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Research paper

Hybridization is limited between two lineages of freeze-resistant *Trichinella* during coinfection in a mouse model

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ABSTRACT

Hybridization between two closely related but distinct genetic lineages may lead to homogenization of the two lineages with potentially novel phenotypes, or selective pressure to avoid hybridization if the two lineages are truly distinct. *Trichinella nativa* and *Trichinella* T6 are zoonotic nematode parasites which can be distinguished genetically despite occasional hybridization. Here, using an experimental murine model, we attempt to determine whether there are barriers to hybridization when sizeable numbers of each lineage are allowed to coinfect a host. Two mice were independently infected with equal numbers of *T. nativa* and T6. The offspring of these coinfections were genotyped at two microsatellite loci and one mitochondrial locus capable of distinguishing *T. nativa* from T6 genotypes. Among larvae in the F1 generation, offspring of every possible mating were encountered. Most larvae (63.6%) derived from *T. nativa* × *T. nativa* matings, while 21.1% of offspring were the product of T6 × T6 matings, and only 15.3% were hybrid offspring of *T. nativa* × T6 crosses, differing markedly from null expectations. In this experimental model, *T. nativa* and *Trichinella* T6 were able to mate, but ratios of offspring indicated pre- or post-zygotic barriers to hybridization that may include assortative mating, genetic incompatibilities, and/or differences in the fitness of offspring. These barriers would limit gene flow between these two lineages in a natural setting, serving as a barrier to their homogenization and promoting their persistence as distinct and separate entities.

1. Introduction

Closely related but genetically distinct biological lineages are sometimes able to interbreed (see Rieseberg and Carney, 1998, Scribner et al., 2001, and Detwiler and Criscione, 2010 for reviews of specific phyla). This may result in gene flow, leading to the homogenization of the two lineages or extirpation of one lineage in favor of the more successful phenotype (Rhymer and Simberloff, 1996). Alternatively, if lineage phenotypes are adaptive, behavioral or physiological barriers may maintain distinctions between lineages (Haldane, 1948). Absolute reproductive isolation would meet the criteria for one influential species concept (Mayr, 1942). Thus, the results of mating between two such lineages may be interpreted along a spectrum from intra-specific population structure to inter-specific reproductive isolation, including the possibility of incipient speciation. Distinguishing among these outcomes requires an understanding of both the biology and demography of the populations in question. Examining the outcomes of experimental crosses between genetically distinct representatives may provide insight into the biological relevance of hybridization and introgression of genes.

Trichinella nativa and the *Trichinella* T6 (henceforth referred to as T6) genotypes are genetically distinct lineages of nematode parasites endemic to the Canadian Arctic, peculiar for their freeze resistance. The two cannot be distinguished from each other by any morphological character (Pozio and Zarlenga, 2005). T6 is currently considered a sister taxon to *T. nativa*, but has not been raised to species status due to its similarities with *T. nativa* (Pozio et al., 1992; Zarlenga et al., 1999, 2001; La Rosa et al., 2003; Reichard et al., 2008). Both lineages are primarily found in wild carnivores, such as foxes and wolves (Kapel, 2000), and complete their life cycle within a single host, with developmental stages being separated only by tissue type (Reichard et al., 2008). Despite their similarities, *T. nativa* and T6 may be differentiated using genetic markers. This implies that there has been a history of separation between the two (Pozio, 2000), most likely during the Pleistocene glaciations (Zarlenga et al., 2006). Previously, T6 was thought to occupy a distinct geographic range from that of *T. nativa*, but the lineages have been found together in particular host populations (Reichard et al., 2008; La Rosa et al., 2003). Furthermore, natural

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T. nativa/T6 hybrids have been observed (La Rosa et al., 2003, Dunams-Morel et al., 2012), raising the possibility of limited gene flow. The extent and biological consequences of introgression between the two lineages remain unknown.

Hybridization and subsequent introgression erodes species boundaries. Dunams-Morel et al. (2012) distinguished natural *T. nativa*/T6 hybrids from true-breeding individuals in each lineage using genetic markers, suggesting that in the studied population, introgression had not been extensive. This may be for lack of opportunity, if the taxa only rarely co-occur (Reichard et al., 2008). Changes in the geographic distribution of lineages, as are expected in changing climates, may provide more opportunities for breeding between closely related lineages, and an increase in gene flow. Increased gene flow between two such lineages might produce a single lineage with greater genetic and/or phenotypic diversity.

By contrast, introgression may be limited, in spite of opportunities for hybridization, by pre- or post-zygotic barriers. La Rosa et al. (2003) showed that when pairs of *T. nativa* and T6 were compelled to hybridize, fewer offspring resulted than in purebred lines of either lineage. Moreover, hybrid F1 individuals descended from *T. nativa* mothers did not produce offspring when mated to each other. Such reproductive penalties should reinforce reproductive isolation between *T. nativa* and T6. Pre-zygotic barriers for parasites include geographic isolation, isolation by host-specificity, and mate recognition systems (Southgate et al., 1998). As *T. nativa* and T6 currently have overlapping geographical ranges and host species, mate recognition provides the most likely pre-mating barrier to hybridization between these two lineages. This process, termed assortative mating, occurs where particular parasites mate preferentially with their own lineage despite access to another competent for hybridization (Wright, 1921). Such behavior reinforces inbreeding, promoting genetic divergence among populations. Differentiation of lineages that occurs due to any form of pre-zygotic isolation may give rise to genetic incompatibilities that reduce the fitness of hybrid offspring, resulting in post-zygotic barriers to hybridization. Adaptations that minimize such maladaptive hybridization would increase a pure-breeding individual's fitness as measured by the average number of viable offspring produced from a mating event (relative fitness). This process of reinforcement may be important in cementing evolved differences between incipient species (Kelly and Noor, 1996; Noor, 1999; Matute, 2010).

Here, using an experimental murine model, we attempt to determine the likely outcome of hybridization between these two lineages when encountered in a well-mixed population. We characterized the nature and frequency of hybridization in experimental coinfections involving many individuals from both the *T. nativa* and T6 lineages, where each parent was capable of "choosing" similar or dissimilar mates. We genotyped offspring of this initial cross to determine the relative contributions of each parental lineage, and to assess the degree of admixture occurring under these experimental conditions. In order to track the inheritance of somatic and maternally inherited genes, we employed two nuclear microsatellite loci and a mitochondrial restriction fragment length polymorphism.

2. Methods

2.1. Sample preparation

In May 2012, two Swiss-Webster mice (NCI) were experimentally infected, each with approximately 420 larvae consisting of equal numbers of *T. nativa* (ISS45) and the T6 genotype (ISS34). Outbred mice were used in order to replicate experiments conducted previously (La Rosa et al., 2003). The introduced larvae migrated to the gut, wherein they matured and mated. The F1 generation then migrated to the animals' skeletal muscle tissue. After 49 days, muscle larvae from one mouse (henceforth referred to as mouse A) were obtained by digesting the skinned and eviscerated carcass in a 1% HCL/1.0% pepsin solution. Immediately after isolation, 500 larvae from this mouse digestion were used to infect 5 uninfected mice in order to follow the success and mating tendencies of the F1 generation. Data from subsequent generations (F2 and F5) are presented as supplemental information as there was no replication of these data. The remaining worms from mouse A (those not used for infection) were saved for genetic analysis. The second mouse (mouse B) was sacrificed 70 days post-infection, and muscle larvae were isolated as before and saved for genetic analysis, but no new mice were infected.

From the F1 generation, individual larvae were isolated from their respective pooled worm suspension (mouse A or B) by micropipette utilizing a dissection microscope for visualization. Individual worms were stored at 4 °C in water until ready for DNA extraction. DNA was purified from these single larvae using the DNA IQ System Tissue & Hair Extraction Kit (Promega Corp.) according to manufacturer recommendations. Individual DNAs from mouse replicates in the F1 generation were kept separate in order to evaluate repeatability of the experimental crosses.

In order to control for fitness differences between the parental strains, reproductive capacity indices (RCI) were calculated from mice infected with either strain alone. In each case, five mice were infected with 500 muscle larvae of *T. nativa* or T6. After six months, muscle larvae were collected from infected mice using the method described above, and pooled in 25 ml water for counting. Three separate counts of a 200 μl sample were conducted to enumerate the muscle larvae collected, and the average was reported. The resulting pools (*T. nativa* or T6) were used to infect an additional five mice, and counts were repeated after seven months of infection. RCI was calculated as the average number of worms recovered from two replicates of each genotype divided by the number of worms used to infect host animals.

2.2. Amplification of loci

2.2.1. Microsatellites

Amplification of two nuclear loci and one mitochondrial locus was attempted for 93 larvae from each pool (93 from mouse A, 93 from mouse B, 93 from the F2 generation, and 93 from the F5 generation). Individual larval DNAs were amplified for 2 microsatellite loci: TP32 and TP47 (Rosenthal et al., 2008). Larval *T. nativa* and T6 parental strains had been previously genotyped and determined to differ at these loci (see Dunams-Morel et al., 2012). Therefore, hybrids could be identified by the coincidence of alleles particular to each parental type. Microsatellite loci were amplified by 20 μl PCR reactions conducted in 96 well plates. Each reaction contained 5 μl of template DNA, 0.5 mM dNTPs, 0.5 μM of each primer, 0.5 U Platinum High Fidelity Taq polymerase (Invitrogen), and 2.5 mM $MgSO_4$ in 1× High Fidelity PCR buffer (Invitrogen). Reactions were subjected to thermal cycling as follows: 94 °C for 3 min, then 40 cycles of 94 °C for 30 s, 53 °C for 45 s and 68 °C for 1 min, with a polishing step of 68 °C for 5 min. In every reaction plate, no template, parental *T. nativa*, and parental T6 controls were included. For size fragment analysis on an ABI 3130 capillary electrophoresis machine, PCR reactions were diluted 1:6 and 1 μl of diluted microsatellite PCR product was mixed with 9 μl HiDi Formamide containing 0.2% GeneScan 500 LIZ molecular weight standards (Applied Biosystems). The resulting size fragment trace files were viewed in Geneious (Biomatters Ltd.), and alleles were called based on fluorescence peaks allowing the software to call each peak in order to avoid human bias.

2.2.2. Mitochondrial DNA

The matrilineage of each larva was determined using a mitochondrial specific restriction fragment length polymorphism (RFLP). Mitochondrial DNA (mtDNA) from each larval DNA extract was amplified using Trich-COB F1 and Trich-seq R3 primers (Lavrov and Brown, 2001) resulting in PCR products of 960 base pairs (bp). Each 20 μl PCR reaction contained 5 μl template, 2.5 mM $MgSO_4$, 0.5 mM dNTPs, 50 nmol of each primer, and 1 unit HiFi Platinum Taq (Invitrogen). Amplified DNA was

subjected to restriction digest using MluC I (New England Biolabs) according to manufacturer's protocol with 5 units of enzyme per restriction digest. Restriction fragment banding patterns were visualized by gel electrophoresis and scored as belonging to *T. nativa* or the T6 lineage mtDNA for each sample. The diagnostic bands were generated by an additional MluC I restriction site in the T6 mtDNA which cuts the largest fragment of the *T. nativa* digest (351 bp) into bands of 184 and 171 bp.

2.3. Analyses

Genotypes were organized by individual and mouse in GenAlEx v. 6.5 (Peakall and Smouse, 2012). Allele frequencies were calculated according to standard procedures. F_{IS} was calculated for each microsatellite locus in GenAlEx. Only individuals successfully genotyped at two or three loci were considered for population genetic analyses, as these individuals could be classified unambiguously as deriving from purebreeding within a lineage or the offspring of hybrid crosses in the F1 generation. Inclusion of those individuals that amplified at only one locus did not change allele frequencies significantly (data not shown).

Multilocus genotypes from the F1 generation were used to infer the parentage of each individual. Progeny of a *T. nativa* mother and a *T. nativa* father harbored only alleles characteristic of that lineage, and individuals possessing only alleles exclusive to the T6 lineage were inferred to have both a T6 mother and a T6 father. Offspring of crossbreeding between *T. nativa* and T6 individuals were expected to be heterozygous at both microsatellite loci. The mtDNA RFLP was used to categorize hybrid offspring derived from *T. nativa* or T6 mothers. These F1 genotypes were used for calculating success rates at genotyping as well as frequency of progeny from every possible mating class.

Larvae from two independent mouse replicates were used to assess the repeatability of the outcome of mating in mixed populations of *T. nativa* and T6. Chi-squared tests were conducted to determine whether the number of offspring in each class of cross differed between mice, and whether the proportion of hybrids deriving from either *T. nativa* or T6 mothers differed.

Proportions of offspring from each type of cross were compared to two a priori models of population mating. The first model assumed random mating between all individuals in the parent population and equal relative fitness for an expectation of equal numbers of offspring from each possible cross. For the second possible outcome, random mating was maintained, but differences in relative fitness as reported by La Rosa et al. (2003) for each type of cross were taken into account, resulting in an expectation of 39% *T. nativa* × *T. nativa*, 27% T6 × T6, 15% *T. nativa* mother × T6 father, and 19% T6 mother × *T. nativa* father offspring. These expectations were calculated by multiplying the random mating frequency (0.25) by the relative fitness reported previously (1.0 for *T. nativa* × *T. nativa*, 0.71 for T6 × T6, 0.39 for T6 father × *T. nativa* mother, and 0.49 for *T. nativa* father × T6 mother).

In order to compare the results reported here with those reported by La Rosa et al. (2003), we use the term relative fitness as the proportion of muscle larvae that were produced by each type of cross. This does not reflect the actual number of newborn larvae produced from each mating (fecundity). Rather, it takes into account the relative fitness of the parents, as only larvae that successfully establish in muscles will be present for mating in the next generation of infection. Observed frequencies of F1 offspring were compared to these expectations using chi-squared tests of population proportions in R.

3. Results

Among 558 attempts to amplify individual loci, 349 were successful (62.5%). TP32, TP47, and mtDNA amplified in 70.4%, 64.5%, and 52.7% of individuals, respectively.

Genotypes from the F1 offspring were used to test our ability to discern alleles at each microsatellite locus. Among the 186 offspring for which genotyping was attempted, 124 F1 progeny (67%) were characterized at two or more loci, and thus deemed suitable for analysis. Multilocus genotypes from 118 of these individuals (95.2%) supported classification as offspring of pure *T. nativa*, pure T6, or hybrid matings with no conflict between any loci. Five of the remaining six multilocus genotypes had conflicting microsatellite genotypes with purebred alleles at one microsatellite locus and hybrid allele combinations at the other microsatellite genotype yielding indeterminate parentage. The final individual was hybrid at the TP32 locus, purebred *T. nativa* at the TP47 locus, and mtDNA characteristic of T6 mothers. All multilocus genotypes with indeterminate parentage were removed from subsequent analyses. Thus, the ability to infer parentage in F1 progeny was nearly, but not entirely, perfect.

Two independent mouse replicates were used to compare consistency of mating proportions within and between *T. nativa* and T6 in coinfections in mice. Mouse A yielded 40 multilocus genotypes and mouse B provided 78 multilocus genotypes amenable to analysis. Following classification of genotypes as deriving from *T. nativa* × *T. nativa*, T6 × T6, or hybrid matings (Fig. 1), there was no significant difference in genotype frequencies between these two mouse host replicates ($X^2 = 0.997$, df = 2, p = 0.607). The multilocus genotypes from both mice were pooled for further analysis. Among 118 larvae genotyped in the F1 generation, offspring of every possible mating were encountered. *T. nativa* × *T. nativa* genotypes were most numerous accounting for 63.6% of all offspring. Individuals derived from T6 × T6 pairs accounted for 21.1% of genotypes. Only 15.3% of offspring were hybrid offspring of *T. nativa* × T6 crosses. Among hybrid offspring that amplified at the mtDNA locus, 30% derived from *T. nativa* mothers and 70% from T6 mothers with no difference between mouse replicates ($X^2 = 0.476$, df = 1, p = 0.490).

Among F1 larvae, there were substantial heterozygote deficits at both microsatellite loci. Inbreeding coefficients (F_{IS}) were 0.438 and 0.600 for TP32 and TP47, respectively. Heterozygotes occurred less than half as frequently as would be expected under Hardy–Weinberg expectations (global $F_{IS} = 0.519$) indicating substantial departures from random mating, equal production of offspring among all crosses, and/or fitness of offspring in the F1 generation.

The observed proportions of offspring from each cross differed from two a priori null expectations (Table 1). Had progeny been produced by random mating and equal relative fitness, half of them would have been hybrid and equal numbers of offspring would have been produced from every class of cross (*T. nativa* breeding with *T. nativa*, *T. nativa* mothers mating with T6 males, *T. nativa* males mating with T6 females, and pure T6 crosses). The actual F1 generation departed markedly from these expectations, harboring a substantial deficit of hybrids and excess of purebred *T. nativa* offspring ($X^2 = 99.4$, p < 1e-15). Thus, biased relative fitness among randomly mating individuals would have resulted in significantly different genotypic distributions (Table 1; $X^2 = 32.6$, p < 1e-6). In particular, we observed only about half as many hybrids of each type as would have been expected and almost twice as many

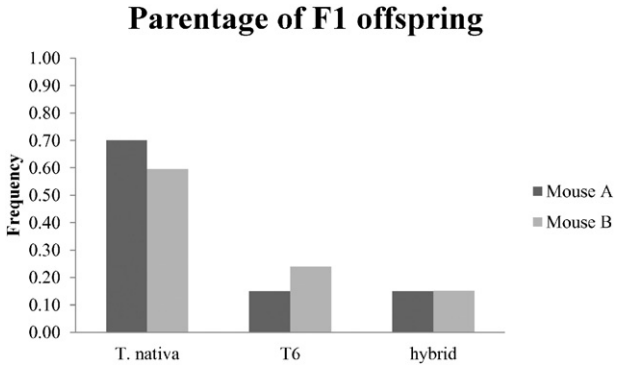

Fig. 1. Frequency of offspring collected from two mice coinfected with equal numbers of *T. nativa* and *Trichinella* T6. There was no significant difference between the proportions present in each mouse ($X^2 = 0.997$, df = 2, p = 0.607).

Table 1
Observed and expected F1 offspring frequencies. Expectations were developed for two null models: random mating with equal relative fitness, and random mating with fitness differences as reported by La Rosa et al., 2003 estimates.

Cross	Observed frequency of F1 offspring	Expected frequency of offspring — Random mating	Expected frequencies after Random mating assuming unequal fitness (La Rosa)
T. nativa$_f$ × *T. nativa*$_m$	0.64	0.25	0.39
T. nativa$_f$ × T6 $_m$	0.05	0.25	0.15
T6$_f$ × *T. nativa* $_m$	0.10	0.25	0.19
T6$_f$ × T6$_m$	0.21	0.25	0.27

Subscripts $_m$ and $_f$ in the cross column refers to male or female parent.

purebred *T. nativa* comprised the F1 population as would have been expected.

Relative fitness of these two lineages in the mouse strain used in this study may differ from what was reported by La Rosa et al. (2003), though both studies were conducted in outbred mice. In two passages of *T. nativa* in mice from the same source, a pool of 14,500 or 18,500 *T. nativa* worms were recovered from 5 mice infected with 500 worms each, yielding an average reproductive capacity index (RCI) of 6.6 worms produced for every worm given to the host. T6 produced 26,325 or 12,500 worms in similar passages for an average RCI of 7.8. While not informative of the relative fitness of hybrids in this particular mouse strain, T6 would be expected to have higher numbers of offspring than *T. nativa* if random mating occurred based on these RCI estimates.

4. Discussion

In experimental crosses in a mouse model system with many individuals from each lineage, *T. nativa* and *Trichinella* T6 were able to interbreed, but *T. nativa* produced many more offspring and contributed far more to the descendent populations at all characterized loci (see Supplemental information). In the first generation, pure-breeding *T. nativa* produced about three times as many progeny as did pure-breeding T6 parents. Furthermore, *T. nativa*/T6 hybrids accounted for only 15% of all offspring, indicating that pure-breeding parents had a greater than 5.6:1 advantage over hybrids. Among the hybrids in the F1 generation, 70% had T6 mothers and 30% *T. nativa* mothers. The observed proportions of offspring from each type of cross did not match a priori expectations for this population.

If *T. nativa* and T6 are different genotypes within the same species, it would be expected that coinfection with an equal number of worms from each lineage would result in one of two outcomes: random mating with equal relative fitness, or random mating with unequal relative fitness (La Rosa et al., 2003). However, crosses using 210 worms of each genotype revealed a dramatic excess of purebred *T. nativa* individuals. This requires explanation. Among the possible causes for this outcome are assortative mating, skewed sex ratios, and fitness differences among offspring.

Pre-zygotic barriers to hybridization may exist between *T. nativa* and T6, such as assortative mating. In the gut, male and female *Trichinella spiralis* are attracted to each other via pheromones. Females have been shown to be more attracted to males than males to females (Bonner and Etges, 1967). Assuming that chemoattractants play a role in mating among all species and genotypes, the observed 5.6:1 ratio of purebred offspring to hybrid offspring suggests that females and males are more strongly attracted to mates within their particular lineage resulting in assortative mating. Furthermore, the offspring of *T. nativa* mothers outnumber the offspring of T6 mothers by 2.4:1, where a ratio of 1.2:1 would have been expected based on previous estimates of relative fitness (La Rosa et al., 2003). This indicates that *T. nativa* may have a more efficient sensory system for attracting and finding mates. However, hybrids with T6 mothers outnumbered hybrids with *T. nativa* mothers by over 2:1, hinting that T6 mothers may not discriminate between T6 and *T. nativa* fathers as faithfully as *T. nativa* mothers. Even so, if assortative mating is solely responsible for the differences in the types of offspring observed in F1 populations, these ratios would indicate that within lineage mating occurred three times for every hybrid mating and that *T. nativa* mothers successfully mated twice as often as T6 mothers. Despite these inferences, assortative mating seems unlikely as a singular explanation for the observed F1 ratios. Pure T6 matings should not decrease because of greater attraction among *T. nativa*, unless chemoattractants from *T. nativa* somehow interfere with T6 signaling. This suggests that other factors contributed to the observed ratios of offspring.

Skewed sex ratios provide another factor that could have contributed to the outcome of the coinfections presented here, particularly the excess of purebred *T. nativa* offspring relative to purebred T6 offspring. *T. spiralis* males are able to impregnate as many as 4 females (Gardiner, 1976), suggesting that males are not a limiting factor in successful infections. In order to explain the excess of *T. nativa* offspring, *T. nativa* females would have to outnumber T6 females by at least 2:1, even after taking differences in expected fecundity into account. This seems unlikely as previous experiments showed that one *T. nativa* isolate from polar bear and one *T. spiralis* isolate had similar sex ratios (1.19 and 1.16, respectively) when passaged through mice (Belosevic and Dick, 1979) suggesting that sex ratios do not vary greatly among species of *Trichinella*. Additionally, sex ratios of *T. spiralis* in the intestine have been consistently shown to be 2:1 females to males (Boyd and Huston, 1954, Gursch, 1949, Rappaport, 1943) in other studies. Nevertheless, it is possible that differing numbers of gravid females among *T. nativa* and T6 could occur. However, while this might explain some of the competitive advantage of purebred *T. nativa* over purebred T6, this still does not explain the excess of hybrids with T6 mothers. Thus, several factors may have contributed to skewed numbers of offspring in the F1 generation prior to fertilization, but other hypotheses are required to fully explain the lack of hybrids in the experiments presented here.

When administered singly, infections of *T. nativa* and T6 resulted in similar larval recoveries in these mice (similar RCI), suggesting comparable relative fitness. Moreover, T6 produced slightly more muscle larvae than did *T. nativa*. This contrasts markedly with the underperformance of T6 when administered in coinfection, where *T. nativa* was the most fit (relative fitness = 1.0), followed by T6 (relative fitness of 0.33), and finally the hybrids, with a relative fitness of 0.16 (T6 mothers) or 0.07 (*T. nativa* mothers). The relative fitness inferred from reproduction in a population of worms may differ from estimates of relative fitness from pairs of worms because there are post-zygotic fitness penalties for T6 and T6:*T. nativa* hybrids after pre-zygotic selection has occurred.

Several post-mating barriers may have contributed to the predominance of *T. nativa* in the F1 generation. Genetic incompatibilities may affect hybrid relative fitness. If there were genetic incompatibilities between *T. nativa* and T6, hybrid larvae may be deficient in any number of characters including maturation rates and ability to penetrate host muscle tissue. These could have significantly reduced the number of hybrid larvae recovered from host tissue. Alternatively, the timing of release of offspring may have exposed later arriving newborn larvae (NBL) to a primed immune system. Belosevic and Dick (1979) showed that NBL production peaked 5–7 days post-infection (dpi) and 6–8 dpi for *T. nativa* and *T. spiralis* isolates, respectively, suggesting variability in timing of release of newborn larvae. If T6 and/or hybrid offspring were released later than *T. nativa* offspring, they might have encountered a host immune system primed for removing NBL from the

bloodstream. Thus, though similar numbers of offspring may have been generated by the differing crosses, late arriving offspring may have encountered stronger host immune response, reducing the viability of T6 and hybrid larvae. Undermining this possibility is the likelihood that, during a synchronic infection, most NBL leave the blood stream within hours of entering via the capillaries. Nonetheless, such factors could conceivably reduce T6 and hybrid offspring ensuing from coinfection. Further experiments would be required to understand the timing and durability of offspring as they relate to the overall infection process.

Taken altogether, *T. nativa* was more successful at producing offspring than either T6 or hybrid crosses. This might explain the observation made by La Rosa et al. (2003) that, when found in sympatry in Alaskan wolves, *T. nativa* outnumbered T6 by 2.2:1. The results here and those from the Alaskan wolves suggest that relative fitness among individual types of crosses is not the only factor influencing the results of hybridization between these two lineages. Assortative mating, sex ratios, and post-zygotic fitness may also be factors that contribute to and maintain barriers to gene flow between *T. nativa* and T6.

In a study involving samples from naturally-infected animals from Nunavut, Canada, Dunams-Morel et al. (2012) identified isolates with nuclear genomes resembling T6 but with mitochondrial genomes characteristic of *T. nativa* reference strains. Here, both nuclear and mitochondrial loci reflected increasing contributions from *T. nativa* parents. It may be that the parasite reference strains used in this study and previous experimental work (e.g. La Rosa et al., 2003) overrepresent biological distinctions between *T. nativa* and T6 in the wild due to the model system utilized, rendering the assortative mating and previously-described partial F1 sterility as mere artifacts of interactions between these genotypes and experimental hosts. However, the small Nunavut sample studied by Dunams-Morel et al. (2012) may be similarly exceptional to an overall trend of T6-biased mitochondrial introgression. It may be that the outcome of any hybridization event between these two lineages depends on the composition of the local assemblage of genotypes, dominated by the lineage that is most prevalent at that particular locality. Broader geographic sampling is needed to evaluate this possibility.

Natural opportunities for hybridization are limited to short windows in time where a host eats flesh infected with either parasite followed by an additional meal infected with the other. In the experimental cross reported here, with many individuals from each lineage simultaneously available as potential mates, F1 larvae derived from every possible type of cross were observed. If only one or a few mating pairs actually engage in the reproductive hybridization event within a new host, as might be expected in a natural infection, the resulting sampling error would tend to disadvantage rare variants. In this system, hybrids and the T6 genotype would be disadvantaged (La Rosa et al., 2003, and this study). Such bottlenecking could, over generations, exacerbate any degree of inbreeding, limiting opportunities for gene flow between *T. nativa* and T6 to cases where nascent infections are picked up from multiple sources within a short time frame.

Frequent hybridization between two closely related lineages can result in a range of evolutionary outcomes, ranging from homogenization to reinforcement of differences between lineages resulting in speciation. If replicated in nature, the tendencies exhibited in our experimental crosses would engender separation of the lineages into distinct biological species or the extinction of the less successful lineage (T6) in coinfections. In the first generation, fewer hybrid offspring were produced (relative to their true-breeding counterparts) than would have been expected in the absence of pre- or post-zygotic penalties. T6 may persist in nature owing to advantages not accounted for in this experiment, which was conducted in a convenient but unnatural laboratory host. In a natural setting, the scarcity of infected hosts coupled with the vastness of endemic geographical regions could account for well-preserved pockets of T6 genotypes. However, and to the extent that this experiment reflects natural circumstances, the tendency against hybridization would limit gene flow between these two lineages, serve as a barrier to their homogenization, and promote their persistence as distinct and separate entities.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.meegid.2015.12.016.

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