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EFFECTS OF CLONALITY IN MULTIPLE INFECTIONS ON THE LIFE-HISTORY STRATEGY OF THE TREMATODE *COITOCAECUM PARVUM* IN ITS AMPHIPOD INTERMEDIATE HOST

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Theoretical models predict that genetic relatedness affects the competition within and between parasite clonal groups sharing a common host. Here, we studied natural and experimental multiple infections of the trematode *Coitocaecum parvum* in its intermediate host. We focused on the effects of clonality on the life-history strategy of parasites competing for resources. *Coitocaecum parvum* can either delay maturation until its amphipod host is ingested by a definitive host, or adopt a progenetic strategy and reproduce inside the amphipod. Within a common host, clonal parasites were more likely to adopt identical life-history strategies than different genetic clones, both in natural and experimental infections. However, when timing of infection and other factors were controlled experimentally, parasites sharing a host were likely to adopt identical strategies regardless of their clonal identity, although pairs of clones were more likely to adopt progenesis than pairs of nonclones. The asymmetries in relative size and egg production between coinfecting parasites adopting the same life-history strategy were slightly, but not significantly, higher between different clones than identical clones. Our results suggest that the dynamics of competition between coinfecting parasites, although influenced by numerous external factors, is also modulated by genetic relatedness among parasites.

KEY WORDS: ***Coitocaecum parvum*, genetic relatedness, microsatellites, multiple infections, parasite life-history strategy, within-host competition.**

In situations in which a single parasite lives in a host, this parasite faces a simple trade-off between damaging its host, therefore destroying its resource supply, and the benefits of rapid growth and/or transmission (Frank 1992). The parasite's optimal strategy can be modulated by both host condition (age, sex, immune resistance, etc.) and the parasite's mode of transmission (Thomas et al. 2002a). For example, if the host has no value to the parasite other than as a food source, the parasite may benefit by quickly consuming all of the host resources, eventually killing it (Parker et al. 2003). In contrast, many parasites have complex life cycles with larval stages using intermediate hosts for both growth and transmission by predation to the next host in the cycle. In theory, because survival of the intermediate host is vital for transmission, a parasite larva should reduce its level of host exploitation to avoid killing the host and, therefore, increase its probability of reaching the next host.

In nature, free-living organisms often become infected by either several parasite species and/or different genetic strains of the same species (Thomas et al. 2002b; Bell et al. 2006; Poulin 2007). Consequently, conflicts of interest are likely to arise when two or more species of parasites share the same intermediate host but do not have the same definitive hosts (Lafferty 1999), or when different strains of the same pathogen have different life-history strategies (Gower and Webster 2005; Bell et al. 2006). Different parasites may thus attach a different value to the survival of their shared host. The presence of other genetic strains of the same

species within a host may influence the strategies adopted by individual parasites (Thomas et al. 2002b; Parker et al. 2003). Because parasites rely only on the limited space and energy available within the host, several individuals using the same host may be faced with resource constraints that could influence their growth rates and life-history strategy (Davies et al. 2002; Brown et al. 2003; Fredensborg and Poulin 2005; Poulin and Lefebvre 2006). Generally, within-host competition is assumed to select for higher levels of host exploitation when host resources are limited. Genetic relatedness may also influence the strategy used by parasites sharing a host: closely related individuals should exploit the resources of a common host in a more cooperative and equitable manner (through kin selection) than unrelated individuals (Frank 1992; Griffin and West 2002). If so, parasites should be able to detect the presence, as well as the number, and genetic relatedness of other parasite individuals within their host, before adopting a particular strategy.

The trematode *Coitocaecum parvum* (Opecoelidae) is a common parasite of freshwater fish in New Zealand (MacFarlane 1939; Holton 1984a), mainly the common bully (*Gobiomorphus cotidianus*). Eggs are released in fish feces and hatch into free-swimming larvae (miracidia). Miracidia penetrate snails in which they multiply and develop into sporocysts. Sporocysts asexually produce free-living cercariae that leave the snail and then enter the amphipod *Paracalliope fluviatilis* in which they encyst as metacercariae in the body cavity. At this stage, metacercariae can adopt two very distinct strategies: they can either await ingestion by a fish in which they will grow, mature, and reproduce (the normal three-host cycle), or keep growing and reach maturity although still inside the amphipod (the progenetic cycle). Field data indicate that, on average, 35% of worms in a well-sampled population adopt the latter strategy (Lagrue and Poulin 2008a). Worms that reach maturity in the crustacean intermediate host reproduce by selfing and lay eggs within their cyst (Holton 1984b; Poulin 2001). After the death of the amphipod host, eggs produced by selfing hatch into larvae that are infective to the snail first host without the need to pass through a fish host. Worms adopting the normal three-host cycle, meanwhile, await ingestion by a fish before they reach maturity and reproduce. There is an obvious situation of conflict in cases in which several worms have to share the same amphipod host but adopt different life-history strategies. On the one hand, individual parasites adopting the classical three-host cycle need their amphipod host to be eaten by a fish; although they are incapable of altering amphipod phenotype in any way that would enhance fish predation, they somehow increase amphipod mortality relative to that of amphipods harboring progenetic conspecifics (Poulin 2001). On the other hand, individuals adopting progenesis would benefit by keeping the amphipod host alive as long as possible to maximize their egg output, meanwhile using most, if not all, of the space and resources available in the host to swiftly grow and reproduce. Therefore, in cases of multiple infections, there is a conflict of interests between the two distinct life-history strategies that is potentially very costly for one of them (see Dezfuli et al. [2001] for an example in acanthocephalans). Their requirements for the intermediate host are very different: long life for egg production in the case of progenetic worms versus ingestion by a definitive host fish in the case of "normal" worms (i.e., worms adopting the three-host cycle). The higher mortality induced by normal worms (see Poulin 2001) is perplexing because when the amphipod host dies before being eaten by a fish, a "normal" worm also dies without any offspring being produced; however, what leads to mortality in the laboratory may be part of a general debilitation of the host that could enhance rates of fish predation in nature. Whatever the causes of the higher host mortality induced by normal worms, it could have negative fitness consequences for progenetic worms sharing the same amphipod.

Cercariae produced by each sporocyst within snails are genetically identical, released in batches of three to 10 individuals, and have a limited capacity of dispersion (they are tailless and must crawl along the substrate, that is, they do not float or swim; MacFarlane 1939). The amphipod hosts also frequently settle upon a snail host for several minutes, coincidentally exposing themselves to infection by clonal cercariae (MacFarlane 1939). Consequently, it is possible that individual metacercariae found coinfecting the same amphipod host are genetically identical clones produced by the same sporocyst. Whether genetic relatedness between co-occurring *C. parvum* influences their developmental strategy remains to be investigated. Clones sharing the same amphipod host may adopt the same strategy because of genetic determinism but also because the inclusive fitness of one individual depends on how its strategy affects the success of its clones as well as its own success. Mechanisms of kin recognition are unknown in trematodes, but perhaps not necessary: other factors, such as nearly synchronous infection, could serve as cues of genetic relatedness for co-occurring parasites and influence their subsequent selection of particular developmental strategies.

Here, we tested the hypothesis that individuals of *C. parvum* sharing the same amphipod intermediate host are more likely to adopt the same life-history strategy if they are genetic clones. We first determined whether identical clones of this parasite can be found sharing the same host in natural infections, and examined whether clonality is associated with the developmental strategies adopted by these parasites in nature. Second, because many variables other than genetic relatedness can influence the development of parasites in naturally infected amphipods, we also used experimental infections, with either single clones or mixtures of clones, to control for these other potential effects on the life-history strategies adopted by parasites.

Materials and Methods

ANIMAL COLLECTION

Naturally infected snails (*Potamopyrgus antipodarum*) and amphipods (*Paracalliope fluviatilis*) were collected from macrophytes along the shoreline of Lake Waihola, South Island, New Zealand, using dip nets in August 2005 and August 2006. Snails and amphipods were kept alive in aerated lake water. Amphipods were never kept more than a week in the laboratory before dissections or controlled infections, because the warmer laboratory temperatures and stress proved to have significant effects on amphipod survival and parasite development (Poulin 2003; Lagrue and Poulin 2007). Infected snails were obtained by selectively choosing individuals that displayed an altered shell shape, a sure sign of infection by *C. parvum* (Lagrue et al. 2007a). Uninfected amphipods used for experimental infections were obtained by inspecting each amphipod under a microscope and discarding all amphipods that showed any sign of infection, that is an opaque mass in the body cavity corresponding to a metacercaria. This method allows the selection of only uninfected individuals with an accuracy of about 95% (Lefebvre and Poulin 2005). Because host condition (age, size, sex) can influence the outcome of within-host competition between coinfecting trematodes (Parker et al. 2003), we used only adult male amphipods of similar size (and therefore age) as carrying hosts in our experimental infections, minimizing the variation in body condition among hosts.

NATURAL INFECTIONS

Amphipods were killed in 70% ethanol to facilitate handling, rinsed in distilled water, and screened under a binocular microscope to visually assess the presence of *C. parvum* metacercariae through the amphipod cuticle (Lefebvre and Poulin 2005). This method kills the amphipod but not the metacercariae within the host; therefore it has no effect on parasite measurements. Because the prevalence of *C. parvum* was low (< 9%; Hansen and Poulin 2006), only amphipods judged as infected were dissected. Amphipods harbor between one and six metacercariae, but the vast majority harbor one or two. When amphipods containing exactly two metacercariae were found, each metacercaria was categorized, based on the presence or absence of eggs, as either "normal" (non–egg-producing) or "progenetic" (egg-producing). These included both eggs released by the metacercaria in its thin-walled cyst and shelled eggs still in utero. Pairs of metacercariae were divided into three classes based on "pair status": progenetic pairs, when both parasites had produced eggs; normal pairs, when neither individual had produced eggs; and mixed pairs, when one individual had already produced eggs but not the other. Metacercariae were then preserved individually in Eppendorf tubes containing 50 μl of 100% ethanol before genotyping.

EXPERIMENTAL INFECTIONS

Cercariae of *C. parvum* were obtained from snails under controlled conditions to ensure that the cercariae used to experimentally infect amphipods were freshly released and, therefore, more likely to penetrate the amphipod. For experimental infections, 40 infected snails were haphazardly chosen from a stock of approximately 100 infected snails and transferred to individual petri dishes filled with 5 mL of filtered lake water. Snails were incubated at 25°C for 20 min under constant light, conditions known to induce cercarial release (Hay et al. 2005). The petri dishes were then screened under a dissecting microscope and the cercariae that were found were transferred to 500 μl Eppendorf tubes using a 20-μl micropipette. Snails were returned to the stock for later use. Three cercariae from either the same individual snail or three different snails were placed in each tube with 2.5 μl of filtrated lake water and an uninfected amphipod was then added. Almost 75% of infected snails from our source population harbor only a single *C. parvum* clone, with the rest usually harboring two or three clones, and no clone ever occurring in more than one snail (Lagrue et al. 2007a); thus, the above procedure was guaranteed to generate both single-clone and mixed-clones infections. Amphipods were left in the tube along with the three cercariae for 5 h, a time after which unsuccessful cercariae stop moving and die. Amphipod survival, at this stage, was over 99%. Amphipods were then separated into groups of seven to nine individuals (mean ± SE: 7.8 ± 0.1). Each group was placed in a plastic Falcon tube filled with 10 mL of lake water, and a strand of macrophyte (*Elodea canadensis*) was added for food; all tubes were maintained in the same water bath, with their position randomized. These conditions should not constrain parasite growth, as food and space were not limiting factors: amphipods were kept at densities much lower than field densities and given a surplus of food. Six hundred and thirty-eight amphipods were experimentally infected for the purpose of this study. They were maintained for 7 weeks, a time sufficient for the metacercariae to grow and adopt one or the other strategy (Lagrue and Poulin 2007). At that point, there were 0–6 amphipods (mean ± SE: 2.6 ± 0.2) left alive per tube; there was no significant difference in survival between amphipods exposed to cercariae from the same snail and those exposed to cercariae from different snails (Fisher's exact test: $\chi^2 = 0.36$, $P = 0.548$). The 201 surviving amphipods were dissected under the microscope to assess their infection status. Because amphipods were experimentally exposed to three cercariae, individuals harbored from zero to three metacercariae: 66 were uninfected, 30 harbored a single metacercaria, 80 had two metacercariae, and 25 had three. There was no significant difference between amphipods exposed to cercariae from the same snail and those exposed to cercariae from different snails in either the probability of becoming infected (Fisher's exact test: $\chi^2 = 0.17$, $P = 0.738$) or the mean number of parasites acquired (Mann–Whitney U-test: $Z = -1.51$,

Table 1. Microsatellite loci developed for this study. Locus name is followed by repeat motif of cloned allele, primer sequences, forward (F) and reverse (R), primer-annealing temperature (T_a), and GenBank accession number.

Locus	Repeat motif	Primer sequences (5′−3′)	T_a (°C)	Accession no.
Cpa-41	$(GA)_{11}(GA)_{19}$	F: CACACCCGTTATATTCAATAC	47	EU203672
		R: CAATATCAGTTTTCTGCTCTC		
Cpa-48	$(GT)_{16}$	F: GAAATGAAATATGGGTATCGTTGTG	64	EU203673
		R: CGTTCGCCATCGACATACAC		
Cpa-60	$(TG)_7(TG)_7(TG)_6$	F: GAGGGCTTTATGTATGTGTG	55	EU203674
		R: CTGATTAGTCTTCTCGCATAG		
Cpa-62	$(AC)_9$	F: GCGTAAAATGGGCGTAAGTG	62	EU203675
		R: GACTTCACGCACCCAGAGTG		
Cpa-63	$(GA)_{22}$	F: CATCCGATTCATCCGATTC	55	EU203676
		R: GGATATCAAAATACTTTGAACGG		
Cpa-64	$(AC)_9$	F: GAATCTGACACTACTGGTCCT	65	EU203677
		R: GTTCGTACTAAAGTTGTGGG		

$P = 0.132$). To avoid any potential confounding effects from competition between individual parasites sharing the same host (see Thomas et al. 2002b; Parker et al. 2003), we only used amphipods with double infections (two metacercariae per amphipod) in our analyses. Parasites found in these experimental infections were measured (length and width) and then processed as described for natural infections. The body surface of each metacercaria was later determined and used as a surrogate for body size. This was done using the formula for an ellipsoid, $(\pi LW)/4$, in which L and W are the length and width of the parasite.

GENOTYPING

A total of 564 metacercariae were recovered from naturally (404 individuals) and experimentally (160 individuals) parasitized amphipods. Because each snail host can contain up to five parasite genotypes (Lagrue et al. 2007a), all metacercariae collected in both natural and experimental infections were genotyped. DNA was first extracted by placing metacercariae individually in Eppendorf tubes with 400 μl of 5% Chelex containing 0.1 mg/mL proteinase K, incubating at 60°C for 2 h and then 90°C for 10 min. The genotypes of metacercariae were then determined using eight of the nine microsatellite loci (Cpa-3, Cpa-4, Cpa-8, Cpa-12, Cpa-19, Cpa-26, Cpa-28, Cpa-29) described in Lagrue et al. (2007b) plus six additional loci developed for this experiment (Cpa-41, Cpa-48, Cpa-60, Cpa-62, Cpa-63, and Cpa-64; see Table 1 for details). All of the 14 loci were used for metacercariae from natural infections whereas individual metacercariae recovered from experimental infections were genotyped at 10 loci (Cpa-3–Cpa-48). The number of alleles per locus, expected and observed heterozygosities, deviations from Hardy–Weinberg expectations for each locus and across all loci, F_{IS} (Weir and Cockerham 1984), and tests of genotypic disequilibrium between all pairs of loci were calculated separately for natural and experimental infections with GENEPOP version 3.4 (Raymond and Rousset 1995) using unique genotypes (identical genotypes are the result of asexual reproduction within snail first intermediate host). Significance of deviations from Hardy–Weinberg expectations was determined using the Markov chain exact probability test of Guo and Thompson (1992) and significance of linkage disequilibrium values was determined with Fisher's exact test as implemented in GENEPOP version 3.4 (both tests used 10,000 dememorizations, 1000 batches, and 10,000 iterations per batch). Alpha significance levels were corrected for multiple simultaneous pairwise comparisons using the sequential Bonferroni approach (Rice 1989) for Hardy–Weinberg (initial $\alpha = 0.0036$ for natural infections and $\alpha = 0.005$ for experimental infections) and disequilibrium (initial $\alpha = 0.0038$ and 0.0056, respectively) analyses. In natural infections, the probabilities of observing at least as many identical parasite genotypes by chance were estimated using the program GenClone1.1 (Arnaud-Haond and Belkhir 2007). GenClone1.1 takes into account departure from Hardy–Weinberg equilibrium when calculating probabilities that two metacercariae possessing the same multilocus genotype are the result of sexual reproduction rather than being truly genetic clones. These probabilities were not calculated for experimental infections as multiple replicates of the same genotypes were expected.

STATISTICAL ANALYSES

Within each type of infection (natural or experimental), the proportions of pairs of clones (two genetically identical metacercariae found in the same amphipod) were compared between pair status classes (progenetic, normal, or mixed) in a pairwise manner using Fisher's exact tests. In the case of experimental infections, the proportions of clone pairs were compared in two ways, first using the total number of nonclone pairs, and then using only nonclone pairs from single-host origin to account for possible effects of snail host origin (because metacercariae in a nonclone pair came either from the same snail or from different snails).

For experimental infections, the effect of life-history strategy on parasite body size was tested using a one-way analysis of variance (ANOVA) with the size of the metacercaria used as the dependent variable. The body area of the parasite was log transformed before analyses to normalize the data. Differences in egg production between progenetic parasites sharing their host with either another progenetic parasite or a normal one were examined using nonparametric Mann–Whitney *U*-tests, with the number of eggs used as the dependent variable; only parasites that had produced at least one egg were included in this analysis. A linear regression between the size of the parasite and the number of eggs produced was also used to assess the effect of parasite size on egg production. Because the size and egg production of parasites sharing the same amphipod host cannot be considered independent, only one randomly chosen individual metacercaria per pair was used in the above analyses.

The effects of genetic relatedness (clone or nonclone) on differences in metacercarial size or in egg production between metacercariae sharing the same amphipod host were tested using nonparametric tests (Mann–Whitney *U*-test); these tests were performed separately for progenetic pairs and normal pairs. We also compared the average body sizes and egg output of pairs of clonal and nonclonal metacercariae sharing the same amphipod host, using Mann–Whitney *U*-tests, to determine whether parasites perform better when co-occurring with clones than with nonclones. Finally, because nonclone pairs in experimental infections can originate from either the same snail or from two different snails (see Materials and Methods), we compared the sizes and egg outputs of nonclone pairs from single and multiple host origins to determine whether a shared snail origin can influence differences in growth and reproduction within a pair.

Table 2. Number of alleles (*A*), observed heterozygosity (H_O), expected heterozygosity (H_E), and F_{IS} for each microsatellite locus and across all loci used in (A) natural infections and (B) experimental infections.

(A)	Locus	*A*	H_O	H_E	F_{IS}
	Cpa-3	5	0.997	0.517	−0.931
	Cpa-4	3	0.950	0.539	−0.764
	Cpa-8	17	0.059	0.859	0.932
	Cpa-12	13	0.086	0.652	0.868
	Cpa-19	14	0.123	0.701	0.825
	Cpa-26	7	0.109	0.396	0.726
	Cpa-28	3	0.122	0.504	0.757
	Cpa-29	2	0.011	0.176	0.937
	Cpa-41	8	0.114	0.652	0.825
	Cpa-48	13	0.056	0.648	0.914
	Cpa-60	5	0.075	0.583	0.871
	Cpa-62	2	0.006	0.365	0.985
	Cpa-63	15	0.069	0.743	0.908
	Cpa-64	2	0.024	0.471	0.950
	All loci	109	0.200	0.558	0.642
(B)	Locus	*A*	H_O	H_E	F_{IS}
	Cpa-3	3	0.984	0.553	−0.786
	Cpa-4	3	0.856	0.557	−0.539
	Cpa-8	15	0.096	0.912	0.895
	Cpa-12	14	0.128	0.844	0.849
	Cpa-19	12	0.168	0.819	0.796
	Cpa-26	7	0.240	0.765	0.687
	Cpa-28	2	0.128	0.485	0.737
	Cpa-29	3	0.016	0.294	0.946
	Cpa-41	8	0.224	0.638	0.650
	Cpa-48	12	0.064	0.847	0.925
	All loci	79	0.290	0.671	0.568

Results

GENETIC DATA

The 14 loci used for this study possessed 2–17 alleles and observed heterozygosities of 0.011–0.997 (Table 2). All loci deviated significantly from Hardy–Weinberg expectations ($P < 0.0001$) in both natural and experimental infections, and significant disequilibrium was detected in 61 of 91 and 26 of 45 pairwise comparisons in natural and experimental infections, respectively. The majority of observed deviations from Hardy–Weinberg expectations and disequilibrium were not unexpected as a high proportion of metacercariae in natural populations produce eggs by selfing in their amphipod host (progenesis). This reproductive strategy can lead to inbreeding and subsequently high F_{IS} values (Jarne and Auld 2006), as detected in our study (overall $F_{IS} = 0.642$ in natural infections and $F_{IS} = 0.568$ in experimental infections), as well as high levels of disequilibrium between loci (Annan et al. 2007). Two loci (Cpa-3 and Cpa-4) displayed excess of heterozygotes, which may reflect the influences of processes such as clonal reproduction by miracidia (Balloux et al. 2003) (although this would affect the entire genome and would likely be exhibited by a majority of loci) or associative overdominance (Ohta and Kimura 1970) on these loci. GenClone1.1 results indicated that pairs of metacercariae with identical genotypes found sharing the same host could be classified as true genetic clones (all P_{sex}-values < 0.05).

MULTIPLE INFECTIONS AND PARASITE LIFE-HISTORY STRATEGY

Amphipod body length did not differ between natural and experimental infections (3.57 ± 0.04 and 3.58 ± 0.03 mm, respectively, for naturally and experimentally infected amphipods; ANOVA, $F_{1,305} = 0.56$, $P = 0.454$). Host individuals used in the experiment thus belong to the same size classes as those found infected in nature.

Table 3. Results of (A) parasite genotyping showing the number of pairs of metacercariae within each "pair status" class for natural infections, and (B) Fisher's exact tests for pairwise comparisons of the proportion of clone pairs (two genetically identical metacercariae in the same host) between pair status classes.

	Number of pairs		
(A) Pair status classes	Total	Clones	Nonclones
Progenetic	49	1	48
Normal	68	4	64
Mixed	85	0	85

(B) Pair status classes compared	χ^2	*P*-value
Normal vs. mixed	5.13	0.0371
Progenetic vs. mixed	1.75	0.3657
Normal vs. progenetic	1.03	0.3004
(Normal + progenetic) vs. mixed	3.72	0.0626

Among the 202 pairs of metacercariae recovered in natural infections, five pairs (2.5%) were found to be genetic clones: four normal and one progenetic (Table 3A). Although not statistically significant, probably because of the small sample size, the proportion of clones was higher in pairs in which both metacercariae adopted the same life-history strategy (normal and progenetic pairs; 4.3%) than in pairs in which the two metacercariae used different strategies (mixed pairs; 0%). There was a significant difference in the proportion of clones between normal (5.9%) and mixed pairs (0%; Fisher's exact test, $\chi^2 = 5.13$, and $P = 0.0371$; see Table 3B for all pairwise comparisons).

Within experimental infections, the proportion of genetic clones was significantly higher among the pairs of metacercariae adopting the same strategy (progenetic or normal: 58.9%; see Table 4) than in mixed pairs (0%). There was a significant difference in the proportion of clones between normal (49%) and mixed (0%), and between progenetic (82%) and mixed pairs (0%; Table 4). In addition, clonal pairs adopted progenesis more frequently than did nonclone pairs (42% of 43 vs. 11% of 37; Fisher's exact test, $P < 0.001$). All trends documented above were similar when nonclone pairs were controlled for host origin (i.e., single or multiple-host origin; see Table 4). In other words, the clear tendency for the proportion of clones to be higher amongst pairs of metacercariae adopting the same strategy than amongst those adopting different strategies is not influenced by whether the comparison is made with nonclones originating from the same snail or from different snails.

SIZE AND EGG PRODUCTION IN EXPERIMENTAL INFECTIONS

Overall, the parasite life-history strategy had a significant effect on the mean body size of *C. parvum* metacercariae: progenetic parasites were significantly larger than normal ones (0.149 ± 0.003 and 0.049 ± 0.003 mm^2, respectively; ANOVA, $F_{1,78} = 98.347$, $P < 0.0001$).

The numbers of eggs produced per progenetic individual were not different between progenetic metacercariae sharing their amphipod host with either a normal or another progenetic worm (14.8 ± 1.9 and 19.3 ± 5.3, respectively; Mann–Whitney *U*-test, $Z = -0.357$, $n = 29$, $P = 0.746$). However, the number of eggs produced increased significantly with the size of the metacercariae ($r = 0.607$, $n = 29$, $P = 0.0005$).

Although tendencies were observed, no significant effect of genetic relatedness was found in differences in egg production

Table 4. Results of (A) parasite genotyping showing the number of pairs of metacercariae within each "pair status" class for experimental infections, and (B) Fisher's exact tests for pairwise comparisons of the proportion of clone pairs (two genetically identical metacercariae in the same host) between pair status classes. The proportions of clone pairs were compared using either the total number of nonclone pairs or only nonclone pairs from single-host origin to account for possible effects of snail host origin.

	Number of pairs				
			Nonclones		
(A) Pair status classes	Total	Clones	Total	Single-host origin	Multiple-host origin
Progenetic	22	18	4	2	2
Normal	51	25	26	11	15
Mixed	7	0	7	4	3

	Total		Single-host origin	
(B) Pair status classes compared	χ^2	*P*-value	χ^2	*P*-value
Normal vs. mixed	6.03	0.0142	7.41	0.0149
Progenetic vs. mixed	15.10	0.0002	14.40	0.0014
Normal vs. progenetic	6.83	0.0079	3.05	0.0749
(Normal + progenetic) vs. mixed	8.92	0.0032	10.84	0.0049

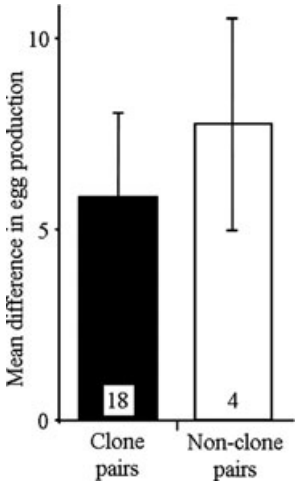

Figure 1. Mean egg production differences (± SE) between metacercariae sharing the same amphipod host for progenetic pairs in experimental infections. Numbers inside bars are sample sizes.

(5.8 ± 0.003 and 7.8 ± 0.006; Mann–Whitney *U*-test, Z = −1.192, $n = 22$, $P = 0.233$; Fig. 1) or size differences (Mann–Whitney *U*-test, Z = −0.784, $n = 22$, $P = 0.433$; Fig. 2) between progenetic metacercariae found in clone and nonclone pairs.

For pairs of normal metacercariae, the mean size difference between parasites sharing the same host was higher when found in nonclone pairs (0.0104 ± 0.0029) than in clone pairs (0.0077 ± 0.0024) although the difference was not statistically significant (Mann–Whitney *U*-test, Z = −1.80, $n = 51$, $P = 0.071$; Fig. 2).

The above comparisons concern differences in size or egg output between metacercariae sharing the same host; we also examined mean values of size and egg output for pairs of metacercariae sharing the same host. There was no significant difference

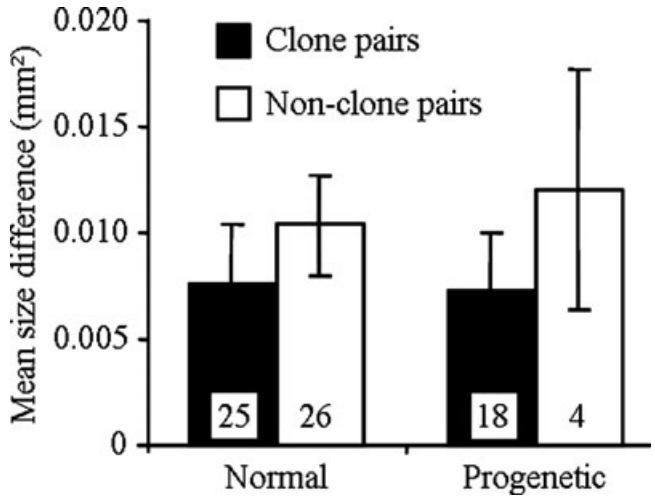

Figure 2. Mean size differences (mm² ± SE) between metacercariae sharing the same host in experimental infections. Numbers inside bars are sample sizes.

in mean body size between clone and nonclone pairs of metacercariae either in normal (0.052 ± 0.006 and 0.045 ± 0.005 mm², respectively; Mann–Whitney *U*-test, Z = 0.782, $n = 51$, $P = 0.434$) or progenetic pairs (0.148 ± 0.005 and 0.150 ± 0.015 mm², respectively; Mann–Whitney *U*-test, Z = 0.255, $n = 22$, $P = 0.798$). Furthermore, there was no difference in mean egg production between clone and nonclone pairs of progenetic parasites (14.9 ± 3.8 and 14.1 ± 3.3, respectively; Mann–Whitney *U*-test, Z = −0.639, $n = 22$, $P = 0.522$).

EFFECTS OF SNAIL ORIGIN

In experimental infections, there was no difference in the proportion of normal, progenetic, and mixed pairs of metacercariae between nonclone pairs originating from either the same snail host or two different snails ($\chi^2 = 1.40$; df = 2; $P = 0.497$). However, in normal individuals, we found a slightly significant size difference between metacercariae from single and multiple-host origin (0.055 ± 0.006 and 0.038 ± 0.004 mm², respectively; Mann–Whitney *U*-test, Z = −2.199, $n = 109$, $P = 0.028$). Finally, no significant effect of single- or multiple-host origin was detected on either size (Z = −0.353, $n = 51$, $P = 0.724$) or egg production (Z = −0.059, $n = 51$, $P = 0.953$) in progenetic individuals.

When comparing infections originating exclusively from single snail hosts, no significant differences were detected in size or egg production between metacercariae found in clonal and nonclonal infections in either normal (body size, Z = −0.675, $n = 36$, $P = 0.5$) or progenetic pairs (body size, Z = −0.611, $n = 20$, $P = 0.541$; egg production, Z = −0.398, $n = 20$, $P = 0.691$). Furthermore, the mean size difference in nonclone normal pairs was not significantly different between pairs from single-host (0.0101 ± 0.0032) and multiple-host origin (0.0106 ± 0.0035; Mann–Whitney *U*-test, Z = 0.389, $n = 26$, $P = 0.697$). Although no significant differences were found, the mean size difference between parasites sharing the same host was lower in clone (0.0076 ± 0.0024) than in nonclone pairs, regardless of the host origin (Z = −1.892, $n = 36$, $P = 0.059$, and Z = −1.151, $n = 40$, $P = 0.249$, for single and multiple host-origin, respectively). No statistical tests were run on progenetic pairs as the sample sizes were too small.

Discussion

Our results show that clonal trematodes do occasionally share the same host in nature, and more importantly, our study is the first to demonstrate that genetic relatedness may influence the life-history strategy adopted by trematode individuals sharing the same host. The fact that clonal parasites co-occurring in the same host adopt the same strategy could be the result of genetic determinism, but is also compatible with a plastic developmental response to being with kin. The genetic diversity of infections is believed to play

a central role in the evolution of pathogen virulence and/or life-history strategies (e.g., Frank 1992; Bell et al. 2006; Michaud et al. 2006). We provide a rare empirical test of this general hypothesis involving a metazoan parasite.

In situations in which genetically diverse infections are common, parasites should evolve to become more virulent (Bell et al. 2006). Consequently, when studying the effect of genetic relatedness on parasite life-history strategy, the first question that needs to be answered is: within a parasite population, what are the relative proportions of genetically diverse versus clonal multiple infections? Previous studies found that, whereas first intermediate hosts are usually infected by only one or a few trematode clones, infections in second intermediate and definitive hosts are characterized by high genetic diversities (Criscione and Blouin 2006; Keeney et al. 2007a,b). Typically, a majority of hosts is infected by combinations of unique genotypes but a few also possess batches of genetically identical parasites, potentially setting the stage for differential interactions within and between parasite strains inside the host (Keeney et al. 2007b). Rauch et al. (2005) suggested that the dispersion abilities of both the parasite larvae and the second intermediate host (a fish in their study) could induce a high spatial dispersion of parasite genotypes within the second intermediate host population. However, the extent of genetic dispersion may vary according to trematode species (because of prevalence in first intermediate hosts, dispersal capacities; Rauch et al. 2005) and/or environmental characteristics (tidal flow, temperature; Keeney et al. 2007a). Contrary to other trematode species in which a high proportion of first intermediate hosts are infected and produce very mobile cercariae (Rauch et al. 2005; Keeney et al. 2007a), the prevalence of *C. parvum* in snail first intermediate hosts is generally low (< 5%; Lagrue and Poulin 2008a) and infected snails produce cercariae with very limited dispersal ability. Both intermediate host species (snails and amphipods) of *C. parvum* also have limited dispersal ability compared to the vertebrate intermediate hosts used by other trematode species; this suggests that the likelihood of second intermediate hosts being exposed to genetically identical cercariae could be greater than in previously documented systems. However, our results are consistent with these earlier studies; we found that only 2.5% of naturally infected amphipods harboring two trematodes contained genetically identical metacercariae. Although the occurrence of identical clones sharing the same host is low, the fact that, within the five pairs of clones, the two coinfecting metacercariae always adopted the same strategy suggests that the genetic relatedness between parasites sharing the same host may still influence their life-history strategy.

Several theoretical models predict that the relatedness of parasites within a common host could induce conditional responses in terms of growth rate and/or life-history strategy (Frank 1992; van Baalen and Sabelis 1995). Such adjustments in life-history strategies would imply that parasites are able to detect coinfecting parasite genotypes and respond accordingly. However, the mechanisms by which individual trematodes assess their genetic relatedness with conspecific individuals sharing the same host remain unclear. First, parasites could use chemical cues to detect the presence and genetic relatedness of competing trematode larvae, either by directly releasing chemical substances within the host that can be recognized by their kin (Taylor et al. 1998; Parker et al. 2003) or by detecting the host immune response to infection assuming that each parasite genotype induces a specific immune response (Puustinen et al. 2004; Jäger and Schjørring 2006). Second, in addition to the genetic relatedness of coinfecting parasites, the timing of infection events, that is whether infections are simultaneous or sequential, could influence the strategy of competing parasites. Although identical clone infections are likely to be synchronous (Keeney et al. 2007b), multiclone infections can arise from a single infection event with genetically different parasite larvae but, because trematode first intermediate hosts are typically infected by one or very few parasite genotypes, the accumulation of different clones by second intermediate hosts is more likely to occur through time (Read and Taylor 2001; Rauch et al. 2005). The delay between two infection events could be used by individual parasites as an indication of genetic relatedness to consequently adjust their growth and life-history strategy. In certain situations, following sequential infections, strong competition can exist and the first inoculum may have a significant advantage (Hood 2003). However, the relative importance of each mechanism cannot be assessed from natural infections because the time gap between each infection event is unknown and many other environmental factors can also modulate parasite life-history strategies.

By using synchronous experimental infections, we were able to test for the true effect of genetic relatedness on parasite life history. Our results show that a large majority of pairs of metacercariae (91%) recovered from experimental infections had adopted the same life-history strategy, regardless of their genetic relatedness, or of whether they originated from the same snail or from different snails. This last point is important, because a clone is associated with one snail only, and thus any effects that parasite genetics and host identity may have are potentially entangled. The analyses we performed rule out any effect of host origins on the subsequent life-history route adopted by metacercariae. Our findings suggest that simultaneity of infection may be an important factor influencing parasite life-history strategy. Still, although infections were synchronized, all parasite pairs (seven of seven) that adopted different life-history strategies were not genetic clones. More importantly, when both metacercariae in an amphipod displayed the same life-history strategy, clonal pairs were significantly more likely to adopt progenesis than nonclonal pairs. As progenesis is the strategy with lower virulence (Poulin

2001), this may be a kin-selected response to the presence of a clone. Therefore, both the synchrony of infection and the genetic relatedness of co-occurring parasites influence the strategy adopted by these parasites. Similarly, Jäger and Schjørring (2006) found that both genetic relatedness and time lag between two infection events by the cestode *Schistocephalus solidus* affected establishment and growth rates within its second intermediate host. Similarly, using only pairs of parasites adopting the same strategy, we found higher size asymmetry, although not quite statistically significant, in nonrelated parasite pairs than in pairs of clones. This trend suggests that the competition between two coinfecting trematode metacercariae is a little bit stronger between genetically different parasites than between true clones.

It has been suggested that host condition (age, size, sex), and consequently the host resources available to pathogens, could be a factor influencing the life-history strategy of trematodes and possibly the outcome of within-host competition between coinfecting parasites (Parker et al. 2003). However, by using only males of similar size (and therefore age) as carrying hosts in our experimental infections, we minimized the variation in body condition among hosts. Still, resource constraints can have an impact on progenetic parasites: metacercariae sharing an amphipod with conspecifics or with other trematode species are not as likely to achieve large sizes as those found singly within the host, and if they become progenetic their egg output will also be influenced by co-occurring parasites (Lagrue and Poulin 2008b). This suggests that host resource availability may affect the reproductive output of progenetic parasites and could possibly have an effect on trematode life-history strategy depending on host body condition and the number of coinfecting parasites.

In conclusion, we showed that, although genetic clones found in coinfection in the natural environment are rare, the genetic relatedness between trematodes sharing the same host seems to affect the life-history strategy of the parasites. Genetic clones were more likely to adopt the same life-history strategy than nonclonal metacercariae. In simultaneous experimental infections, parasites infecting the same host are likely to adopt the same strategy regardless of their genetic clonality. Still, relatedness matters among metacercariae sharing the same host: clonal pairs were more likely to adopt the progenetic strategy than nonclonal pairs. However, we did not find significantly larger size and egg production asymmetries in nonclonal parasite pairs than in pairs of clones, indicating that genetic relatedness does not greatly affect parasite growth. Earlier, we observed that external cues associated with the presence of fish-definitive hosts also affect the life-history strategy adopted by *C. parvum* (Poulin 2003; Lagrue and Poulin 2007); combined with the present results, this means that the flexible developmental strategy of the parasite is shaped by a range of factors. We cannot determine the relative importance of each of these factors from our results. Nevertheless, they underline the fact that within-host interactions between conspecific parasites are modulated by several factors, including genetic relatedness.

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