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Accelerated deactivation of *Myxobolus cerebralis* myxospores by susceptible and non-susceptible *Tubifex tubifex*

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ABSTRACT: In the 1990s, the *Tubifex tubifex* aquatic oligochaete species complex was parsed into 6 separate lineages differing in susceptibility to *Myxobolus cerebralis*, the myxozoan parasite that can cause whirling disease (WD). Lineage III *T. tubifex* oligochaetes are highly susceptible to *M. cerebralis* infection. Lineage I, IV, V and VI oligochaetes are highly resistant or refractory to infection and may function as biological filters by deactivating *M. cerebralis* myxospores. We designed a 2-phased laboratory experiment using triactinomyxon (TAM) production as the response variable to test that hypothesis. A separate study conducted concurrently demonstrated that *M. cerebralis* myxospores held in sand and water at temperatures ≤15°C degrade rapidly, becoming almost completely non-viable after 180 d. Those results provided the baseline to assess deactivation of *M. cerebralis* myxospores by replicates of mixed lineage (I, III, V and VI) and refractory lineage (V and VI) oligochaetes. TAM production was zero among 7 of 8 Lineage V and Lineage VI *T. tubifex* oligochaete groups exposed to 12 500 *M. cerebralis* myxospores for 15, 45, 90 and 135 d. Among 4 mixed lineage exposure groups, TAM production averaged 14 641 compared with 2 202 495 among 12 groups of Lineage III oligochaetes. Among the 6 unexposed Lineage III experimental groups seeded into original Phase 1 substrates for the 45, 90 and 135 d treatments during the Phase 2 portion of the study, TAM production was reduced by 98.9, 99.9 and 99.9%, respectively, compared with the average for the 15 d exposure groups. These results are congruent with the hypothesis that Lineage V and Lineage VI *T. tubifex* oligochaetes can deactivate and destroy *M. cerebralis* myxospores.

KEY WORDS: *Myxobolus cerebralis* · *Tubifex tubifex* · Myxospore deactivation · Biological filtering

INTRODUCTION

Whirling disease (WD) of salmonid fishes is caused by the myxozoan parasite known as *Myxobolus cerebralis* (Markiw & Wolf 1983). First observed in Germany in 1893 (Plehn 1905), the life cycle of *M. cerebralis* remained an enigma until the early 1980s, when it was shown to include 2 obligate hosts, with infection alternating between a salmonid fish such as rainbow trout *Oncorhynchus mykiss* and *Tubifex tubifex*, an aquatic oligochaete (Wolf & Markiw 1984). During the first 9 decades of the 20th century, the *M. cerebralis* parasite was primarily considered a threat for the cold water aquaculture industry

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(Bartholomew & Reno 2002) as there were no documented cases of population-level impacts in aquatic ecosystems where the parasite was enzootic among wild salmonids.

In the early 1990s, evidence of epizootic WD infections with population-level impacts among wild salmonids began to appear in the western US. The first case was on the upper Colorado River in 1993 and 1994, where it was shown that recruitment failure among wild rainbow trout had begun in 1991. By the fall of 1995, the loss of 4 successive year classes had resulted in a rapidly decreasing, senescent rainbow trout population, with few fish less than 30 cm in length (Nehring & Walker 1996). The wild rainbow trout population in a 3.2 km reach of the Gunnison River was estimated at 11 100 fish ≥15 cm in the late 1980s, before the *M. cerebralis* parasite became enzootic in 1993. After 10 consecutive years of complete loss of rainbow trout fry recruitment to WD infection, the wild rainbow trout population in 2003 was estimated to be 86 fish (Nehring 2006). Population-level impacts have been documented on more than 20 major trout streams in Colorado (Nehring & Thompson 2001, Nehring 2006), including wild rainbow trout, brook trout *Salvelinus fontinalis* and cutthroat trout *O. clarki* ssp. populations in both lakes and streams in wilderness areas at elevations ranging from 2500 m to more than 3700 m (Nehring 2010). Arising from melting snows at an elevation of more than 3500 m, the Middle Fork of the South Platte River supported sympatric populations of wild brook trout and brown trout in the early 1990s, prior to the time when the *M. cerebralis* parasite became enzootic. After establishment of the parasite, the biomass of juvenile and adult brook trout declined by 94 % between 1998 and 2002 (Nehring 2006). *M. cerebralis* has been enzootic in Lower Square Top Lake (LSTL) since the mid-1990s (Schisler 1999). Located at an elevation of 3677 m, 1 yr survival of cutthroat trout fry stocked into LSTL is ~1 % (Nehring 2010).

Similarly, WD infections have had devastating impacts on wild rainbow and cutthroat trout in other areas of North America. *M. cerebralis* infections were first documented in the Madison River in Montana in 1994, where precipitous declines in wild rainbow trout numbers had begun in 1991, resulting in more than a 90 % reduction in the wild rainbow trout population by the mid-1990s (Vincent 1996a,b). The wild rainbow trout population in Rock Creek in west–central Montana experienced a precipitous decline after the *M. cerebralis* parasite became enzootic in the late 1990s (Granath et al. 2007). Dramatic declines in the spawning runs of adfluvial Yellowstone cutthroat (YC) trout *O. clarki bouvieri* have been documented in Pelican Creek, Clear Creek and Bridge Creek draining into Yellowstone Lake in Yellowstone National Park since the presence of the *M. cerebralis* parasite was documented in the late-1990s (Koel et al. 2005, 2006, 2007, 2010, Gresswell 2011). The spawning run into Pelican Creek, which numbered nearly 30 000 fish in 1981, was non-existent by 2004. Prior to the detection of lake trout *Salvelinus namaycush* and the *M. cerebralis* parasite in the 1990s, the spawning run of YC trout in Clear Creek averaged almost 44 000 fish over a 15 yr period from 1977 to 1992 (Gresswell et al. 1994) and peaked at more than 70 000 fish in 1978 (Koel et al. 2005). By 2001–2004, the run averaged 3828 (Koel et al. 2005) and it declined to 471 in 2006, a 99 % reduction from the 15 yr historical average and the lowest in 60 yr of record (Koel et al. 2007). Similarly, the spawning run of YC trout in Bridge Creek dropped from 2363 fish to 1 fish in the 6 yr period from 1999 to 2004 (Koel et al. 2005). While some investigators hypothesize that predation by non-native lake trout is the primary factor in the decline of the YC trout population in Yellowstone Lake (Williams et al. 2014), other investigators consider WD a major contributor to the decline (Murcia et al. 2015).

Concerns over the environmental and ecological impacts of WD on wild salmonids across western North America led to wide-ranging research efforts directed at all aspects of the etiology and epidemiology of the *M. cerebralis* parasite that began in the mid-1990s. Potential impacts on the many subspecies of native cutthroat were of special concern. These research efforts were focused on both aspects of the parasite life cycle. Application of polymerase chain reaction (PCR) technology (Andree et al. 1998, Baldwin & Myklebust 2002, Cavender et al. 2004) molecular biology (Sturmbauer et al. 1999, Beauchamp et al. 2001) and other state-of-the-art analytical techniques led to rapid advances and a deeper understanding of the etiology of *M. cerebralis* in the aquatic oligochaete host *T. tubifex* (Gilbert & Granath 2001, Beauchamp et al. 2002, 2005, 2006, Nehring et al. 2013, 2014, 2015). Beauchamp et al. (2001) developed mitochondrial 16S ribosomal DNA markers to parse the *T. tubifex* species-complex into 6 distinct lineages. Subsequent studies demonstrated that the Lineage III *T. tubifex* worms are highly receptive to *M. cerebralis* infection and prodigious producers of the fish-infective triactinomyxon (TAM) actinospores even at a low dose rate of 50 *M. cerebralis* myxospores worm^{-1} (Nehring et al. 2014). Lin-

eage I *T. tubifex* can be infected but only produce TAMs when exposed to dose rates ≥1000 myxospores worm^{-1} (see Table 6 in Nehring et al. 2014). Numerous studies have repeatedly shown *T. tubifex* Lineages IV, V, and VI from all across western North America to be refractory to *M. cerebralis* infection (DuBey & Caldwell 2004, DuBey et al. 2005, Arsan et al. 2007, Baxa & Hedrick 2008, Hallett et al. 2009, Zielinski et al. 2011, Nehring et al. 2013, 2014). Lineage II tubificids, originally described as a European species (Sturmbauer et al. 1999, Beauchamp et al. 2001), to our knowledge have never been collected in North America or tested for susceptibility to the *M. cerebralis* parasite.

Given that *T. tubifex* oligochaetes belonging to Lineages I, IV, V and VI have been shown to be highly resistant or refractory to infection by the *M. cerebralis* parasite, many investigators have wondered whether or not these worms might be capable of consuming and deactivating *M. cerebralis* myxospores. Deactivation of myxospores in the natural environment by these tubificids could reduce or eliminate *M. cerebralis* infections where they were the dominant oligochaete (Beauchamp et al. 2002, 2005, 2006, Kerans et al. 2004, Baxa et al. 2008, Nehring et al. 2013). The objective of our study was to answer that question.

MATERIALS AND METHODS

Source(s) of experimental oligochaetes

The Mt. Whitney Lineage III worms used in the study were originally obtained from collaborators at the University of California, Davis, and subsequently maintained, cultured and used as our laboratory controls for comparative purposes in this study and previous research investigations (Nehring et al. 2013, 2014, 2015). All of the other *Tubifex tubifex* exposure groups were collected from 4 streams and 1 reservoir in Colorado that had been the subject of previous research efforts (Nehring et al. 2013, 2014, 2015). Progeny from each of these collections were maintained and held in 13 l aquaria supplied with aerated, dechlorinated tap water for 1 yr or longer in order to acquire the number of experimental worms needed for this study. A minimum of 100 worms from each exposure group were screened by quantitative PCR (qPCR) assay at the beginning of the experiment to determine the lineage composition and demonstrate that they were not infected with the *Myxobolus cerebralis* parasite prior to experimental exposure. Worms were fed ad libitum with a formulation of 0.2 g of dehydrated Spirulina discs, Tetramin™ tropical fish granules and ALGAMAC 2000™ in a ratio of 6:3:1 by weight, which was ground to a fine powder with a small commercial coffee bean grinder.

Study design

To determine whether non-susceptible lineages (I, V and VI) of *T. tubifex* deactivate *M. cerebralis* myxospores, a 2-phase experimental design was employed. For Phase 1 (P1), 4 groups of 250 worms from 6 separate source populations were placed in 1 l containers with 240 g of sterilized white arena sand (≤1 mm grain size) and 750 ml of aerated, dechlorinated tap water, at an approximate density of 34 600 oligochaetes m^{-2}. This density is at the lower end of the range observed among *T. tubifex* oligochaete populations observed in organically rich aquatic habitats in Colorado (Nehring et al. 2013). The *M. cerebralis*-susceptible (Lineage III) populations were from Mt. Whitney, California, and Parachute Creek and Cross Creek in Colorado. The mixed lineage (I, III, V and VI) population came from the Eagle River in Colorado, and the non-susceptible lineage (V and VI) worms came from the Williams Fork River and Windy Gap Reservoir in Colorado. The worms were fed weekly with the ration described above. Diurnal water temperatures fluctuated by 1–2°C. Water temperatures ranged from 5 to 15°C during the 9 mo long test period. Each P1 container was inoculated with an estimated 12 500 *M. cerebralis* myxospores, a dosage of 50 myxospores oligochaete^{-1}. The myxospores were harvested from *M. cerebralis*-infected rainbow trout held at the Fish Health Research Laboratory at the University of California, Davis. The spores were separated from macerated fish tissues and concentrated by plankton centrifuge (O'Grodnick 1975), stored under refrigeration at 4°C, enumerated by microscope in a hemocytometer chamber to determine the spore density (n µl^{-1}), refrigerated in an aqueous solution and shipped overnight to Colorado. Each exposure container was inoculated with the appropriate volume of myxospores using a calibrated micropipette.

The 4 prescribed time treatments for the P1 exposure periods were 15, 45, 90 and 135 d, after which the worms were transferred to new containers filled with clean, sterilized white arena sand and aerated, dechlorinated tap water that had not been inoculated with myxospores. Weekly estimation of TAM production continued for the remainder of the experiment.

At the end of each prescribed time treatment during the P1 exposure and at the termination of the experiment, all of the worms were separated from the sand substrate in the following manner. The contents of each exposure container were placed in a clean, white porcelain dissection pan. All worms were separated from the substrate using disposable pipettes with a 1 mm diameter bore and placed into a glass petri dish and rinsed with dechlorinated tap water after which the worms were transferred to new containers for the duration of the experiment. The original arena sand from each P1 container, together with all dechlorinated rinse water from the porcelain dissection pan and the glass petri dish, were returned to the original P1 container to insure that no remaining myxospores were lost during the worm separation process. The blood red color of the worms viewed against the white arena sand substrate and white porcelain dissection pan made it easy to separate all tubificid worms (no matter how small) using disposable pipettes. There were 24 containers of 250 worms for each of the 2 phases of the experiment.

After removal of all of the exposed worms from the containers at the end of the initial prescribed exposure period for the P1 phase of the experiment, the second (P2) phase of the experiment commenced. Two hundred and fifty unexposed Lineage III worms from Parachute Creek were stocked into the P1 containers, still containing all of the sand/substrate and *M. cerebralis* myxospores remaining from the original exposure but none of the original *T. tubifex* worms. These containers were then referred to as the P2 exposures.

Monitoring for initiation of TAM release began in all containers at 60 d post-exposure (PE). Initiation of TAM release began 75–90 d PE. TAM production was estimated once each week in all replicates for 120–150 d, or until the weekly TAM release was less than 0.5% of the total TAM production for that container. Total TAM production was determined by summing the weekly estimates of TAM production for each container. This study was completed over a 9 mo period. At the termination of the experiment the large worms in each container were counted. Numbers of small juvenile worms produced during the experimental exposure were estimated by doing 3 replicate counts of 1000 juvenile worms, obtaining 3 wet weights (mg), and then getting a total wet weight for all juvenile worms from each container.

Estimates of TAMs produced in this experiment were also compared to those collected in a separate experiment conducted concurrently (Nehring et al. 2015) to account for any degradation of myxospores over time that might confound the results of this study. In that experiment, 2 replicate containers of 12 500 *M. cerebralis* myxospores were held in identical conditions for 0, 15, 30, 45, 90, 120 and 180 d prior to being exposed to 250 Lineage III *T. tubifex* worms. The TAM numbers produced from these 14 time-delayed replicate exposures were evaluated along with P2 exposures from this experiment to provide a more direct assessment of TAM production when considering age of myxospores with and without time-delays prior to exposure to *T. tubifex*.

TAM filtration and quantification

Except for minor modifications, we used the protocol of Thompson & Nehring (2000) to estimate the weekly TAM production for each replicate. The air supply to each replicate was turned off for a minimum of 1 h prior to collection of the filtrate to avoid loss of myxospores. This allowed any myxospores that might have been suspended in the water column to settle onto the sand substrate. Approximately 95% of the water from each container was gently decanted off and passively filtered through a form-fitted, cone-shaped, 20 µm mesh screen with a top width of 100 mm that was inserted into a perforated plastic funnel. After filtration was complete, the cone-shaped screen was removed, inverted, and the material retained by the screen was back-washed through a funnel and collected in the sample collection jar. Filtrate volumes generally ranged from 15 to 50 ml. Five 2 ml aliquots were withdrawn by micropipette and placed into 10 ml scintillation tubes and stained with 60 µl of saturated aqueous crystal violet stain. A single 160 µl sub-sample was drawn by micropipette from each scintillation tube, placed onto a gridded petri dish, covered with a disposable coverslip and scanned by stereo-zoom microscopy for TAMs, with a magnification range of 20 to100×.

In previous studies (Thompson & Nehring 2000, Nehring et al. 2003, 2014) it was determined that high densities of TAMs in a filtrate often leads to clumping and entanglement of the TAMs which could result in an overestimation of the true density and total number of TAMS produced. To avoid this problem in these situations, the concentrated filtrate was diluted with distilled water to a volume that would result in less than 100 TAMs being enumerated in a single 160 µl sub-sample. This eliminated this potential source of error in the TAM enumeration and quantification process. In previous studies (Nehring et al. 2014) it was sometimes necessary to

dilute a single filtrate with 20 l of distilled water to keep the number of TAMs observed in a single 160 µl sub-sample ≤100.

Statistical methods

The statistical analyses were performed with Proc GLMSELECT in SAS® system software (SAS® Institute 2013). TAMs produced, the response variable in the study, were natural log transformed for this analysis to linearize the data and reduce heteroscedasticity. A set of models was fit to determine which of the variables had the greatest effects on TAM production in P2 of the trials. Variables tested included pre-exposure of the substrate containing myxospores to *T. tubifex* oligochaetes during the P1 treatments, lineage of the worms used in the pre-exposure, source of the worms used in the pre-exposure, age of myxospores in days, and interaction terms of these factors. Models were ranked using Akaike's information criterion corrected for small sample sizes (AICc) to choose the best-fitting model without over-parameterization from among the candidates (Burnham & Anderson 2002).

RESULTS

The results of the statistical analyses are summarized in Table 1. Although not a part of this investigation, the age of *Myxobolus cerebralis* myxospores prior to initial exposure to Lineage III *Tubifex tubifex* worms has a significant negative effect on TAMs produced (Nehring et al. 2015). The myxospore-aging study was run concurrently with this experiment under identical conditions and provides the baseline for comparison of the results presented in this paper. A model with both time after exposure ($p < 0.001$), and pre-exposure of sediment containing the myxospores to *T. tubifex* ($p < 0.05$), along with an interaction effect of the 2 parameters ($p < 0.001$), was the most parsimonious model (Table 1). This model (Table 2), with an adjusted R^2 of 0.66, clearly demonstrates the significant decrease in the total number of TAMs subsequently produced by naïve Lineage III *T. tubifex* introduced during the P2 phase of the study compared to the TAM production in treatment groups with no pre-exposure, with the difference becoming greater with time (Fig. 1). Pre-exposure to both susceptible and non-susceptible lineages of *T. tubifex* affected the production of TAMs among all the naïve *T. tubifex* Lineage III (P2) treatment groups similarly. Lineage and source of these *T. tubifex* did not have a strong enough effect to be included in the final model, indicating that any *T. tubifex* type will have the effect of reducing available *M. cerebralis* myxospores for later consumption by new naïve Lineage III *T. tubifex* oligochaetes. These results substantiate the hypothesis that resistant lineages of *T. tubifex* can deactivate *M. cerebralis* myxospores.

TAM production among the various containers and treatments are summarized in Table 3. These data indicate that 15 d exposure during P1 was an inadequate period of time for any of the exposed worms to consume a substantial portion of the myxospores. The estimated average total TAM production among the unexposed Lineage III worms seeded into the original substrates during P2 was 814 722, ranging from 129 630 to 2 809 274 TAMs compared with an estimated average total TAM production of 381 693 among the 6 P1 15 d exposure groups. These results indicated that many myxospores remained in the

Table 1. Model parameters and corrected Akaike's information criterion (AICc) results for candidate models (N = 38 for all models). Means square error (MSE), number of parameters including intercept (*K*), and adjusted R^2 values are also included. Age of myxospores in days was natural log transformed in all models shown

Model	MSE	*K*	R^2	AICc
Source	20.278	6	0.05	163.5656
Type	20.934	4	0.02	161.2209
Pre-exposure, Source	17.458	7	0.18	159.9018
Pre-exposure, Type	16.518	5	0.22	153.9191
Pre-exposure	15.450	2	0.27	146.6807
Source, Days	12.219	7	0.43	146.3431
Type, Days	13.339	5	0.37	145.7953
Days	13.671	2	0.36	142.0325
Pre-exposure, Source, Days	10.878	8	0.49	144.1447
Pre-exposure, Type, Days	10.320	6	0.51	137.9014
Days, Pre-exposure	9.753	3	0.54	130.6354
Days, Pre-exposure, Days × Pre-exposure	7.218	4	0.66	120.7615

Table 2. Parameter estimates for selected model. Days in model are log-transformed

Parameter	df	Estimate	SE	*t*	p
Intercept	1	23.109697	2.677702	8.63	<0.0001
No pre-exposure	1	−6.879007	3.174617	−2.17	0.0373
Days	1	−3.989610	0.658535	−6.06	<0.0001
Days × No pre-exposure	1	2.884410	0.791249	3.65	0.0009

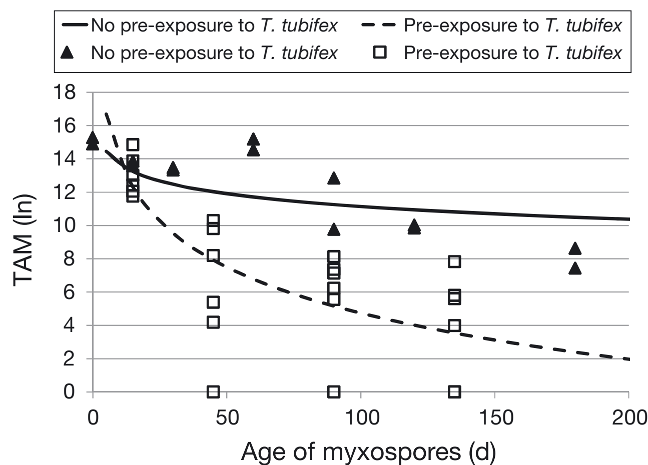

Fig. 1. Raw data points (solid triangles) and model results (solid trend line) represent triactinomyxon (TAM) production as a function of age of the myxospores at initial exposure to naïve *Tubifex tubifex*, demonstrating the rate of natural degradation of the myxospores with increasing time delay (Day 0 to 180 d) prior to worm exposure from the Nehring et al. (2015) study. The open squares (Phase 2 [P2] raw data points from Table 3) and model results (dashed trend line) indicate the TAM production among the 6 individual P2 treatment groups of Lineage III worms introduced at 15, 45, 90 and 135 d after the completion of the Phase 1 exposures

sand from the P1 15 d exposures after the worms were removed to be subsequently consumed by the newly introduced naïve Lineage III *T. tubifex* oligochaetes during the P2 exposure period (Table 3). However, unexposed pure Lineage III worms for P2 seeded into the original substrate(s) from the 45 d P1 exposure produced an average of 8600 TAMs, compared to an average of 814 722 TAMs produced by the Lineage III oligochaetes for the 15 d replicates, a reduction of 98.9%. Similarly, estimates of total TAM production during the P2 phase of the experiment among the unexposed Lineage III oligochaetes seeded into original P1 substrates for the 90 d and 135 d replicates were reduced by 99.8 and 99.9%, respectively, compared to the average for the 15 d replicates (Table 3). All of these results validate the theory that the highly resistant (Lineage I) and non-susceptible (Lineages V and VI) *T. tubifex* oligochaetes de-activate *M. cerebralis* myxospores. Among the 2 *M. cerebralis* myxospore replicates incubated in sand and water for 90 d prior to exposure to Lineage III *T. tubifex* oligochaetes in the myxospore-aging study (Nehring et al. 2015) TAM production was 17 385 and 378 838, averaging 198 112. In contrast, among the 6 P2 90 d containers of Parachute

Table 3. Estimated cumulative number of triactinomyxon (TAMs) produced by 250 *Tubifex tubifex* of various lineages from 6 sources exposed to 50 *Myxobolus cerebralis* myxospores at intervals of 15, 45, 90 and 135 d and then removed from the Phase 1 (P1) container and placed into a new container with sterile sand and water and no *M. cerebralis* myxospores. For Phase 2 (P2), 250 Lineage III *T. tubifex* oligochaetes not previously exposed to *M. cerebralis* myxospores were placed into the P1 containers with the original substrate (and any remaining myxospores) from which the P1 worms were removed. Water in all replicates was decanted off once weekly, filtered and concentrated through a 20 µm mesh screen, and TAM production was estimated

Worm source	Lineage(s)	Time of initial exposure (d)			
		15	45	90	135
P1 TAM production					
Mt. Whitney, CA	Pure III	930518	10276062	2625737	2268858
Parachute Creek, CO	Pure III	1250159	2935463	4316683	877347
Cross Creek, CO	Pure III	108391	69713	59061	711947
Eagle River, CO	I, III, V, VI	1092	34238	2231	21001
Williams Fork River, CO	Pure V	0	8439[a]	0	0
Windy Gap Reservoir, CO	Pure VI	0	0	0	0
Avg. pure III TAM production		763023	4427079	2333827	1286051
Avg. P1 TAM production		381693	2220653	1167285	646526
P2 TAM production					
Mt. Whitney/Parachute Crk	Pure III	2809274	0	3386	2514
Parachute Crk/Parachute Crk	Pure III	173594	66	260	54
Cross Creek/Parachute Crk	Pure III	452283	220	506	334
Eagle River/Parachute Crk	Pure III	1070609	18281	0	0
Williams Fork/Parachute Crk	Pure III	252942	29413	1264	0
Windy Gap/Parachute Crk	Pure III	129630	3623	1574	270
Avg. P2 TAM production		814722	8601	1165	529

[a]At the end of the experiment, quantitative PCR testing of 250 large adult worms and 50 juvenile worms in this container detected only Lineage V DNA in the samples tested. However, the Williams Fork River source population has had a very small number of Lineage III worms and occasionally produced low numbers of TAMs in previous exposure experiments (see Nehring et al. 2014)

Creek Lineage III *T. tubifex* worms, TAM production ranged from zero to 3386, and averaged 1165, a reduction of 99.4% (Table 3). Among the 3 P2 90 d mixed and refractory lineage treatment groups, TAM production ranged from zero to 1574, averaging 946 TAMs. If these worms were not de-activating *M. cerebralis* myxospores, then TAM production should have been similar to that observed in the 90 d myxospore-aging replicates (198112 TAMs) in the Nehring et al. (2015) study. Once again, these results are congruent with the hypothesis that the mixed lineage Eagle River exposure group containing Lineages I, III, V and VI and refractory Lineages V and VI *T. tubifex* worms from the Williams Fork River and Windy Gap Reservoir did de-activate *M. cerebralis* myxospores.

It is especially noteworthy that no TAM production was ever observed among 7 of 8 P1 containers for the Lineage V and Lineage VI treatment groups. The minimal amount of TAM production observed in the 45 d P1 containers of Williams Fork River Lineage V worms was due to a very small number of Lineage III worms (perhaps 1 to 3 among the 250 worms) introduced at the beginning of the experiment from the original culture. TAM production among all P2 Lineage III oligochaetes was significantly lower across all exposures for the 45, 90 and 135 d treatment groups, compared to the Mt. Whitney, Parachute Creek and Cross Creek pure Lineage III-P1 oligochaetes for the same time periods (Table 3). These results are additional evidence corroborating the hypothesis that the Lineages I, V and VI *T. tubifex* worms deactivate and destroy *M. cerebralis* myxospores.

At the termination of P1 and P2 of this experiment, a minimum of 100 large, adult worms and 50 juvenile worms from each container were euthanized and tested by qPCR to determine the approximate lineage composition of the exposed groups and also test for evidence of *M. cerebralis* DNA. Those screenings verified that the lineage composition in all treatment groups at the end of the experiment was in agreement with the same qPCR test results performed on the individual cultures prior to the start of the experiment. At the end of the experiment, we tested 5 aliquots of 50 adult worms and one 50 worm aliquot of juvenile worms from the 45 d P1 Williams Fork River Lineage V exposure group to see if Lineage III DNA could be detected, given that an estimated 8439 TAMs were produced (Table 3). Surprisingly, no Lineage III DNA was detected. However, there were more than 8000 worms estimated in this exposure group at the end of the experiment, and it would have been cost prohibitive to test them all by qPCR to determine what percentage of the DNA in the replicate was from Lineage III worms.

More worms were removed from the exposure containers at the end of the P1 and P2 phases in 43 of the 48 containers than the 250 worms per container at the start of the experiment.

The average number of worms among the 48 exposure containers was 4079 at the end of the experiment. The greatest number of worms in one container was estimated at more than 9400. The vast majority of these worms were small juvenile worms. The estimated number of worms observed among the unexposed negative control groups of the Parachute Creek and Mt. Whitney Lineage III containers 4709 and 6472, respectively. The low dose exposures of 50 myxospores worm^{-1} in our experiment did not negatively affect the fecundity and reproduction of any of the worms, as evidenced by the large increases in the number of worms over the 9 mo exposure period, even though the Lineage III worms were heavily infected by the *M. cerebralis* parasite. Other investigators have shown that higher dose rates and poorer nutrition can reduce fitness among *M. cerebralis*-susceptible tubificids, resulting in poor growth rates, lower fecundity and parasitic emasculation in some instances (Shirakashi & El-Matbouli 2009).

At the end of the experiment, qPCR screening for DNA of the *M. cerebralis* parasite was also completed on a minimum of two 50 worm aliquots of adult worms and one aliquot of 50 juvenile worms from each exposure container. In all instances, DNA of the *M. cerebralis* parasite was present in all aliquots of adult worms from containers with Lineage III worms from both the P1 and P2 phases of the experiment. No *M. cerebralis* DNA was detected in any aliquot of juvenile worms for any lineage. Likewise, no *M. cerebralis* DNA was detected in any of the 8 exposure groups of Williams Fork River (Lineage V) and Windy Gap Reservoir (Lineage VI) from the P1 phase of the experiment. Taken together these results suggest that all of the myxospores had been consumed by the 250 adult worms in each container within the first 60–90 d of exposure, prior to the onset of natural reproduction and hatching of the worm cocoons.

DISCUSSION

At the end of the 20th century, the development of DNA markers that parsed the *Tubifex tubifex* species-complex into 6 distinct lineages made it possible to test for differences in vulnerability to the *Myxobo-*

lus cerebralis parasite (Beauchamp et al. 2001). Subsequent investigations demonstrated that fewer *M. cerebralis* TAMs were produced by mixed cultures of *T. tubifex* oligochaetes that contained worms belonging to Lineages I, III, V and VI than pure cultures of the *M. cerebralis*-susceptible Lineage III worms (Beauchamp et al. 2005, 2006, Nehring et al. 2013, 2014). Lineage III *T. tubifex* worms are prodigious producers of *M. cerebralis* TAMs even at a dosage of 50 *M. cerebralis* myxospores worm^{-1} (Nehring et al. 2014). In contrast, studies all across western North America have repeatedly demonstrated that *T. tubifex* oligochaetes belonging to Lineages IV, V and VI are refractory to *M. cerebralis* infection (DuBey & Caldwell 2004, DuBey et al. 2005, Arsan et al. 2007, Baxa & Hedrick 2008, Hallett et al. 2009, Zielinski et al. 2011, Nehring et al. 2013, 2014, 2015). Likewise, *Limnodrilus hoffmeisteri* and *Ilyodrilus templetoni* are also refractory to the *M. cerebralis* parasite (Kerans et al. 2004). These 2 species of tubificids are closely related to *T. tubifex* and often occur in sympatry with them in microhabitats laden with organically rich fine sediments in both lakes and streams.

Some investigators have suggested that tubificids that are not susceptible to infection by the *M. cerebralis* parasite might be capable of deactivating *M. cerebralis* myxospores in the aquatic environment (Kerans et al. 2004, Beauchamp et al. 2005, 2006, Baxa et al. 2008, Nehring et al. 2013). However, none of those studies were designed to directly address that possibility. The outcome of our investigation indicates that *T. tubifex* oligochaetes belonging to Lineages V and VI do deactivate and destroy *M. cerebralis* myxospores. It is very likely that Lineage I *T. tubifex* worms do as well when the level of exposure is low or moderate, i.e. ≤250 myxospores worm^{-1} (Kerans et al. 2005, Nehring et al. 2014). However, because no replicates of pure Lineage I oligochaetes were tested in the study, some uncertainty with respect to that lineage remains. The Eagle River treatment group (Table 3) was a mixed culture of *T. tubifex* oligochaetes belonging to Lineages I, III, V and VI. Lineage I worms from the Gallatin River in Montana, did produce a few TAMs when the dosage was 1000 myxospores worm^{-1} (Kerans et al. 2004). Likewise, 77 % of Lineage I oligochaetes from Windy Gap Reservoir, Colorado, that were exposed to 6000 myxospores worm^{-1} did release some TAMs (Beauchamp et al. 2002).

During the 1990s, numerous investigations that included water filtration studies to estimate TAM production (Thompson & Nehring 2000, Nehring et al. 2003), aquatic oligochaete studies (Zendt & Bergersen 2000) and sentinel fish exposure experiments (Thompson et al. 1999, 2002) demonstrated that Windy Gap Reservoir (WGR) in Grand County, Colorado, was a major point source of *M. cerebralis* infectivity and a primary factor in the WD epizootic that decimated the wild rainbow trout population in the upper Colorado River downstream of the reservoir (Nehring & Thompson 2001, Nehring 2006). Between April 1997 and March 1998 and April 1998 and March 1999, total annual numbers of TAMs in the discharge from WGR were estimated to be 960×10^9 and 1.8×10^{12}, respectively (Nehring et al. 2002). Estimates of average TAM density in the discharge from WGR ranged between 3.5 and 7.1 l^{-1} over the 4 yr period from 1997 through 2000 and then declined to 1.2 and 0.25 l^{-1} in 2001 and 2002 (Nehring et al. 2013). Initially, these steep declines in TAM production were thought to be linked to low spring runoff levels that resulted from the severe drought during those years. However, estimates of average TAM densities in the discharge of WGR never again reached 1 l^{-1} from 2002 through June 2006, when monthly water filtration studies were terminated. Even though spring stream flows in 2003 and 2005 returned to levels observed in the late 1990s, estimates of average TAM densities ranged between 0.25 and 0.85 l^{-1} over the 5 yr period from 2002 through 2006 (Nehring et al. 2013).

Two separate studies in 1998 (Zendt & Bergersen 2000, Nehring et al. 2003) assessed the distribution and density of the tubificid worm population and the spatial and temporal distribution of *M. cerebralis* TAM production in WGR during the period of high levels of TAM discharge.

The 1998 studies provided a baseline data set for comparing the spatial and temporal distribution of tubificid oligochaete population structure and the corresponding TAM production during the period of low TAM levels seen in WGR between 2001 and June 2006 (Nehring et al. 2013). A truncated version of Table 6 from Nehring et al. (2013) (our Table 4) is included here to facilitate an easier and clearer understanding of the spatial and temporal changes in the oligochaete population structure in WGR between 1998 (during the period of high TAM production) and 2004 and 2005, subsequent to the dramatic declines in TAM production that began during the drought years of 2001 and 2002 and continued through 2004 and 2005.

Intensive core sampling studies were initiated in 2004 and 2005 to ascertain whether or not there had been a shift in the *T. tubifex* worm population structure that might be linked to the dramatic decline

Table 4. Estimated relative abundances of aquatic oligochaetes and *Tubifex tubifex* worms belonging to Lineages I, III, V and VI in Windy Gap Reservoir in 1998, 2004 and 2005 as determined by intensive core sampling studies. Density estimates are standardized as number per square meter

Year	Oligochaetes m^{-2}		Core	Estimated densities by lineage			
	Total	Haired	samples	I	III	V	VI
1998	45537	34702	1012[a]	4773	13562	390	16077
2004	60173	42982	25	9069	3310	12637	17966
2005	97373	88657	190	31694	12579	8116	36268

in TAM production that began in 2001 and continued into 2006 (Nehring et al. 2013). Results of that study indicated that the estimated densities of the *M. cerebralis* resistant Lineages I, V and VI *T. tubifex* worms were much higher in 2004 and 2005 compared with the period between 1998 and 2001. In contrast, the estimated densities of Lineage III *T. tubifex* oligochaetes were essentially unchanged between the 2 periods (see Table 6 in Nehring et al. 2013 and Table 4). In 2005, the combined densities of the Lineages I, V and VI oligochaetes comprised approximately 86% of the *T. tubifex* worm population. When the other species of aquatic oligochaetes that occur in WGR (primarily *Limnodrilus hoffmeisteri* and *Ilyodrilus templetoni*) that are also refractory to the *M. cerebralis* parasite (Kerans et al. 2004) are included, the non-susceptible oligochaete population had a 7.7-fold advantage in density over the Lineage III population, comprising an estimated 95% of the total oligochaete population in the lake. *L. hoffmeisteri* and *I. templetoni* comprised ~9% of the total oligochaete population in WGR during the 2005 study (Nehring et al. 2013). Moreover, *M. cerebralis* infection prevalence among *T. tubifex* oligochaetes was estimated at 2.7% in May 1998, when the average TAM density for the year was estimated at 7.12 l^{-1}, compared with an infection prevalence of 0.3% in May 2005, when the average TAM density was estimated at 0.85 l^{-1}. The results of the laboratory exposures in this study demonstrating that the highly resistant Lineage I and Lineages V and VI oligochaetes that are refractory to *M. cerebralis* infection provide corroborating evidence that the large increases in density of Lineages I, V and VI worms in WGR were most likely deactivating and destroying *M. cerebralis* myxospores. The 7.7-fold advantage in density among the non-susceptible oligochaetes in WGR in 2005 was most likely responsible for the 90% decline in TAM production in the reservoir and the corresponding reduction in *M. cerebralis* infection prevalence in the worm population.

The results of the WGR studies (Nehring at al. 2013) together with the findings from the laboratory experimental exposures in this study are congruent with the hypothesis that those oligochaetes that have high resistance or are refractory to infection by the *M. cerebralis* parasite do deactivate *M. cerebralis* myxospores and are capable of reducing or eliminating *M. cerebralis* infections in the natural environment where Lineages I, V and VI oligochaetes occur. It is possible that large numbers of oligochaetes that are highly resistant (Lineage I) or refractory (Lineages V and VI) to the *M. cerebralis* parasite could be reared and introduced into WD positive streams or lakes where benthic sampling demonstrates that only allopatric populations of Lineage III *T. tubifex* oligochaetes exist. This might prove to be an inexpensive management tool that would potentially have few or no negative environmental consequences or economic side effects. In select riverine or lacustrine aquatic ecosystems it might even be possible to break the life cycle of the *M. cerebralis* parasite.

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