2.13								
FUM	4.2.1.2			+					+
G-3-PDH	1.2.1.12								
G-6-PDH	1.1.1.49				/				
GDH	1.4.1.2								
GOT	2.6.1.1								
GOX	1.1.3.15						/		
GPI	5.3.1.9				/				
IDH	1.1.142	+		+	+	+	/	/	
LAP	3.4.11.1		/	+	+	+	/	/	
MDH	1.1.1.37	+		+	+	+	/	/	+
ME	1.1.1.40			/			/		
NP	2.4.2.1								
ODH	1.1.1.1								
PER	1.11.1.7			/					
PGM	5.4.2.2				/				
XDH	1.2.1.37								

habitat types using starch gel electrophoresis and for *T. tubifex* lineage using cellulose acetate electrophoresis. Starch gel electrophoresis used seven diagnostic allozyme stains to analyze *T. tubifex* lineages from cultures of collections from deep and shallow habitats in the San Juan River tailwater.

Results

Three polymorphic allelic loci were exhibited using starch gel electrophoresis (IDH, LAP, and MDH) (Table 4.2). Of the seven allozymes screened ACON, αEST, FUM, and GPI were not resolvable. The IDH locus exhibited a dimeric allele as well as two alleles with no apparent heterozygote allele. The MDH locus exhibited a dimeric allele as well as one additional allele. The LAP locus exhibited a monomeric allele where the heterozygous form was identified. Both IDH and MDH dimeric allele frequencies in deep and shallow habitats significantly departed from Hardy-Weinberg expected frequencies, however, the LAP locus exhibited allele frequencies that were in equilibrium with Hardy-Weinberg expected frequencies (Table 4.3). Heterogeneity of allele frequencies among populations was analyzed by Chi-Square (χ^2) tests. Detectable differences in allele frequency differences were found between tubificids from deep and shallow habitats for the LAP locus but no differences were observed for the IDH and MDH loci (Table 4.4).

Cellulose acetate electrophoresis used TG and CAAPM buffer systems and nine allozyme stains to characterize genetic differentiation among *T. tubifex* lineages.

Table 4.2. Allele frequencies of three allozyme loci (isocitrate dehydrogenase, IDH; leucine , LAP; malate dehydrogenase, MDH) using starch gel electrophoresis for *Tubifex tubifex* collected in deep sites (> 1 m) and shallow sites (< 1 m) in the San Juan River, New Mexico tailwater.

	Allele Frequency					
Locus - Site	1	2	3	4	5	N
IDH - Deep	0.051	0.010	0.717	0.141	0.081	99
IDH - Shallow	0.007	0.014	0.490	0.000	0.490	147
LAP - Deep	0.692	0.308				26
LAP - Shallow	0.531	0.469				32
MDH - Deep	0.092	0.411	0.028	0.468		141
MDH - Shallow	0.049	0.431	0.049	0.472		144

Table 4.3. Chi square analysis comparing observed allozyme (isocitrate dehydrogenase, IDH; leucine , LAP; malate dehydrogenase, MDH) genotype frequencies using starch gel electrophoresis to Hardy-Weinberg expected genotype frequencies of *Tubifex tubifex* collected from deep and shallow habitats in the San Juan River, New Mexico tailwater.

Locus/habitat	df	χ^2	Probability
IDH/Deep	2	33.52	<0.001
IDH/Shallow	2	40.47	<0.001
MDH/Deep	2	22.84	<0.001
MDH/Shallow	2	11.20	0.004
LAP/Deep	2	1.202	0.601
LAP/Shallow	2	1.28	0.529

Table 4.4. Chi square analysis comparing allozyme (isocitrate dehydrogenase, IDH; leucine aminopepsidase, LAP; malate dehydrogenase, MDH) allele frequencies using starch gel electrophoresis of *Tubifex tubifex* collected from deep and shallow habitats in the San Juan River, New Mexico tailwater.

Allele	df	χ^2	Probability
IDH_1	2	2.980	0.225
IDH_2	1	0.585	0.445
LAP	2	6.406	0.041
MDH_1	2	2.745	0.253

Six polymorphic allelic loci were exhibited (ACON, α EST, IDH, LAP, PGM) and three monomorphic allelic loci were exhibited (FUM, GPI, ME) (Table 4.5). Many of the specimens did not provide staining results for all loci using cellulose acetate gels resulting in small sample sizes for many of the allozymes.

The MDH locus exhibited a dimeric allele as well as one other allele and the LAP locus exhibited a monomeric allele where the heterozygous form was identified. The IDH locus exhibited two alleles with no apparent heterozygote individuals. In contrast, using starch gel electrophoresis, this locus exhibited a dimeric allele and two monomeric alleles. Furthermore, cellulose acetate electrophoresis revealed six additional diagnostic loci. Polymorphism was exhibited by the ACON locus with two alleles and the PGM locus with three alleles, and α EST, FUM, GPI, and ME loci were all monomorphic. Only the LAP locus for lineage VI exhibited a heterogeneous genotype and was in Hardy-Weinberg equilibrium ($\chi^2 = 2.185$, $P < 0.3353$). Heterogeneity of allele frequencies among lineages was analyzed by Chi-Square (χ^2) tests. Detectable differences were found between lineages III and VI for the LAP and MDH loci but no differences were observed among ACON, IDH, or PGM loci (Table 4.6). Nei's (1972) genetic identity (I) and genetic distance (D) (Table 4.7) was calculated for three diagnostic loci from the cellulose acetate data to examine the relationship of *T. tubifex* lineages III and VI to other tubificids. Allele frequencies for IDH, GPI and PGM loci were available for comparison of *T. tubifex* and the closely related tubificid *Potamothrix moldaviensis* collected in the Great Lakes

Table 4.5. Allele frequencies of 7 allozyme loci (aconitase, ACON; carboxylesterase, α EST; fumarate hydratase, FUM; glucose 6-phosphate isomerase, GPI; isocitrate dehydrogenase, IDH; leucine , LAP; malate dehydrogenase, MDH; malate dehydrogenase $NADP^+$, ME; phosphoglucomutase; PGM) using cellulose acetate electrophoresis of *Tubifex tubifex* lineages III and VI collected in the San Juan River, New Mexico tailwater.

	Allele Frequency				
Loci - Lineage	1	2	3	4	N
ACON - III	0.667	0.333			18
ACON - VI	0.857	0.143			14
α EST - III	1.000				12
α EST - VI	1.000				12
FUM - III	1.000				9
FUM - VI	1.000				11
GPI - III	1.000				18
GPI - VI	1.000				18
IDH - III	0.212	0.788			33
IDH - VI	0.250	0.750			32
LAP - III	1.00				48
LAP - VI	0.122	0.878			49
MDH - III	0.926	0.074			26
MDH - VI	0.269	0.154	0.462	0.038	24
ME - III	1.000				12
ME - VI	1.000				12
PGM - III	0.900	0.050	0.050		24
PGM - VI	0.750	0.125	0.125		20

Table 4.6. Chi square analysis comparing five allozyme loci (aconitase, ACON; isocitrate dehydrogenase, IDH; leucine , LAP; malate dehydrogenase, MDH; phosphoglucomutase; PGM) allele frequencies from cellulose acetate gel electrophoresis of *Tubifex tubifex* lineages III and VI collected in the San Juan River, New Mexico tailwater.

Allele	df	χ^2	Probability
ACON	1	1.524	0.217
IDH	1	0.131	0.717
LAP	2	97.0	>0.001
MDH	3	26.697	>0.001
PGM	2	1.65	0.438

Table 4.7. Nei's (1972) genetic identity (I, above diagonal) and genetic distance (D, below diagonal) and for *Tubifex tubifex* lineages III (*T. t.* III) and VI (*T. t*. VI) from the San Juan River, New Mexico tailwater and *T. tubifex* (*T. t.)* and *Potamothrix* moldaviensis (*P. m.)* from the Great Lakes (Weider 1992). Allele frequency data of glucose 6-phosphate isomerase (GPI), malate dehydrogenase (MDH), and phosphoglucomutase (PGM) from cellulose acetate electrophoresis were analyzed with Popgene version 1.31 (Yeh and Yang 1999).

Population	*P. m.*	*T. t.*	*T. t.* III	*T. t*. VI
P. m.	*****	0.5545	0.5119	0.5658
T. t.	0.5897	*****	0.8314	0.7678
T. t. III	0.6696	0.1846	*****	0.8838
T. t. VI	0.5696	0.2642	0.1235	*****

(Weider 1992). Two loci were in common with other studies using starch gel electrophoresis; thus, those were not used for calculating genetic distance. The analysis used the program Popgene version 1.31 (Yeh and Yang 1999) and Nei's (1972) genetic distance (D) and unweighted pair-group method with arithmetic averaging (UPGMA) to produce a dendrogram of phylogenetic relationships (Figure 4.1).

Discussion

Tubifex tubifex is not an ideal organism for starch gel electrophoretic analysis. Of the 23 allozymes screened for analysis, only three yielded resolvable alleles with starch gel electrophoresis (IDH, LAP, and MDH) and nine with cellulose acetate (ACON, α EST, FUM, GPI, IDH, LAP, ME, and PGM). Anlauf (1997) was successful in obtaining six allozyme loci (α EST, GPI, IDH, MDH, ME, and PGM) with starch gel electrophoresis to differentiate among European populations of *T. tubifex*. The PGM locus was used to differentiate among Anlauf's (1997) ecological lineages. Sturmbauer et al. (1999) used three of the allozyme loci identified in Anlauf's (1997) study (GPI, IDH, and MDH) with starch gel electrophoresis to differentiate cadmium resistance among European populations of *T. tubifex*. In the work by Sturmbauer et al. (1999), the GPI locus differentiated mitochondrial lineages I and IV from lineages II, III, and V. In comparison, the starch gel electrophoresis of *T. tubifex* from the San Juan River collections only exhibited diagnostic alleles from the IDH, LAP, and MDH loci and was unable to resolve the potentially diagnostic

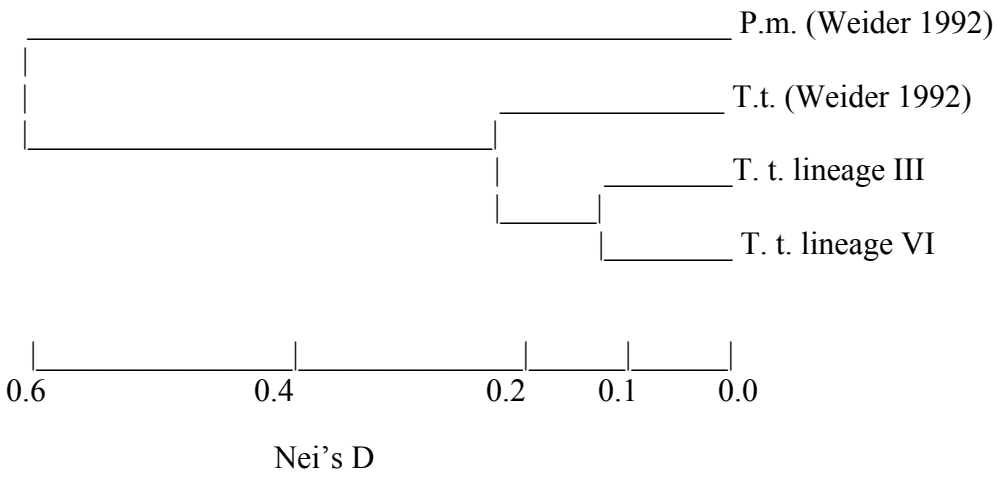

Figure 4.1. Dendrogram of Nei's (1972) genetic distance (D) for *Tubifex tubifex* lineages III (T. t. III) and VI (T. t. VI) from the San Juan River, New Mexico and *T. tubifex* (T. t.) and *Potamothrix* moldaviensis (P. m.) from the Great Lakes (Weider 1992). Allele frequency data of GPI, MDH, and PGM from cellulose acetate electrophoresis were analyzed with Popgene version 1.31(Yeh and Yang 1999) using the unweighted pair-group method with arithmetic averaging (UPGMA).

GPI and PGM loci. Many of the electrophoretic gels for the IDH, LAP and MDH loci also did not produce resolvable staining results which may suggest the lack of sufficient homogenate for analysis. Furthermore, the GPI and PGM loci were diagnostic using cellulose acetate suggesting insufficient homogenate with starch gel electrophoresis.

Allele frequencies of the LAP locus did provide results which were congruent with *T. tubifex* mitochondrial lineage distributions among deep and shallow habitats in the San Juan River. The cellulose acetate electrophoresis results revealed that the LAP locus alleles differentiated between lineage III and VI. The homozygote allele 'A' was exhibited only within lineage III where the heterozygote 'AB' and homozygote 'B' alleles were only exhibited within lineage VI. The LAP heterozygote allele 'AB' associated with lineage VI was exhibited with starch gel electrophoresis only from shallow habitat *T. tubifex* samples. DuBey and Caldwell (2004) reported lineage VI was the dominant lineage in shallow habitats, thus it follows that lineage VI may be under positive selection in shallow habitats for the LAP heterozygote. Selection for genotypes may result in a deviation from Hardy-Weinberg equilibrium (Baker 2000). However, LAP was shown to be in Hardy-Weinberg equilibrium among tubificids in deep and shallow habitats and among lineage VI. The fixed LAP allele in lineage III may not indicate reproductive isolation as fixed allelic differences may indicate local adaptation or genetic drift. However, assortative mating (pre-mating isolation) may occur between lineages. *Tubifex tubifex* are sessile organisms and most probably mate with nearby individuals

residing in the same microhabitat. Sympatric speciation may occur if assortative mating occurs with the accumulation of genetic changes at loci within that population (Rice 1987).

Other loci exhibiting heterozygotes were IDH and MDH using starch gel electrophoresis and both were not in Hardy-Weinberg equilibrium. Non-random mating, selection for particular genotypes, and strong gene flow from adjacent populations are probable causes of deviation Hardy-Weinberg equilibrium (Baker 2000). Asexual organisms are often not in Hardy-Weinberg equilibrium because of non-random mating of genetically different clones (Hebert and Beaton 1993). *Tubifex tubifex* are hermaphrodites and can reproduce by parthogenesis (Poddubnaya 1984) and the departure from Hardy-Weinberg equilibrium exhibited may infer asexual reproduction within certain habitats. Furthermore, because the *T. tubifex* populations analyzed were collected within the same water body, strong gene flow from adjacent populations may have occurred. This explanation is supported by the effects of scouring flow on tubificid habitat in the San Juan River (DuBey and Caldwell 2004). The physical disturbance caused by scouring flow may have redistributed *T. tubifex* among habitats.

Many populations of *T. tubifex* in North America were established after the retreat of ice sheets resulting in insufficient time for mutations to be fixed within the populations. Therefore, it may be inappropriate to use measures of population structure such as F-statistics (Wright 1965, 1978) or Slatkin's (1985) estimator of population cohesiveness as they rely on mutation fixation within the populations.

Thus, measures of heterogeneity and genetic differentiation were used among populations from distinct habitats and among genetic lineages of *T. tubifex*. Heterogeneity of allele frequencies (χ^2 tests) among populations collected from deep and shallow habitats and between lineages III and VI showed detectable differences for both the LAP and MDH loci. Furthermore, Nei's (1972) genetic distance (D) of 0.1235 using the cellulose acetate allele frequency data for GPI, MDH and PGM loci revealed *T. tubifex* lineage III and VI were more similar to each other than the *T. tubifex* population from the Great Lakes (Weider 1992). Finally, all *T. tubifex* populations were distinct from *P. moldaviensis*. The genetic distance exhibited among lineages may be compared to Schmidt and Westheide's (2000) report of a genetic distance of 0.17 for two terrestrial enchytraeids within a cryptic species complex which are reproductively isolated (*Enchytraeus variatus* and *E. crypticus*). Thus, the genetic distance between lineages may be sufficient to hypothesize that the lineages are cryptic species.

A wide range of susceptibility of *T. tubifex* to *M. cerebralis* may exist within lineages susceptible to infection. Beauchamp et al. (2002) reported that *T. tubifex* from one lineage (V) was resistant to infection by *M. cerebralis.* When subjected to experimental challenges of *M. cerebralis* lineage VI resisted *M. cerebralis* infection while those in lineage III exhibited infection (DuBey et al. 2005). Furthermore, lineage VI also exhibited wider tolerance to experimental conditions than lineage III which corresponded with earlier field observations of DuBey and Caldwell (2004).

These findings may, in part, be explained by sympatric cryptic populations of *Tubifex* species that are either resistant or susceptible to *M. cerebralis* infection.

There is support for significant genetic differentiation between resistant lineage VI and susceptible lineage III. The electrophoretic data for the LAP locus is fixed for lineage III and differentiates it from the sometimes heterozygous lineage VI and there is genetic distance of 0.1235 (Nei 1972) between the lineages. The genetic differentiation demonstrated through this survey and others coupled with resistant and susceptible lineages, in fact, indicate that lineage V and VI may be cryptic species. Thus, the existence of cryptic species of *Tubifex* may provide a key component to understanding the etiology of whirling disease. However, crossbreeding studies of *T. tubifex* are complicated by the fact that the species are hermaphroditic and can reproduce through parthogenesis. Development of genetic markers (e.g. randomly amplified polymorphic DNA, microsatellites) that distinguish parentage of progeny in well designed crossbreeding studies may offer resolution to this question.

CHAPTER 5: DISTRIBUTION OF *TUBIFEX* *TUBIFEX* LINEAGES AND ASSOCIATED HABITAT VARIABLES IN HEADWATER SYSTEMS OF NEW MEXICO

Introduction

The *Myxobolus cerebralis* parasite has a two host life cycle involving separate stages of sporogony in each host (Wolf and Markiw, 1984; El-Matbouli and Hoffman 1989). The definitive host of *M. cerebralis* is the freshwater oligochaete *Tubifex tubifex* and the obligate host is a salmonid fish. The novel pathogen hypothesis (Grenfell and Gulland 1995), in part, predicts limited host resistance when pathogens are spread into areas where the hosts have little or no defense history against the pathogen. *Myxobolus cerebralis* qualifies as a novel pathogen with its recent introduction into North America from Europe in the 1950's. The devastating effects of whirling disease on wild salmonid populations was not fully realized until its discovery in the inter-mountain west with the sudden collapse of many wild rainbow trout populations (Vincent 1996, Hedrick et al. 1998). The presence of the *M. cerebralis* was confirmed in New Mexico rainbow trout populations in the spring of 1999 (Hansen 2002). The parasite's pathogenic effects can decimate salmonid young of year cohorts (Rognlie and Knapp 1998). Thus, the most devastating potential of the disease in New Mexico lies in the threat it poses to native salmonid populations that rely on natural reproduction.

New Mexico has two native trout, the Rio Grande cutthroat (*Oncorhynchus clarki virginialis*) and Gila trout (*O. Gilae*). These native fish currently occupy only a fraction of their historic range (Mello and Turner 1980; Sublette et al. 1990) and are isolated to headwater systems in New Mexico (Paroz et al. 2002). Rio Grande cutthroat trout are considered a species at risk by the State of New Mexico and the U.S. Fish and Wildlife Service and the Gila trout is federally endangered. Populations of Rio Grande Cutthroat trout and Gila trout are typically located in small, headwater streams and are often isolated from other core populations and non-native trout by migrational barriers.

Tubifex tubifex is a cosmopolitan species, however, little is known about their distribution and abundance in high altitude headwater systems where they may co-occur with native trout. *Tubifex tubifex* are typically associated with sediments characterized by fine substrates and feed on the bacteria associated with organic matter (McMurtry et al. 1983). *Tubifex tubifex* are abundant in habitats degraded by siltation, nutrient enrichment, or low oxygen levels, however, populations can also be found in oligotrophic conditions typical of high altitude headwater streams in the western United States (Zendt and Bergersen 2000).

Beauchamp et al. (2002) reported four lineages of *T. tubifex* (I, III, V, and VI) at two sites on the Colorado River, Colorado. The authors also observed varied susceptibility of the lineages to experimental infection of *M. cerebralis*. Recently, three lineages (I, III, and VI) were identified in sympatric populations from the tailwater of the San Juan River, New Mexico (DuBey and Caldwell 2004). No

infection was detected in lineages I and VI in contrast to lineage III. Furthermore, lineage III exhibited higher infection levels in pool (3.0%) than in riffle habitats (0.5%), demonstrating varied infection presumably due to different environmental conditions. Lineage VI dominated riffle reaches while lineages I, III, and VI were observed in pool habitats. Varied *M. cerebralis* infection levels in *T. tubifex* have also been observed in populations from different geographical areas (Stevens et al. 2001; Beauchamp et al. 2002) as well as different lineage assemblages. (Beauchamp et al. 2002). For example, Beauchamp et al. (2002) observed varying infection levels within waters exhibiting varying sediment loads which supported different lineage compositions. The varying infection levels, in part, may have been due to the presence of resistant *T. tubifex* lineage V (Beauchamp et al. 2002). Resistant worms may ingest and inactivate *M. cerebralis* spores**,** thus, effectively removing spores from the habitat by acting as biological filters and preventing contact with susceptible lineages (El-Matbouli et al. 1999b).

Recent laboratory experiments have also demonstrated varied lineage resistance to infection with *M. cerebralis* and different survival and fecundancy rates (DuBey et al. 2005). Lineage III exhibited infection levels of 4.3% at 5^{o}C, 3.3% at 17^{o}C and 0% at 27^{o}C while lineage VI resisted infection by *M. cerebralis* at all temperatures. Competition of *T. tubifex* lineage VI with other lineages for resources may serve to decrease overall infection levels among *T. tubifex* populations, thereby reducing both triactinomyxon production and the occurrence of whirling disease

among susceptible salmonids. Thus, the habitat and lineage of *T. tubifex* are important in characterizing prevalence of whirling disease.

Little is known about *T. tubifex* distribution and abundance in headwater systems to assess the risk for the spread of *M. cerebralis* to trout populations. Thus, the objectives of this study were to characterize *T. tubifex* distribution, habitat variables, presence of native and non-native trout, and the incidence of *M. cerebralis* in headwater systems in New Mexico. Furthermore, as *T. tubifex* lineage resistance to *M. cerebralis* varies, the lineage composition of headwater populations were characterized to accurately assess the risk to native trout. This survey for *T. tubifex* characterized supporting habitat in an effort to characterize components of the risk for disease introduction to native trout populations and increase our understanding of lineage distribution and genetic variability for this cryptic species.

Methods

In 2002, thirteen study reaches were established in eleven streams in the Carson (CNF), Cebola (CBNF), and Santa Fe (SFNF) National Forests and on the Vermejo Park Ranch. In 2003, eleven study reaches were established in ten streams on the Carson and Santa Fe National Forests. The study area included stream systems within mountain regions of New Mexico above 1700 meters. Montaine areas in New Mexico have a variable climate with cool summers and moderate winters. Mean daily air temperature ranges from -31.7^{o}C to 10.0^{o}C in winter and from -1.1^{o}C to 35^{o}C in summer. Mean annual precipitation varies from 25.4 cm to 88.8 cm per

year, with the greater amount falling at higher elevations. Sample collection sites were established in 2002-2003 within the study area on streams where *M. cerebralis* has been established and/or on streams inhabited by Rio Grande cutthroat trout populations, adjacent to Rio Grande cutthroat trout populations, or potential reintroduction areas for the species.

Sampling of New Mexico headwater streams in 2000 and 2001 showed highly variable systems in relation to *T. tubifex* habitat with an average of 10% of reach samples containing tubificids**.** A Stratified Random Sample design was developed to characterize these highly variable headwater systems. The experimental design uses a sample size for detection of rare species by the following formula:

$$n = (-1/m) \ln \text{beta},$$

where m is 10 % of samples contain *T. tubifex* and beta is a 5% type II error, (n = 29.957) (Green & Young 1993). Thus, 30 quantifiable core samples were taken from quadrats allocated among strata of potential tubificid habitat within each study reach in the headwater surveys for a representative sample of the oligochaete community and to provide sediments for measurement of organic carbon (ASTM 2000). In addition, physical and chemical parameters were collected at the substrate level to reflect environmental conditions for *T. tubifex*. Depth (m) was measured with a meter stick, velocity (m/s) was measured with a Marsh-McBirney direct-reading flow meter, temperature and dissolved oxygen (mg/L) were measured with a dissolved oxygen meter (Yellow Springs Instrument, OH), and turbidity (NTU) was measured with a turbidity meter (H F Instruments). All meters were calibrated daily. Water

samples were collected with a Kemmerer vertical water sampler to assess nitrite (mg/l), sulfates (mg/l) and biochemical oxygen demand (BOD). Water was stored in 300 ml glass BOD bottles and analyzed in accordance to Standard Methods for the Examination of Water and Wastewater (APHA 1999). Nitrite (mg/L) and sulfates (mg/L) were measured using a HACH spectrophotometer (DR/2010) and HACH methods 8507 and 8155, respectively.

To quantify habitat variables in headwater systems, 100 m study reaches were established in each study stream and sampled using transect methodologies as described by Platts et al. (1983). Study reach length was measured along the thalweg using a metric surveyor's tape. Measurements of channel and stream width, aquatic habitat types, velocity, and substrate were taken at 3 transects placed across the stream perpendicular to flow at pool, riffle, and run or glide habitat types. Gradient of each study section was measured using a handheld clinometer and stadia rod. Habitat types within each study reach were categorized as pool, glide, run, or riffle and measured to the nearest decimeter across the horizontal portion of the transect (Bisson et al. 1982; Herger et. al. 1996; Sponholtz and Rinne 1997). Pools were defined as deepwater habitats relative to adjacent habitats, little or no surface flow, substrate consisting of sediments finer than adjacent faster-flowing habitats, and a mean water column velocity of <10 cm/s. Glides were defined as habitats with moderate water depth, low turbulence, and a mean water column velocity of 10-20 cm/s. Runs were defined as areas of moderate depth, a mosaic of substrate types such as sand, gravel, or cobble, moderate surface agitation, and a mean water column

velocity of 21-60 cm/s. Riffles were defined as areas with relatively shallow water depth, broad surface agitation, substrate consisting of gravel, cobble with interspersed boulders and a mean water column velocity > 60 cm/s.

Mean water column velocity was measured at six tenths of the water depth at one-fourth, one-half, and three-quarters the stream width using a Marsh-McBirney direct-reading flow meter at each transect. Stream depth was measured to the nearest centimeter using a meter rule. Stream depth was measured at one-fourth, one-half, and three-fourths the stream-width distance across each transect. The three measurements were then totaled and divided by four to account for the zero depths at the stream shore where the water surface and the bank meet. Substrate was characterized using the pebble count procedure described by Bevenger and King (1995) and classified according to the Modified Wentworth Particle Size Scale (Bovee and Cochnauer 1977). Riparian vegetation was measured with a meter stick. All vegetation overhanging the stream bank of providing thermal cover for the stream was considered in riparian vegetation measurements.

Biomass and abundance were sampled for all fish species in accessable headwater reaches prior to habitat sampling. Study reaches were blocked with 6 mm mesh nets at each end and sampled by three-pass depletion electrofishing. Total lengths (mm) and weights (g) of all fishes captured were recorded and evaluated for whirling disease by morphological criteria. If surveyed fish were identified with the clinical symptoms of whirling disease they were collected along with all fish

mortalities for genetic (Andre et al. 1998) and histological confirmation of *M. cerebralis* infection.

Benthic samples were processed to establish oligochaete density, community structure, and percentage of *T. tubifex* within that community. Oligochaetes from each sample were microscopically sorted and identified using a dissecting microscope into morphological groups. Tubificidae with hair chaetae (morphology consistent with *T. tubifex)* were sorted and enumerated.

Triactinomyxon screening was performed on sub-samples of *T. tubifex* for *M. cerebralis* infection and definitive diagnosis of infection through genetic analysis (Andre et al. 1998). These *T. tubifex* were evenly distributed in a sorting tray containing a grid and individuals randomly selected and placed in 24 cell-well plates filled with well-water. The *T. tubifex* were cultured at 15^{o}C for 3 d. Water from each well was viewed daily by phase-contrast microscopy for the presence of TAMs (Markiw 1989). *Tubifex tubifex* found producing TAMs were genetically tested for confirmation of *M. cerebralis* infection using molecular PCR markers developed by Andre et al. (1998) and were genotyped for lineages I, III, V, and VI utilizing the primers and protocol developed by Beauchamp et al. (2002).

Multiple regression analysis was used to relate tubificid density to water quality parameters and habitat variables. Mallow's Cp statistic and Akaike's information criterion were used for model selection. Statistical analysis were performed using the software SAS Version 9.1 (SAS 2002).

Results

Field surveys collecting *T. tubifex* samples, fish population data, water quality samples and measurements, and habitat parameters were conducted at 24 sites on 21 headwater streams (Appendix B). *Tubifex tubifex* populations were identified at 12 of the 24 sites. Population densities ranged from 17 to 1600 per square meter and are considered generally low. Populations of *T. tubifex* were found along the entire elevation range of the study area.

Multiple regressions were used to analyze the relationship between *T. tubifex* density and physical parameters (Appendix C) and water quality measurements (Appendix D). The independent variables conductivity, nitrites, sulfates, % silt in sediments (silt), turbidity and gradient were used in the analysis. The independent variable biochemical oxygen demand (BOD), % carbon in sediments (carbon), were excluded as they exhibited correlations which were contrary to biological significance. Analysis of normality revealed a *T. tubifex* density outlier that skewed the residuals, thus, the data from that site were not included in analysis. Mallow's Cp statistic and Akaike's information criterion were used to identify the best three independent variable model ($R = 0.6080$, $P < 0.0004$) to model *T. tubifex* density. Silt was the best single predictor of *T. tubifex* density ($F_{1,22} = 29.11$, $P < 0.0001$).

Tubifex tubifex density = – 17.297 + (silt*1.161) + (conductivity*-0.08165) + (sulfate*-0.988)

Two *T. tubifex* lineages were identified by 16S mtDNA molecular markers (Beauchamp et al. 2002) among the headwater samples. The headwater streams were

dominated by the presence of lineage III which was found in all streams containing *T. tubifex* populations. In contrast, lineage VI was only found at three sites in the Jemez Mountains and were sympatric with lineage III. All *T. tubifex* tested for lineage were observed for three days for TAMs. No TAMs were observed, thus, the *T. tubifex* were not genetically screened for *M. cerebralis* infection.

Surveys of fish abundance were completed on 17 sites within 15 streams (Table 5.1). The habitat and water quality surveys showed that all of the reaches sampled have the potential of supporting native trout. Furthermore, the fish surveys showed an age class distribution which indicates reproducing trout populations within each of these systems. No fish were found within sites on the upper Cow Creek (above the Viveash fire in 2000) and Elk Creek (within the Viveash fire area) in the Pecos drainage (SFNF), and McCrystal Creek (intermittent flow due to drought) in the Canadian drainage (CNF). Jacks Creek in the Pecos drainage (SFNF) was sampled in 2002 and 2003 and exhibited a reduced RGCT population in 2003. Jacks Creek was reported to have gone dry in the upper reaches after the 2002 survey and the lower density was likely due to drought conditions. Populations of Rio Grande cutthroat trout were also found on the upper Doctor Creek, Cabresto Creek, Columbine Creek, Commanche Creek, and the Middle Ponil. In the Pecos drainage, Panchuela Creek, Cave Creek, and the lower Doctor Creek site, and Columbine Creek in the Rio Grande drainage had reproducing brown trout populations above barriers. The Costilla Creek sites were on the Vermejo Park Ranch in the Rio Grande drainage. These sites were established in conjunction with a RGCT re-introduction effort in

Table 5.1. Headwater streams surveyed in 2002 and 2003 for occurrence of salmonid species.

River	Location	Watershed	Species
Bluewater Creek	Cebola National Forest	Rio Puerco	Rainbow trout
Cabresto River	Carson National Forest	Rio Grande	Rio Grande cutthroat, brown, brook, and rainbow trout
Cave Creek	Santa Fe National Forest	Pecos	Brown trout
Cebolla Creek	Santa Fe National Forest	Guadalupe	Rio Grande cutthroat trout – above Brown trout - below
Columbine Creek	Carson National Forest	Rio Grande	Rio Grande cutthroat, brown trout
Commanche Creek	Carson National Forest	Rio Grande	Rio Grande cutthroat trout
Costilla Creek	Vermejo Park Ranch	Rio Grande	Rio Grand Cutthroat, Rio Grande cutthroat x rainbow, rainbow, and brook trout
Cow Creek	Santa Fe National Forest	Pecos	No fish
Doctor Creek	Santa Fe National Forest	Pecos	Rio Grande cutthroat trout – above Brown trout - below
Elk Creek	Santa Fe National Forest	Pecos	No fish
Jacks Creek	Santa Fe National Forest	Pecos	Rio Grande cutthroat trout
McCrystal Creek	Carson National Forest	Canadian	No fish
Middle Ponil Creek	Carson National Forest	Canadian	Rio Grande cutthroat trout (Hybrids?)
Pancuella Creek	Santa Fe National Forest	Pecos	brown trout
San Antonio River	Santa Fe National Forest	Jemez	brown trout

2002. These sites had reproducing populations of brook trout, rainbow trout, rainbow x RGCT hybrids, and RGCT (personal communication C. Kruse, Turner Endangered Fund).

None of the fish sampled throughout the surveys exhibited clinical signs of whirling disease. All survey mortalities (n = 20) were genetically tested for confirmation of *M. cerebralis* infection using molecular PCR markers developed by Andre et al. (1998) and no infection was detected in any of these fish.

Discussion

Of the 24 sites sampled, only than half supported populations of *T. tubifex*. The absence of *T. tubifex* at many sites may reflect the dynamic nature of headwater streams in arid land systems. Headwater streams in arid lands typically are driven by spring snowmelt events which can result in a 100-fold increase over mid-summer base flow. Highly variable flow regimes directly affect benthic community structure by altering suitable habitat through changes in water velocity (Anderson 1982; Holden et al. 1980). High flows tend to increase the turbidity and scour the substrate which displace segments of the benthic community including *T. tubifex (*Radford and Hartland-Rowe 1971). The absence of *T. tubifex* at many sites may be attributed in part to physical displacement of small populations due to increased velocity during seasonal high flow events and extirpation events due to drought. Summer base flow in many of these headwaters were affected by drought conditions at the time of this survey. Many of the streams scheduled to be sampled in 2002 and 2003 had no base flow with little suitable aquatic habitat for *T.*

tubifex. Systems experiencing these conditions may have been recently repopulated with founder events, thus, may also exhibit low *T. tubifex* population densities.

Population densities exhibited were generally low (17 to 1600 *T. tubifex*/m^2). The size of the water body is often positively correlated to depth and *T. tubifex* density has been shown to increase with depth (Robbins et al. 1989). *Tubifex tubifex* population densities exhibited in larger water bodies are typically higher than those exhibited in this study (Table 5.2). However, the *T. tubifex* densities exhibited in this study were consistent with peak population densities within a smaller water system on the Cache la Poudre River, Colorado, which exhibited *M. cerebralis* infection (50 oligochaetes/m^2) with up to 50 percent *T. tubifex* (Allen and Bergersen 2002).

This study revealed that increases in *T. tubifex* density were positively correlated with siltation. These findings are similar to other studies showing an increase in tubificid densities with sediment accumulation (Robbins et al. 1989; Zendt and Bergersen 2000; Peralta et al. 2002). Sediments tend to accumulate in deeper waters, thus, increasing optimum habitat for the species (Robbins et al. 1989). There was an expectation that *T. tubifex* occurrence and density would highly correlate with organic matter in sediments (Robbins et al. 1989; Peralta et al. 2002; DuBey and Caldwell 2004). However, a very weak positive correlation ($R = 0.060$, $P = 0.785$) between organic matter and *T. tubifex* density was exhibited at headwater sites.

In contrast to *T. tubifex* lineage distributions seen in larger water systems, only two *T. tubifex* lineages (III and VI) were identified among the headwater sites with lineage VI sympatric with lineage III at only three sites. *Tubifex tubifex* collected at

Table 5.2. Tubificid population levels exhibited in large water bodies.

Site	Species	Individuals/m^2	Author
Vistula River, Poland	*T. tubifex*	300 to 2,370	Dumnica (2002)
Crater lakes, Mexico	*T. tubifex*	349 to 1242	Peralta et al. (2002)
Lake Erie	Tubificids (*T. tubifex*, *Limnodrillus hoffmeisteri*, *Quistradrilus multisetosus*)	6,600 to 55,300	Robbins et al. (1989)
Colorado River, Colorado	*T. tubifex*	6,582 to 6,698	Zendt and Bergersen (2000)
San Juan River, New Mexico	Tubificids (tubificids with chaetae consistent with *T. tubifex*)	57 to 123,029	DuBey and Caldwell (2004)

M. cerebralis infected sites on the Colorado River, Colorado, exhibited resistant lineages V and VI and susceptible lineages I and III (Beauchamp et al. 2002). Furthermore, in the Navajo Dam tailwater of the San Juan River, New Mexico exhibited susceptible *T. tubifex* lineage I and III and the resistant lineage VI (DuBey and Caldwell 2004).

Data on lineage composition of natural populations of *T. tubifex* are limited. Thus, it is difficult to infer that populations with either a single lineage (III) or sites with sympatric lineages (III and VI) as exhibited in the headwater sites and many of the San Juan River sites are the norm for arid systems. However, headwater systems in arid lands often experience harsh scouring and dewatering conditions which can lead to the expatriation of *T. tubifex* populations. Subsequent repopulation of these waters via founder events may explain the predominance of single maternal lineage populations exhibited in this survey.

Although the novel pathogen hypothesis predicts limited host resistance when pathogens are spread into areas where the hosts have little or no defense history against the pathogen (Grenfell and Gulland 1995), host resistance was not observed in this study. *Myxobolus cerebralis* was not detected among *T. tubifex* or from fish collected during the headwater surveys. The parasite may not be able to establish itself at the low densities exhibited by both *T. tubifex* and fish at a majority of headwater sites. However, nearly all of the age-0 trout were infected with *M. cerebralis* in the Cache la Poudre River, Colorado which exhibited *T. tubifex* densities comparable to those observed here (Allen and Bergersen 2002). These authors

hypothesized, if introduced, the parasite can persist and cause reduced juvenile trout recruitment in cold, oligotrophic, sediment poor, high-gradient streams. Thus, the potential for establishment of *M. cerebralis* in New Mexico headwater stream exists where both hosts co-occur and habitat conditions are favorable.

APPENDICES

Appendix A. Starch gel electrophoresis system recipes for electrode and gel buffers used in genetic screening of *Tubifex tubifex*.

System Number	Buffer	Electrode Components per liter	Gel Components per liter
2	Tris-citrate - Lithium Hydroxide, pH 8.1	Tris 5.58 g LiOH 0.1 g Citric acid 1.44 g Boric acid 1.19 g	Tris 6.2 g Citric acid 1.6 g pH 8.4
3	Discontinuous Tris-citrate, pH 8.2	Boric acid 18.55 g Sodium Hydroxide 2.40 g	Tris 9.21 g Citric acid 1.05 g pH 8.7
4	Continuous Tris citrate, pH 6.3	Tris 27 g Citric acid 18.07 g	Tris 0.97 g Citric acid 0.63 g pH 6.3
5	Continuous Tris citrate, pH 8.0	Tris 83.2 g Citric acid 30 g	Tris 2.77 g Citric acid 1.1 g pH 8.0
6	Tris-versene-borate pH 8.0	Tris 60.6 g EDTA 6.0 g Boric acid 40.0 g	Tris 6.06 g EDTA 0.6 g Boric acid 4.0 g pH 8.0
9	Tris-maleate pH 7.4	Tris 12.1 g EDTA 3.72 g Maleic acid 11.6 g Magnesium chloride 2.03 g	Tris 1.21 g EDTA 0.37 g Maleic acid 11.6 g Magnesium chloride 0.203 g pH 7.4
10	Tris-citrate pH 7.0	Tris 4.55 g Citric acid 2.625 g	Tris 1.511 g Citric acid 0.875 g pH 7.0
12	Citric acid-morphaline pH 6.1	Citric acid 8.4 g Tritrate N-(3-aminopropyl) morpholine to pH 6.1(~19.0 ml)	Citric acid 0.42 g Tritrate N-(3-aminopropyl) morpholine to pH 6.1(~0.4 ml)

Appendix B. New Mexico headwater streams sampled for *Tubifex tubifex*, site location (UTM, NAD83 and elevation (m), and *T. tubifex* density (#/m2) and lineages found.

River/ location/ watershed	East UTM(m)	North UTM(m)	Elevation (m)	*T. tubifex* density #/m2	*T. tubifex* lineage
Bluewater Creek, Cebola NF, Rio Puerco	760331	3902161	2287	58	III
Cabresto River, Carson, Rio Grande	455984	4065466	2610	0	
Cave Creek, Santa Fe NF, Pecos	437054	3967477	2699	1600	III
Cebolla Creek, upper & lower, Santa Fe NF, Jemez	349192 348233	3980143 3978127	2469 2451	11 0	III, VI
Columbine Creek, Carson, Rio Grande	453894	4057724	2678	0	
Commanche Creek, Carson, Rio Grande	471979	4076017	2727	0	
Costilla Creek, upper & lower, Vermejo Ranch, Canadian	478057 477608	4093739 4090318	3124 3022	0 8	III
Cow Creek, Santa Fe NF, Pecos	447904	3958732	3135	0	
Doctor, upper & lower, Santa Fe NF, Pecos	436489 436635	3958397 3958428	2433 2498	0 0	
E. F. Jemez River, Santa Fe NF, Jemez	436471	3958478	2511	187	III, VI
Elk Creek, Santa Fe NF, Pecos	447569	3953973	2922	0	
Jacks Creek, , Santa Fe NF, Pecos	440524	3965300	2559	153	III
Jemez River, Santa Fe NF, Jemez	350860	3965980	2070	51	III
McCrystal Creek, Carson, Rio Grande	489006	4070640	2485	0	
Middle Ponil, Carson, Rio Grande	480393	4070565	2958	0	
Pancuella Creek, Santa Fe NF, Pecos,	438700	3967463	2696	170	III
Pecos/Dalton Day Use/Pecos river	438123	3945419	2997	0	
Puente Blanco, Santa Fe NF, Jemez	356468	3956977	2433	187	III, VI
Rio de las Palomas, Santa Fe NF, Guadalupe	338937	3990548	2759	17	III
Rio de las Vacas, Santa Fe NF, Guadalupe	335707	3987642	2766	34	III
San Antonio River, Santa Fe, Jemez	351127	3973059	2403	17	III

Appendix C. Physical and biochemical parameters headwater stream study sites sampled for *Tubifex tubifex*.

River/Watershed	BOD_5 (DO mg/l)	Organic Matter (%)	Temperature °C	Silt (%)	Gradient (%)
Bluewater Creek, Rio Puerco	1.2	2.75	10.9	20	5
Cabresto River, Rio Grande	0.8	1.1	13.4	8.3	7
Cave Creek, Pecos	1.32	0.89	11.9	10	3.5
Cebolla Creek, upper, Jemez	1.0	0.72	16.7	10	3.5
Cebolla Creek, lower, Jemez	0.1	0.45	20.9	6	2.5
Columbine Creek, Rio Grande	0.5	0.42	9.2	5	8
Commanche Creek, Rio Grande	0.7	0.73	12.1	7	2
Costilla Creek, upper, Canadian	0.65	1.21	17	6	3
Costilla Creek, upper, Canadian	0.85	0.85	15.5	10	3
Cow Creek, Pecos	0.58	0.32	10.4	6	6
Doctor Creek, upper, Pecos	0.3	0.58	12	4	7
Doctor Creek, lower, Pecos	0.5	0.43	11.9	10	7
E. F. Jemez River, Jemez	0.33	1.05	15.5	25	3
Elk Creek, Pecos	0.37	0.68	17.3	6	6
Jacks Creek, Pecos	0.80	1.28	13.0	24	6
Jemez River, Jemez	0.33	0.74	15.5	12	6
McCrystal Creek, Rio Grande	0.51	0.66	17.6	3	4
Middle Ponil, Rio Grande	0.43	0.32	15.9	18	6
Pancuella Creek, Pecos	1.43	0.44	13.1	32	5
Pecos, Dalton Day Use, Pecos	3.35	2.4	16.8	28	3
Puente Blanco, Jemez	0.94	1.05	10.8	23	3
Rio de las Palomas, Guadalupe	0.44	2.76	15.5	5	6
Rio de las Vacas, Guadalupe	0.57	0.58	9.7	18	4
San Antonio River, Jemez	0.364	1.91	12.8	10	5

Appendix D. Water quality parameters at headwater stream study sites sampled for *Tubifex tubifex.*

River/Watershed	Dissolved Oxygen (mg/l)	pH	Conductivity (µohms)	Nitrite (mg/l)	Sulfate (mg/l)	Turbidity (NTU)
Bluewater Creek, Rio Puerco	6.34	8.78	561	1.8	27	1.5
Cabresto River, Rio Grande	6.32	8.65	183	1.4	18	3.2
Cave Creek, Pecos	9.2	8.53	94	1.9	17	0.9
Cebolla Creek, upper, Jemez	6.4	7.85	99	1.2	0	0.9
Cebolla Creek, lower, Jemez	6.7	7.77	100	1.2	1	1.6
Columbine Creek, Rio Grande	7.55	9.2	159	0.9	7	0.7
Commanche Creek, Rio Grande	5.9	9.43	160	1.1	14	1.0
Costilla Creek, upper, Canadian	7.6	8.22	160	0.7	8	1.0
Costilla Creek, upper, Canadian	7.9	8.2	150	1.3	11	0.6
Cow Creek, Pecos	7.28	8.9	242	0.8	0	0.8
Doctor Creek, upper, Pecos	7.05	9.08	76	0.7	14	0.4
Doctor Creek, lower, Pecos	6.3	8.53	103	0.9	15	0.7
E. F. Jemez River, Jemez	7.48	8.1	143	0.9	8	6.2
Elk Creek, Pecos	5.96	9.07	233	0.7	0	0.8
Jacks Creek, Pecos	7.7	8.62	300	1	22	0.5
Jemez River, Jemez	7.6	8.43	157	1.2	15	3.8
McCrystal Creek, Rio Grande	6.15	9.03	136	0.8	0	0.7
Middle Ponil, Rio Grande	5.98	8.76	402	0.9	11	1.1
Pancuella Creek, Pecos	6.8	9.45	158	1.1	9	0.9
Pecos, Dalton Day Use, Pecos	7.14	8.42	261	1.1	17	4.9
Puente Blanco, Jemez	6.84	8.07	103	0.9	0	6.0
Rio de las Palomas, Guadalupe	7.9	8.7	308	1	0	0.4
Rio de las Vacas, Guadalupe	7.26	8.9	70	1.1	0	1.1
San Antonio River, Jemez	6.76	8.6	137	1	12	5.8

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