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ORIGINAL PAPER

Arrested development of the myxozoan parasite, *Myxobolus cerebralis*, in certain populations of mitochondrial 16S lineage III *Tubifex tubifex*

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Abstract Laboratory populations of *Tubifex tubifex* from mitochondrial (mt)16S ribosomal DNA (rDNA) lineage III were generated from single cocoons of adult worms releasing the triactinomyxon stages (TAMs) of the myxozoan parasite, *Myxobolus cerebralis*. Subsequent worm populations from these cocoons, referred to as clonal lines, were tested for susceptibility to infection with the myxospore stages of *M. cerebralis*. Development and release of TAMs occurred in five clonal lines, while four clonal lines showed immature parasitic forms that were not expelled from the worm (non-TAM producers). Oligochaetes from TAM- and non-TAM-producing clonal lines were confirmed as lineage III based on mt16S rDNA and internal transcribed spacer region 1 (ITS1) sequences, but these genes did not differentiate these phenotypes. In contrast, random amplified polymorphic DNA analyses of genomic DNA demonstrated unique banding patterns that distinguished the phenotypes. Cohabitation of parasite-exposed TAM- and non-TAM-producing phenotypes showed an overall decrease in expected TAM production compared to the same exposure dose of the TAM-producing phenotype without cohabitation. These studies suggest that differences in susceptibility to parasite infection can occur in genetically similar *T. tubifex* populations, and their coexistence may affect overall *M. cerebralis* production, a factor that may influence the severity of whirling disease in wild trout populations.

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Introduction

Myxobolus cerebralis is a myxozoan parasite that infects several species of trout, char, and salmon, which can result in a syndrome known as whirling disease (Hofer 1903; Hoffman 1990). The parasite has two obligatory hosts: a susceptible salmonid fish and a susceptible aquatic tubificid *Tubifex tubifex* (Markiw and Wolf 1983; Wolf and Markiw 1984; Wolf et al. 1986). *M. cerebralis* infections have been detected among salmonid populations from 24 states of the USA, and whirling disease has been implicated in declining populations of wild rainbow trout *Oncorhynchus mykiss* in the intermountain west region (Nehring and Walker 1996; Vincent 1996; Hedrick et al. 1998; Nickum 1999). The elucidation of the two-host life cycle of *M. cerebralis* (Markiw and Wolf 1983, Wolf and Markiw 1984) ushered in subsequent discoveries of similar life cycle strategies for at least 34 other myxosporean parasites that also involve alternating stages found in fish and oligochaete hosts (Kent et al. 2001; S. Atkinson, personal communication).

The emergence of the parasite and disease among wild trout populations in the USA has stimulated research on the effects of *M. cerebralis* infection on both hosts (Hedrick et al. 1998; Hedrick and El-Matbouli 2002). The effects of *M. cerebralis* infections on susceptible fish hosts have been reviewed by several authors, although many of the mechanisms underlying the virulence of the parasite are poorly understood (Hoffman 1990; Hedrick et al. 1998; El-Matbouli et al. 1999; Rose et al. 2000; MacConnell and Vincent 2002). While infectivity and development of the parasite in the oligochaete have been examined (El-Matbouli et al. 1999; Gilbert and Granath 2001; Granath and Gilbert 2002), less is known of the response to infection in the oligochaete compared to the fish host.

Currently, six known lineages (I–VI) of *T. tubifex* have been described based on mitochondrial 16S ribosomal DNA (mt16S rDNA) sequences (Sturmbauer et al. 1999; Beauchamp et al. 2001). The responses to experimental *M. cerebralis* infections differ among four of the lineages of *T. tubifex* so far examined (Beauchamp et al. 2001, 2002). Many strains of oligochaetes representing lineage III, and from diverse geographic areas, have been examined for susceptibility to *M. cerebralis*, and differences in overall parasite production have been reported (Stevens et al. 2001; Kerans et al. 2004; Beauchamp et al. 2005; Rasmussen et al. 2007). In contrast, oligochaetes grouped into lineages V and VI and several strains from lineage I appear completely or partially resistant to *M. cerebralis* infections (Beauchamp et al. 2005, 2006; Rasmussen et al. 2007). Susceptibility to infections with the parasite has not been examined in *T. tubifex* from lineages II. Recent exposure trials with lineage IV worms from the state of Alaska showed resistance to the parasite (Arsan et al. 2007).

In the current study, we report the derivation of both triactinomyxon (TAM)-producing, defined as the susceptible phenotype, and non-TAM-producing (resistant phenotype) oligochaete populations from individual cocoons collected from a group of known TAM-producing lineage III *T. tubifex*. The overall parasite production after *M. cerebralis* exposures of mixtures of susceptible and resistant populations derived from these cocoons were then examined and compared to non-cohabited susceptible populations. The results from these studies suggest that oligochaete–oligochaete interactions, even among genetically similar populations, may have pronounced effects on parasite production after exposure to *M. cerebralis*. These interactions and the subsequent effects on parasite production in naturally occurring *T. tubifex* populations may be an additional mechanism that affects the severity of whirling disease in wild trout populations.

Materials and methods

Screening of TAM-producing oligochaetes and propagation of pure cultures

A mixed population of oligochaetes from Mt. Whitney State Fish Hatchery, Independence, California was brought to the Fish Health Laboratory at the University of California, Davis in May 1999. Worms (20.0 g=8,000 worms) were randomly separated from the stock population and exposed to *M. cerebralis* myxospores (1,200 spores/worm) obtained from infected rainbow trout to confirm their susceptibility to the parasite (Beauchamp et al. 2006). At the peak of TAM production (ca 4 months post-exposure), oligochaetes (n= 50) were placed individually into multi-well plates, incubated at 15°C, and monitored for TAM releases for 2 days. Oligochaetes that were producing TAMs were removed, pooled into an aerated plastic container with sand substrate, covered with dechlorinated tap water, and maintained at room temperature (18°C). This TAM-producing population produced hundreds of cocoons after 7 months, 12 of which were randomly picked and placed individually in containers with dechlorinated tap water and sand substrate. After 1–2 weeks of incubation at 15°C, young worms were hatched from nine cocoons (2, 4, 5, 7, 8, 9, 10, 11, 12) with initial progeny numbers of 33, 29, 6, 17, 25, 28, 23, 60, and 18, respectively. Cocoons 1, 3, and 6 produced few young worms or failed to hatch, and hence, were discarded. At 6 months to 1 year after hatching from the cocoons, progeny from the original cocoons were observed in the nine remaining groups, and these populations were labeled as clonal lines 2, 4, 5, 7, 8, 9, 10, 11, and 12. Although *T. tubifex* are functional hermaphrodites, the exchange of sperm among individuals in a population may occur (Baldo and Ferraguti 2005). The cocoons used in our study were derived from TAM-producing individuals, but the origin of the fertilizing sperm involved in cocoon formation is unknown. Progeny from the nine populations from the original cocoons were raised in isolation as pure cultures and referred to in the current study as "clonal lines". Oligochaetes from each clonal line used in the current studies have been maintained in the laboratory since 1999. The number of worms increased in each clonal line over time, most likely through parthenogenesis, a reproductive strategy that Baldo and Ferraguti (2005) found commonly employed by laboratory populations of *T. tubifex*. Oligochaetes were fed dehydrated *Spirulina* sp. and "Algamac" (Bio-Marine, Hawthorne, CA, USA) once a week and exposed to a photoperiod cycle of 14-h light and 10-h dark using a 50-W fluorescent bulb at room temperature.

Genetic analysis of oligochaetes

Oligochaetes (n=30–50) were collected from stock populations of the nine clonal lines. Genomic DNA was isolated from individual worms with the DNeasy tissue kit (Qiagen, Valencia, CA, USA). Genetic lineages of each clonal line were examined initially with the *T. tubifex*-specific primers (Beauchamp et al. 2001) followed by the mt16S rDNA lineage-specific markers (Beauchamp et al. 2002).

Potential genetic variation among the worm populations was assessed using a randomly amplified polymorphic DNA (RAPD) polymerase chain reaction (PCR; Welsch and McClelland 1990). Random primers (n=6) from the RAPD analysis bead kit (Amersham Biosciences, Piscataway, NJ, USA) were evaluated. Primer 4 (5′-AAGAGC CCGT-3′) was used in subsequent testing of oligochaetes by RAPD PCR, as it showed consistent banding patterns of amplified DNA within and between individuals from the different clonal lines. Dehydrated beads contained reagents for a 25-µl volume PCR including thermostable polymerases, dNTPs, bovine serum albumin, and buffer (Amersham Biosciences). Sterile Dnase-free water, an arbitrary primer or primer 4 (25 pmol) and template DNA (10 ng) were added to the bead and mixed gently. Amplification of DNA was achieved with the following conditions (Amersham Biosciences): 45 cycles of denaturation at 95°C for 1 min followed by primer annealing at 36°C for 1 min and extension at 72°C for 2 min. Banding patterns of randomly amplified DNA were visualized and analyzed in 2% agarose gels.

Genetic sequences based on a portion of the mt16S rDNA were determined from five worms from susceptible clonal lines 7, 10, and 12 and from resistant clonal line 9. A PCR assay using the mt16S rDNA lineage III specific primer (5′ TTA TCA CCC CCA AAC TAA AAG ATA 3′) and reverse primer (5′ TAA RCC AAC ATY GAG GTG CCA 3′) was initially performed to amplify a 147-bp product (Beauchamp et al. 2001, 2002). The PCR products were purified (QIAquick PCR purification kit, Qiagen), and a portion of the mt16S rDNA (364 bp) was directly sequenced. Automated sequencing was conducted with the amplification primers in both directions using an ABI 377 DNA sequencer (Perkin-Elmer Life and Analytical Sciences, Wellesley, MA, USA). Sequences were aligned with Mac DNASIS v3.7 (Hitachi Software Engineering America, Cascade, CO, USA) and Clustal V (Higgins and Sharp 1989).

The ITS1 region of worms from susceptible clonal lines 4, 10, and 12 and from resistant clonal lines 2, 5, and 9 was amplified, cloned, and sequenced as previously described (Kerans et al. 2004), except that the zero blunt cloning kit with vector pCR4Blunt-Topo (Invitrogen, Carlsbad, CA, USA) was used. The ITS1 sequences were then compared to consensus ITS1 sequences from *Limnodrilus hoffmeisteri* (GenBank AF361102-03), *Ilyodrilus templetoni* (GenBank AF362435), and *T. tubifex*. All *T. tubifex* ITS1 sequences available from GenBank were then aligned using Clustal X (Thompson et al. 1997) using multiple pairwise and multiple alignment gap penalties that included the following combinations 10/10, 15/15, and 25/25. Alignments were analyzed by maximum parsimony, maximum likelihood, and neighbor joining using PAUP version 4.0 (Sinauer Associates, Sunderland, MA, USA).

Susceptibility of oligochaete populations to *M. cerebralis*

Susceptibility of the oligochaete clonal populations to *M. cerebralis* was determined by the production of TAMs after experimental exposures to1,000 myxospores/worm following the procedures described by Beauchamp et al. (2006). The myxospores were left in the experimental container with the worms throughout the duration of the study. Exposure trials were conducted on two occasions, as the time required to obtain sufficient individuals (n=100) from bulk cultures of each clonal line varied. Clonal lines 7, 9, 10, and 12 were tested with four replicates of 100 worms each. The remaining clonal lines 2, 4, 5, 8, and 11 were exposed in the same manner, but with only one replicate of 100 worms. Myxospores were freshly isolated from the cranial cartilage of experimentally infected rainbow trout and added to the worms according to procedures previously described (El-Matbouli and Hoffmann 1998). In both exposure trials, one control group (n=100 worms) from each clonal line was not exposed to the parasite. Susceptibility to *M. cerebralis* was evaluated by enumeration of TAM releases once a week between 3 and 7 months post-exposure. The enumeration of TAMs by filtration of water from each clonal group followed procedures previously described by Beauchamp et al. (2002). Early observation that worms from clonal line 9 failed to produce TAMs at 4–5 months post-exposure prompted a second testing. In that study, two separate groups (n=50) of clonal line 9 worms were exposed to high doses (5,000 and 10,000 myxospores/worm) and then evaluated for TAM release beginning at 3 months until 6 months post-exposure. The sediments from the non-TAM-producing clonal groups were also examined for TAMs in fecal packets (Gilbert and Granath 2001) at 6 months post-exposure. The presence of parasite stages both in TAM-producing and non-TAM-producing worms was assessed in hematoxylin and eosin (H&E)-stained histological sections and confirmed by in situ hybridization (Antonio et al. 1998) from worms (n=10/group) collected at 7 months post-exposure.

Cohabitation of resistant and susceptible strains

The interaction of oligochaetes from TAM- and non-TAM-producing lineage III clonal lines was evaluated in two cohabitation studies under conditions similar to those described by Beauchamp et al. (2006). In the first study, worms from resistant and susceptible clonal lines were mixed and immediately exposed to the parasite. In the second study, the clonal lines were cohabited for 1 month and then exposed to the parasite. In the two cohabitation trials, 100 worms from resistant clonal line 9 were mixed with equal numbers of worms from susceptible clonal line 10. Susceptible clonal line 12 was used in study 2, as sufficient numbers of clonal line 10 were not available at that time. A group of susceptible worms (clonal line 10 or 12, $n=100$) not cohabited with resistant clonal line 9 was also exposed to the parasite. Two replicates of cohabited and non-cohabited groups were exposed by placing myxospores (500/worm) into the container (10×10×9 cm) with the worms. The worms and myxospores were left in contact for the duration of both studies. Production of TAMs was evaluated from these groups beginning at 3 months post-exposure until the end of the study at 8 months post-exposure. Control groups of each clonal line not exposed to the parasite were also included. Decrease in TAM production was calculated from the difference in the number of TAMs produced in the cohabited and non-cohabited groups using the equation:

$$\frac{\text{No. TAMs in susceptible clonal line} - \text{No. TAMs in resistant + susceptible clonal lines}}{\text{No. TAMs in susceptible clonal line}}$$

The prevalence of worms releasing TAMs in the cohabited and non-cohabited groups was evaluated at 137 days post-exposure by randomly separating 24 worms from each replicate. The worms were placed individually into multi-well plates with dechlorinated tap water, screened for TAM releases for 2 days at 15°C, and then returned to their respective containers.

Lineage (mt16S rDNA) was determined for 20–40 worms sampled from worms remaining after 8 months of cohabitation (64 and 72% survivors for study 1 and 2, respectively) with the lineage-specific PCR (Beauchamp et al. 2002). A RAPD PCR (Welsch and McClelland 1990, Amersham Biosciences) was utilized to differentiate the relative proportions of each clonal line in mixed groups after 1-month cohabitation and before exposure to the parasite (cohabitation study 2, $n=10$) and again at the end of both cohabitation studies ($n=20$–40). Infections with the parasite were assessed from individual worms by PCR in both trials using the methods described by Andree et al. (1998).

Statistical analyses

A software program SAS Version 8.1 (SAS Institute, Cary, NC, USA) was used for comparisons of TAM production among the different clonal lines using a one-way analysis of variance and Tukey's honest significance difference test on log-transformed data. A one or two-sample t test on log-transformed responses was used to evaluate TAM production in the cohabitation experiments. A Fisher sign test (one or two-sided) was used to determine if the proportion of resistant worms was 50% of remaining worms at the end of the cohabitation tests. The prevalence of TAM-producing (releasing) worms was analyzed using a chi-squared test of independence. Statistical significance of all tests is at the 5% level, with results considered significantly different when $p<0.05$.

Results

Susceptibility of clonal lines to *M. cerebralis*

In the first exposure experiment, TAMs were released from clonal lines 7, 10, and 12 between 90 days and 8 months post-exposure to *M. cerebralis* myxospores. The number of TAMs produced, which ranged from 59,028 to 112,662 (Table 1), was not significantly different among these clonal lines. In contrast, no TAMs were observed from clonal line 9 which was significantly different ($p<0.0001$)

Table 1 Susceptibility and prevalence of infections to *M. cerebralis* in different clonal lines of lineage III *T. tubifex*

Clonal line	Total no. TAMs	No. worms positive for *M. cerebralis* DNA/ No. worms examined by PCR
Exposure 1		
9	0B[a]	9/20
7	59,028A	20/20
10	66,900A	20/20
12	112,662A	20/20
Exposure 2		
2	0	9/20
5	0	14/20
8	0	15/20
4	17,494	20/20
10	20,340	20/20
11	15,985	20/20

Triactinomyxon stages (TAMs) represent the mean total number produced between 90 days to 8 months post-exposure. Exposure 1: $n=100$ worms/clonal line exposed to 1,000 myxospores/worm. Total no. TAMs calculated as mean of four replicates/clonal line. Exposure 2: $n=100$ worms/clonal line exposed to 1,000 myxospores/worm. Total no. TAMs calculated from one replicate group.

[a] Number of TAMs released do not differ statistically if denoted by same letter.

from the other clonal lines tested. Subsequent testing of worms from clonal line 9 at even higher parasite doses (5,000 and 10,000 myxospores/worm) also resulted in the absence of TAM releases. In the second exposure, clonal lines 4, 10, and 11 produced TAMs, while clonal lines 2, 5, and 8 failed to release any TAMs (Table 1). Triactinomyxon stages were not found in fecal packets of non-TAM-releasing clonal lines examined at 8 months post-exposure. Statistical analyses were not conducted in the second exposure study due to the absence of replicate groups in the clonal lines used.

Infections with *M. cerebralis* as determined by PCR were present in all worms examined that produced TAMs and from worms that did not release any TAMs from the susceptible clonal lines 7, 10, 12, 4, and 11 that were exposed to myxospores. Parasite DNA was detected in fewer individuals from the resistant (non-TAM-producing) clonal lines 2, 5, 8, 9 than the susceptible clonal lines after myxospore exposures (Table 1). In the resistant clonal lines, the parasite failed to develop into mature sporogonic forms and release TAMs, although early and developmental stages were observed in histological sections of worms at 3 months post-exposure. Arrested development of the *M. cerebralis* in these worms is the likely cause for the subsequent lack of TAM release seen in the exposure experiments. These early and developmental stages were confirmed by in situ hybridization as being *M. cerebralis* (Fig. 1).

Genetic analysis of TAM- and non-TAM-producing oligochaetes

Oligochaetes (n=30–50/group) examined from stock populations of the nine clonal lines were confirmed as *T. tubifex* as shown by the amplification of a 364-bp product using the *T. tubifex*-specific primers (Beauchamp et al. 2002). Furthermore, both TAM- and non-TAM releasing populations showed amplification products of 147 bp consistent with genetic lineage III using the mt16S lineage-specific primers (Beauchamp et al. 2002).

The RAPD PCR using primer 4 (5′-AAGAGCCCGT-3′) provided unique banding patterns for resistant clonal lines 2, 5, 8, and 9 as compared to susceptible clonal lines 4, 7, 10, 11, and 12 (Fig. 2). These banding patterns were consistently observed using primer 4 and were applied to examine the proportions of susceptible and resistant worms in cohabitation studies. Analysis using four other RAPD primers, however, showed banding patterns that looked nearly identical with one to four bands that were shared between TAM- and non-TAM producing lineage III *T. tubifex*, suggesting a low level of genetic variation may be present between these clonal lines.

Direct sequencing of a 364-bp portion of the mt16S rDNA of oligochaetes from susceptible clonal lines 7, 10, and 12 and from resistant clonal line 9 revealed few differences when compared to sequences of lineage III oligochaetes from Mt. Whitney, CA, USA (GenBank AF326037–AF326046, Beauchamp et al. 2001). The only consistent difference was the presence of a guanine (G) at position 98 for resistant clonal line 9, compared to adenine (A) for susceptible clonal lines 7, 10, and 12.

Two types of ITS1 sequences were obtained from TAM-producing clonal lines 4 (GenBank EF467063-64 and EF467060), 10 (GenBank EF467061-62 and EF467059), and 12 (GenBank EF467057-58). A shorter consensus sequence (783 bp) was obtained from five clones that differed from each other at ten nucleotide positions with two single nucleotide insertion/deletion events. A longer consensus sequence (848 bp) was obtained from three clones with differences at eight nucleotide positions, and one clone had a 6-bp deletion when aligned with the two other sequences. The 848-bp consensus sequence from the susceptible clonal lines was similar to the Mt. Whitney *T. tubifex* sequence previously reported by Kerans et al. (2004). This result was expected, as the clonal lines were derived from single cocoon cultures of Mt. Whitney, CA worms. Two types of ITS1 sequences were also obtained from the resistant clonal lines 2 (GenBank EF467050 and EF467055), 5 (GenBank EF467051 and EF467054), and 9

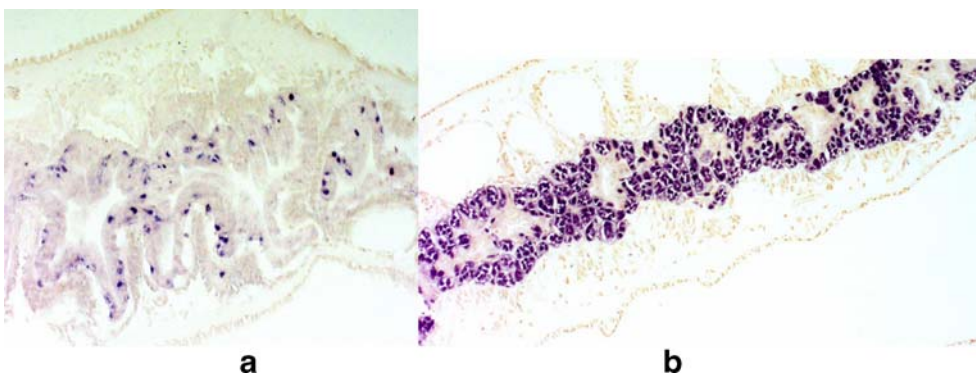

Fig. 1 Early developmental stages of *M. cerebralis* in lineage III *T. tubifex*-resistant clonal line 9 (**a**) compared to fully-developed sporogonic stages in susceptible clonal line 10 (**b**) at 3 months post-exposure to parasite myxospores as detected by in situ hybridization

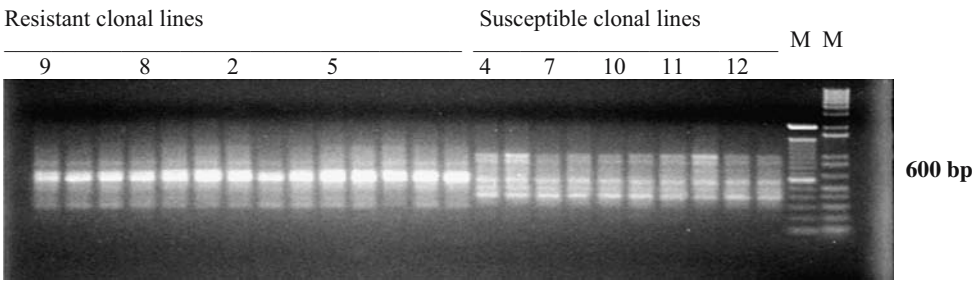

Fig. 2 RAPD banding patterns of resistant (9, 8, 2, 5) and susceptible (4, 7, 10, 11, 12) clonal lines of lineage III *T. tubifex*. Amplification products were generated with primer: 5′-AGAGCCCGT-3′. M indicates lanes with 100-bp and 1-kb ladders

(GenBank EF467052-53 and EF467056). A 792-bp consensus sequence obtained from six clones differed at 14 nucleotide positions, and a single clone had a 774-bp sequence. Both of these ITS1 sequences resembled those from the TAM-producing cultures obtained from the Madison River, MT and Logan River, UT (Rasmussen et al. 2007) more than Mt. Whitney, CA ITS1 sequences. In fact, the 792-bp sequence was nearly identical to the Madison River, MT consensus sequence with only one nucleotide difference.

No significant relationships segregating the resistant clonal lines from those susceptible to *M. cerebralis* infection were found when the nucleotide differences in the ITS1 sequences were examined. Furthermore, phylogenetic analysis indicated that all ITS1 sequences from the resistant and susceptible clonal lines clustered with those representing other *Tubifex* individuals from 16S mt lineage III. Trees using different alignment parameters as well as other phylogenetic methods (maximum likelihood and neighbor joining) had the same topology, indicating that the ITS1 sequences were unable to differentiate between the resistant and susceptible clonal lines.

Production of TAMs in cohabited and non-cohabited *T. tubifex strains*

In cohabitation study 1 (immediate parasite exposure after cohabitation), the number of TAMs produced in the cohabited groups (23,359) was marginally significant (p= 0.0281) to TAM numbers from the unmixed susceptible group (60,787 TAMs) using a one-sided t test of log-transformed TAM numbers (Table 2). Production of TAMs was decreased by 61.6% when resistant and susceptible strains were combined (Table 2). The prevalence of TAM-releasing worms at 4.5 months post-exposure was significant between the cohabited groups (20.8%) and unmixed susceptible group (87.5%) using a chi-squared test of independence (p<0.0001).

In the second cohabitation experiment, TAM production was also decreased (74.2%) when both phenotypes were cohabited for a month before parasite exposure (Table 2). The total number of TAMs produced was marginally significant (p=0.0312) in cohabited groups (10,382 TAMs) compared to the unmixed susceptible group (40,227 TAMs) using a one-sided t test (Table 2). The prevalence of TAM-releasing worms at 4.5 months post-exposure was not significantly different between the cohabited groups (25%) and unmixed susceptible group (58.4%) using a chi-squared test of independence (p>0.05).

Genetic analysis of worms from cohabitation experiments

At 8 months post-exposure to the parasite, all 20 oligochaetes assayed from both replicates of mixed resistant and susceptible worms (cohabitation study 1) were confirmed as lineage III using the lineage-specific markers. By RAPD PCR, 11 and 9 worms (replicate 1) had amplicons consistent with resistant and susceptible worm phenotypes, respectively (Table 3). The Fisher sign test was used to determine if the proportion of resistant worms was 50%. The result was insignificant for a two-sided (p=0.8238) or one-sided (p=0.4119) test. In replicate 2, 25 of the 40 worms examined had RAPD banding patterns consistent with resistant worms, while the remaining 15 resembled the characteristic of susceptible worms (Table 3). The Fisher sign test showed that these figures were also insignificant

Table 2 Production of triactinomyxon stages (TAMs) of *M. cerebralis* in cohabited and non-cohabited clonal lines of lineage III *T. tubifex*

Cohabited	Non-cohabited	TAM decrease[a] (%)	p value from t test
Resistant + susceptible	Susceptible		
Study 1[b]			
Clonal lines 9+10	Clonal line 10		
Rep 1: 20,757	73,059		
Rep 2: 25,961	48,515		
Mean: 23,359	60,787	61.6	0.028
Study 2[c]			
Clonal lines 9+12	Clonal line 12		
Rep 1: 13,495	32,694		
Rep 2: 7,270	47,760		
Mean: 10,382	40,227	74.2	0.031

[a] $\frac{\text{No. TAMs in susceptible clonal line} - \text{No. TAMs in resistant+susceptible clonal lines}}{\text{No. TAMs in susceptible clonal line}} \times 100$

[b] Cohabitation and immediate exposure to 500 myxospores/worm

[c] One-month cohabitation before exposure to 500 myxospores/worm

Table 3 Composition of resistant and susceptible lineage III *T. tubifex* and prevalence of infection at 8 months after cohabitation and exposure to *M. cerebralis* myxospores

Clonal line	Composition of susceptible and resistant worms (RAPD)[a]				Infection prevalence *M. cerebralis* DNA (PCR)	
	Replicate				Replicate	
Study 1[b]	1		2		1	2
Cohabited clonal lines						
Susceptible clonal 10	9/20[c]	$p=0.82$[e]	15/40	$p=0.16$	9/9[d]	15/15
Resistant clonal 9	11/20		25/40		10/11	20/25
Non-cohabited clonal 10	20/20		20/20		20/20	20/20
Study 2[b]						
Cohabited clonal lines						
Susceptible clonal 12	14/40	$p=0.08$[e]	15/40	$p=0.16$	14/14	15/15
Resistant clonal 9	26/40		25/40		25/26	20/25
Non-cohabited clonal 12	20/20		20/20		20/20	20/20

[a] The relative proportion of each clonal line was differentiated by RAPD PCR after cohabitation.
[b] Immediate exposure to *M. cerebralis* myxospores in cohabited worms (study 1) or 1 month cohabitation before exposure (study 2) to 500 myxospores/worm. Susceptible clonal line10 or 12 worms ($n=100$) were cohabited with equal numbers of resistant clonal line 9 worms.
[c] Number of worms identified as susceptible or resistant clonal line as determined by RAPD PCR.
[d] Number of worms positive for *M. cerebralis* DNA as determined by PCR
[e] p value from Fisher sign test to determine if the proportion of resistant worms is 50%; all values are insignificant as a two-sided test.

either as a two-sided ($p=0.1539$) or as a one-sided ($p=0.0769$) test. Thus, in both trials, the proportion of resistant worms was not statistically different from 50%. The presence of *M. cerebralis* DNA was not statistically different from worms identified by RAPD analyses as susceptible (clonal line 10) or resistant (clonal line 9) after 8 months of cohabitation (Table 3).

In study 2, all of the 40 oligochaetes examined from the cohabited group in both replicates were confirmed as lineage III using the lineage-specific markers. As determined by RAPD PCR banding patterns, 26 worms were classified as resistant and the remaining 14 as susceptible (replicate 1, Table 3). The Fisher sign test showed marginal insignificance as a two-sided test ($p=0.0807$), but significant as a one-sided test ($p=0.0404$). The higher proportion of resistant worms in the second replicate (25/40) was marginally insignificant using either a two-sided ($p=0.1539$) or a one-sided ($p=0.0769$) test. The prevalence of worms with *M. cerebralis* DNA was not significantly different between resistant (clonal line 9) and susceptible (clonal line 12) distinguished by RAPD analysis at 8 months post-cohabitation (Table 3). There was no evidence of *M. cerebralis* DNA among any oligochaete groups examined that were not exposed to the parasite in both trials.

Oligochaetes in both replicate groups ($n=20$ in each replicate) from the unmixed (not cohabited) susceptible strains (clonal line 10 or clonal line 12) were all identified as lineage III using the lineage-specific markers. In addition, all unmixed worms from clonal lines 10 and 12 that were exposed to myxospores were positive for parasite DNA by PCR (100%, Table 3). Examination of individual worms in these unmixed groups by RAPD PCR also revealed banding patterns identical to those of uninfected susceptible worms.

Discussion

Genetic markers that provide methods for grouping of *T. tubifex* are currently being employed in studies assessing the important role of this host on the effects of *M. cerebralis* on wild trout populations. These markers exploit sequence differences found in the mt16S rDNA, 18S rRNA gene, the ITS1 region, or throughout the oligochaete genome (Sturmbauer et al. 1999; Beauchamp et al. 2001; Kerans et al. 2004; Rasmussen et al. 2007). Unfortunately, none of these genetic markers is based directly upon DNA regions likely to target genes that may influence resistance or susceptibility to *M. cerebralis* infection, and thus, assessing the response to the parasite ultimately relies upon infectivity trials where worms are exposed to myxospores and then evaluated for parasite production. Despite this limitation, observational and experimental studies indicate that worms susceptible to the parasite are most likely to be found in mt16S DNA lineage III (Beauchamp et al. 2002, 2005, 2006; Kerans et al. 2004; Steinbach-Elwell et al. 2006; Rasmussen et al. 2007). In the current study, we demonstrate that differences in susceptibility can occur even among *T. tubifex* laboratory populations derived from cocoons of adult mt16S lineage III worms actively releasing the parasite. Both the mt16S DNA and ITS1 sequences were ineffective in discerning resistant (non-TAM-producing) and susceptible (TAM-releasing) populations of *T. tubifex* in our study. However, RAPD analyses

using primer 4 distinguished the resistant (non-TAM-producing) from susceptible (TAM-releasing) phenotypes within worms grouped together in the mt16S lineage III. RAPD analysis may therefore be a convenient tool for predicting susceptibility of oligochaetes in known discrete *T. tubifex* populations such as the laboratory groups in our study. In contrast, the use of RAPD analyses to distinguish among less related groups of oligochaetes is more difficult.

The interactions between these closely related genetic lineage III worms may influence overall productivity of the parasite, and thus, modulate the severity of whirling disease among wild trout populations in those aquatic systems. Variations in susceptibility to *M. cerebralis* are known to occur among populations of lineage III *T. tubifex* originating from diverse geographic locations among strains that generally have shown significant genetic differences (e.g., by RAPD; Stevens et al. 2001; Beauchamp et al. 2002, 2005; Kerans et al. 2004) and even among genetically similar populations of lineage III oligochaetes (Rasmussen et al. 2007). The severity of infections to *M. cerebralis* is most often assessed by the production and enumeration of TAMs released from *T. tubifex* over a defined period after exposure to myxospores (Gilbert and Granath 2001; Stevens et al. 2001; MacConnell and Vincent 2002; Kerans et al. 2004; Beauchamp et al. 2005, 2006; Steinbach-Elwell et al. 2006). Infections with the parasite can also be assessed in the oligochaete by detection of *M. cerebralis* DNA by PCR or in situ hybridization, with the advantage that the latter method allows observation of developmental stages of the parasite beneath the intestinal mucosa of the worm (Antonio et al. 1998; Rognlie and Knapp 1998).

Some of the mt16S rDNA lineage III clonal populations in our study showed arrested parasite development, and thus, none of the stages commonly released into the lumen of the gut and then expelled from the worm (i.e., TAMs) occurred. The stage of parasite arrestment was considerably further along in development in these worms than the very early stages found after experimental exposures of known resistant lineage V worms. In lineage V *T. tubifex*, myxospores that were ingested hatched and released their infectious germ cells. The germ cells then invaded the intestinal mucosa but failed to develop further and were quickly eliminated (M. El-Matbouli, unpublished data). In our study, numerous early developmental stages (El-Matbouli et al. 1999) persisted in the resistant lineage III worms throughout the trial as shown by in situ hybridization (Fig. 1). Thus, there appears to be at least two different means by which resistant worms can alter the course of *M. cerebralis* infections, with the end result being that TAM stages infectious for young fish are not released. The first is that after spore ingestion and penetration into the gut lumen, the infection is aborted, and parasites are eliminated as described above for lineage V worms (M. El-Matbouli, unpublished data). The second results in arrested development of early stages of the parasite that persist, never fully maturing to stages that would be released into the gut lumen (i.e., non-TAM-producing lineage III worms in the current study). In both cases, the effects of the resistant worms on the parasite propagation may be viewed as providing biological filtration where myxospores are ingested and hatched but never develop fully to mature stages for release. This process effectively removes myxospores of *M. cerebralis* from the substrate, with the presumed effect that overall infectivity is reduced as well as the severity of whirling disease in wild trout populations sharing this environment (Beauchamp et al. 2006).

As *T. tubifex* is an abundant component of the benthic fauna in freshwater communities, controlling the effects of whirling disease in these habitats is an enormous undertaking (El-Matbouli and Hoffmann 1998). The use of biological control to regulate parasitic infections of fish has been suggested (Costello 1996; Slootweg et al. 1994), and initial experimental trials with *M. cerebralis* have evaluated the effects of mixing *T. tubifex* of different lineages with varying susceptibilities (Beauchamp et al. 2006). In the current study, we suggest that similar effects in reducing overall parasite infectivity with *M. cerebralis* may occur even when cohabiting different phenotypes from the same lineage. A reduction in parasite production (TAMs released) with mixed *T. tubifex* lineages was not observed in a study reported by Steinbach-Elwell et al. (2006). A key difference between that study and those of Beauchamp et al. (2006) and that in our current study was duration of exposure of oligochaetes to the myxospores. In the Steinbach-Elwell et al. (2006) study, there was a single brief exposure to myxospores compared to a continuous exposure in the other studies. This brief exposure likely did not allow ample time for worms to interact with the myxospores, particularly with resistant phenotypes that would have inactivated (biofiltered) the myxospores, and thus, prevented their eventual contact and development in susceptible oligochaetes (Hedrick and El-Matbouli 2002).

In conclusion, our study demonstrates that both susceptible (TAM-producing) and resistant (non-TAM-producing) worms can arise even from known mt16S DNA lineage III TAM producers. Although current genetic markers are convenient tools for grouping *T. tubifex*, their application as indicators of susceptibility to *M. cerebralis* is limited. The mt16S rDNA and ITS1 sequences did not distinguish between resistant and susceptible phenotypes likely because these genes are not directly related to those involved in the mechanisms that determine resistance or susceptibility. The utility of RAPD analysis to distinguish between phenotypes was demonstrated in this study, and the application of this approach is effective particularly when dealing with well-defined genetic populations of *T. tubifex*. Variations in infection prevalence and parasite production may occur in

T. tubifex as influenced by differences in geographic strains, genotype composition, or shifts in lineage composition (Kerans et al. 2004; Beauchamp et al. 2005, 2006; Nehring et al. 2005). Our current study demonstrates that even genetically similar *T. tubifex* can exhibit different responses to infections with the parasite, an effect recently described also by Rasmussen et al. (2007). The combined effects of interactions between and among *T. tubifex* in different genetic lineages should therefore be considered as among the many factors that modulate the severity of whirling disease among wild trout populations.

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