Effects of Temperature, Photoperiod, and Myxobolus cerebralis Infection on Growth, Reproduction, and Survival of Tubifex tubifex Lineages

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Abstract.—Tubifex tubifex is the definitive host for Myxobolus cerebralis, the causative agent of whirling disease in salmonid fish. Several mitochondrial lineages of T. tubifex exhibit resistance to M. cerebralis infection. Release of the triactinomyxon form of the parasite from T. tubifex varies with water temperature; however, little is known about the interactive effects of temperature and photoperiod on the susceptibility of T. tubifex lineages to M. cerebralis infection. In addition, the environmental effects on the growth, reproduction, and survival of T. tubifex lineages are unknown. Monocultures of lineages III and VI were subjected to infection (0 and 500 myxospores per worm), a range of temperatures (5, 17, and 27°C), and various diurnal photoperiods (12:12, 14:10, and 16:8 dark : light) over a 70-d period by using a split–split plot experimental design. Lineage VI resisted infection by M. cerebralis at all temperatures, whereas lineage III exhibited infection levels of 4.3% at 5°C, 3.3% at 17°C, and 0% at 27°C. Lineage VI exhibited significantly higher adult survival, weighed more initially, gained more weight, and had higher natality (production of immature tubificids) than did lineage III regardless of temperature, photoperiod, or infection treatment. There was no detectable effect of lineage, infection, or photoperiod on cysting. Both lineages III and VI cysted at 5°C but not at 17°C or 27°C. Competition between lineage VI and other lineages for resources may serve to decrease the overall infection levels among T. tubifex populations, thereby reducing both triactinomyxon production and the occurrence of whirling disease among susceptible salmonids.

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; Whipp et al. 2004). The devastating effects of whirling disease on wild salmonid populations were not fully realized until its discovery in the intermountain west in association 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 exhibiting 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 dif-
ferences 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. Those 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 responses presumably attributable 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, 15, and 20°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, whereas nonviable degenerating *M. cerebralis* spores were detected at 30°C (El-Matbouli et al. 1999). 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 our research was to characterize the responses of two lineages of *T. tubifex* found in the San Juan River tailwater (DuBey and Caldwell 2004) to a range of photoperiod and thermal regimes and determine whether infection by *M. cerebralis* would affect growth and survival of lineages.

**Methods**

*Tubifex tubifex* are pathogenic (Podubnaya 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 with a polymerase chain reaction (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 defleshed by heating at 45°C, and flesh and noncartilaginous material were manually removed. The cartilaginous material was pulverized and centrifuged to concentrate spores into a pellet. The pellet was resuspended and spores were quantified by microscopic examination and counting 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 by using a split–split plot experimental design. Each aquarium was divided in half with clear Plexiglas. 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; the nonchallenged *T. tubifex* tubs were placed in the other half to ensure independence of the treatment and control groups.

A range of temperatures (5, 17, and 27°C) were selected to represent the optimum temperature at which *T. tubifex* is commonly found. Each temperature treatment was applied to three aquaria at three separate locations. Treatment at 5 ± 1°C was conducted in a Living Stream (Frigid Units, To
ledo, Ohio) in a laboratory with a temperature-controlled refrigeration unit (model DI-500; Aquarium Systems, Mentor, Ohio). Treatment at 17 ± 1°C was conducted within the same laboratory on a bench top. Treatment at 27 ± 1°C was conducted in a walk-in Sheer environmental chamber having a UP 780 digital control system to maintain the desired temperature (Yokogama Corporation of America, Newton, Georgia). Chamber temperature was measured with a National Institute of Standards and Testing–certified sensor (Model MRHT3-2-1-D; General Instruments, Woburn, Massachusetts).

For each photoperiod treatment, fluorescent lamps provided approximately 50 lm/cm² at the water surface of each aquarium. The photoperiod treatment for two of three aquaria (at each location) was manipulated by placing darkened Plexiglas covers on top of each aquarium to achieve the desired dark: light exposure (14:10, 16:8). The aquarium exposed to 12 h 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 by the UP 780 digital control system in the environmental chamber. Water temperature (°C) and light intensity (lm) were measured continuously by Hobo recorders (model H8; Onset Computer Corp., Bourne, Massachusetts).

All tubificids were fed an aqueous solution of ground spirulina (0.5 mg dry weight of spirulina per tubificid) twice weekly. Temperature (°C) and dissolved oxygen (mg/L; Yellow Springs Instrument, Yellow Springs, Ohio) were monitored daily, and pH (pHPlus; LaMotte, Chestertown, Maryland), nitrite (NO₂⁻, mg/L), and ammonia (NH₃-N, mg/L), were monitored in tubs from all aquaria each week throughout all treatments for the experiment duration. Nitrate and ammonia were determined by the diazotization and salicylate methods, respectively (Model DR/2010 spectrophotometer; Hach Company, Loveland, Colorado). Water in tubs was changed weekly with aerated well water. The experiment was run for 70 d.

At 70-d postexposure, 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 by using a gridded sorting tray. The tubificids were placed in centrifuge tubes, frozen with liquid nitrogen, and stored at −70°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 noninfected treatments. The infection level for each treatment combination was computed by dividing the total number of infected worms by the total number of worms examined (lineage-specific) from each aquarium half.

The presence or absence of M. cerebralis infection was determined with a nested PCR amplification of 18S rRNA. The PCR methodology for M. cerebralis used DNA template from phenol extractions, 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 of 1× 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, the interaction between these two being used as the error term. The infected and noninfected 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 (α = 0.10) by using a comparisonwise rate of α = 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**

Because survival differed among individual tubs, the infection levels for treatment combinations are based on a 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, whereas no infection was detected in lineage VI (Table...
Polymerase chain reaction diagnostics detected no infection in *T. tubifex* of either lineage from the nonchallenged group. Under experimental conditions in this study, lineage VI exhibited detectably higher survival (*F* _sub_120 = 100.7; *P* < 0.0001) than lineage III (Table 2). Of the 72 tubs containing lineage III, 9 exhibited complete worm 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 α = 0.026 level (*F* _sub_2,4 = 7.83; *P* = 0.041), survival was consistently higher at 17°C. No effect of photoperiod (*F* _sub_2,4 = 0.69; *P* = 0.55) or infection (*F* _sub_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), which was significant (*F* _sub_1,106 = 25.47; *P* < 0.0001). Interaction between infection and lineage was detectable (*F* _sub_1,106 = 5.69; *P* = 0.019). Infected individuals of lineage III gained more weight than did the uninfected individuals. Lineage VI worms, however, had the same weight gain regardless of parasite challenge. Although temperature effects were not detectable in adult tubificids (*F* _sub_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* _sub_1,6 = 0.03; *P* = 0.87). The lack of a detectable exposure 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 (exposed: lineage III, 0.013 individuals/d; lineage VI, 0.026 individuals/d; unexposed: lineage III, 0.021 individuals/d; lineage VI, 0.034 individuals/d). Natality was greater in lineage VI than in lineage III (*F* _sub_1,106 = 19.32; *P* < 0.0001). The degrees of freedom in this error term were lower because 14 of the tubs had complete mortality and therefore were excluded from analysis. Although there was no effect on natality from photoperiod (*F* _sub_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; *F* _sub_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* _sub_1,1 = 1.24; *P* = 0.4662). However, there was a detectable effect of lineage (*F* _sub_1,43 = 21.35; *P* < 0.0001) on the mean weight of immature tubificids, lineage VI having higher mean weight (1.28 mg/worm, SE = 0.15) than lineage III (0.82 mg/worm, SE = 0.06).

Cysting was observed only at 5°C. It was seen in both lineages and was similar throughout all photoperiods (20–22 cysted worms). The cysting rate for all surviving adult tubificids was 3.9%. None of the cysted individuals was infected according to PCR screening.

**Discussion**

We found that lineage VI exhibited wider tolerance to experimental conditions than lineage III did, corresponding to earlier field observations of DuBey and Caldwell (2004). Lineage VI resisted
M. cerebralis infection, whereas lineage III individuals exhibited infection. Previous experimental exposure studies have 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). Our observed infection levels for lineage III (4.3% at 5°C, 3.3% at 17°C, and 0% at 27°C) were generally low compared with the wide range of infection levels in T. tubifex from West Virginia reported by Blazer et al. (2003). They reported infection levels from an experimental challenge dose of 350 myxospores per worm that ranged from 8.3% to 16.7% at 9°C, from 6.3% to 22.9% at 13°C, from 12.5% to 33.3% at 17°C, and 0% at 20°C. A wide range of infection levels was 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 1 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 by a dramatic decrease in abundance in one of two populations from different geographical origins when subjected to M. cerebralis challenges of 50, 500, and 1000 myxospores per worm (Stevens et al. 2001). Although these authors did not distinguish mitochondrial lineage differences among populations, 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°C and 12°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 statistically detectable in our study; however, survival was consistently higher at 17°C, which suggests 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 have been made among ecological lineages of T. tubifex (Anlauf 1994), T. tubifex forms (Podubnaya 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 the growth, reproduction, or survival of lineage III worms. However, infection had a negative effect on population growth rate (fecundity) in two of three geographical populations of T. tubifex with rates for the exposed population that ranged from approximately 0.001 to 0.009 individuals/d and for the unexposed population from 0.002 to 0.013 individuals/d (Kearns et al. 2004). In addition, both of our exposed and unexposed treatment groups exhibited higher population growth rates at 17°C. The lack of a detectable exposure effect on natality in our study reflected negative growth rates for both the 5°C and 27°C treatment groups.

Bonomi and DiCola (1980) observed that within same-age cohorts of T. tubifex, the faster-growing

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<td>5 v</td>
<td>0.5 (0.48; 12)</td>
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wors matured earlier, remained fecund longer, and produced more young. Thus, the observed reduction in growth, reproduction, and survival of lineage III adults (compared with lineage VI) may result from lineage differences. If the 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°C. Bonomi and DiCola (1980) observed a positive correlation of egg production in *T. tubifex* at temperatures ranging from 5°C to 20°C. Density was shown to affect natality in experimental cultures of *T. tubifex* (Bonomi and DiCola 1980). We did not manipulate density here; however, lineage VI in our study had higher survival rates of adults and produced more young per adult than did lineage III. This suggests that the differences in natality are attributable to lineage differences and not density dependence. We observed densities in tubs as high as 482 young (mean weight, 1.88 mg) and 21 adult worms (mean weight, 7.86 mg) within lineage VI, whereas the high densities of lineage III were 177 young (mean weight, 1.52 mg) and 17 adult worms (mean weight, 7.14 mg). These adult mean weights were higher than the means for field-collected worms, (mean weight, 1.52 mg) and 17 adult worms (mean weight, 7.14 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%; only in the 5°C treatment was cysting observed and 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. Survival strategy was demonstrated experimentally, the cysted *T. tubifex* surviving lower experimental temperatures than those that did not cyst (Anlauf 1990). 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). Because the tailwater exhibited a relatively constant temperature (7–9.5°C) through the seasons, 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 that the cysted ecological lineage exhibited higher weights and greater egg production than the other ecological lineages. Although lineage VI exhibited higher weights, greater weight gains, and greater egg production than lineage III, both lineages of *T. tubifex* cysted in our study. Additional work is needed to better understand the environmental pressures associated with cysting of *T. tubifex*.

In other susceptibility experiments, TAM production by tubificids was directly related to disease severity in juvenile rainbow trout (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. (1999) found that 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). Perhaps resistance of a *T. tubifex* lineage to infection may serve to mitigate the prevalence of *M. cerebralis* in infected systems. Competition between resistant *T. tubifex* and other lineages may reduce the incidence of infection by eliminating spores from the habitat that would otherwise be available to susceptible *T. tubifex*.

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