The influence of clonal diversity and intensity-dependence on trematode infections in an amphipod

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SUMMARY

Individual animals are often infected not only by different parasite species, but also by multiple genotypes of the same parasite species. Genetic relatedness among parasites sharing a host is expected to modulate their strategies of resource exploitation, growth and virulence. We experimentally examined the effects that genetic diversity and infection intensity had on host mortality, infectivity and growth of the marine trematode *Maritrema novaezealandensis* in amphipod hosts. The presence of 2 versus 1 parasite genotype during infection did not influence subsequent host mortality, had different effects on infectivity among genotypes and did not influence growth or variation in parasite growth. Density-dependent growth reductions revealed that the number of parasites infecting a host was more important than their genetic relatedness. Temperature, host size, and host sex influenced the degree to which density-dependent factors affected parasite growth. Our results suggest that the effects of parasite relatedness vary among parasite genotypes in this trematode species, and reveal that many factors play an important role during parasite development and transmission.

Key words: amphipods, clonality, growth, *Maritrema novaezealandensis*, microsatellites.

INTRODUCTION

A fundamental property shaping the ecology and evolution of animal life history strategies as well as their social and reproductive behaviour is the genetic relatedness among interacting individuals (Hamilton, 1964; Dugatkin, 1997; Hughes *et al.* 2008). The relatedness of individuals can determine the selective advantages or disadvantages that different life-history strategies and behaviours confer to the in-dividuals adopting them. For example, Hamilton's (1964) theory of kin selection demonstrates how organisms gain inclusive fitness via the reproduction of related individuals and has been used to explain the evolution of a diversity of biological phenomena and behaviours such as altruism and selfishness (Griffin and West, 2002).

The relatedness of interacting parasites may play an important role in many aspects of their biology, including their infectivity to hosts (Jager and Schjorring, 2006; Rauch *et al.* 2008) and resource utilization within hosts (Frank, 1992, 1996; Parker *et al.* 2003). Parasite populations are typically div-ided into infrapopulations consisting of all of the conspecific individuals infecting the same individual host (Margolis *et al.* 1982), which therefore share an intimate association with each other. The fate of the host affects all individual parasites in this host; it is their habitat as well as their only resource. The growth and host exploitation strategy adopted by an individual parasite affects its individual fitness, but if it is closely related to conspecific parasites in the same host, its actions also affect its inclusive fitness. Individual strategies have consequences for the group, and it is in this context that parasite evolution occurs. Many models suggest that virulence and growth rate of microparasites are affected by within-host parasite genetic variability (Frank, 1996; Chao *et al.* 2000; Brown *et al.* 2002). For macroparasites that do not multiply within the host, relatedness among parasites sharing a host may also matter, in-fluencing both patterns of resource use and growth (Parker *et al.* 2003).

The life cycles of trematodes (Platyhelminthes, Subclass Digenea) typically include 1 or more in-termediate hosts with larval parasites often achieving their final transmission to a vertebrate definitive host through predation (Bush *et al.* 2001). Larval trema-todes are aggregated among their intermediate hosts, forming discrete groups within intermediate hosts of 1 to many individuals. Within molluscan first inter-mediate hosts, asexual reproduction can produce thousands of genetically identical larvae (cercariae) that leave the molluscan host and infect second in-termediate hosts (becoming metacercariae). This sets the stage for metacercariae to share second inter-mediate hosts with genetically identical as well as genetically distinct conspecifics. The majority of empirical studies on trematodes have examined schistosomes that are atypical digeneans in being dioecious and having cercariae shed from snails that

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directly penetrate definitive hosts. These studies suggest that mixed-genotype infections within snails are characterized by increased parasite reproduction rate and virulence (potentially resulting from increased host defence costs), but that less virulent genotypes may have a competitive advantage when multiple infections are common (Davies *et al.* 2002; Gower and Webster, 2005). In addition, competition among co-infecting *Diplostomum pseudospathaceum* genotypes produced lower parasite loads in fish second intermediate hosts (Rauch *et al.* 2008). These results indicate that the genetic diversity of trematode infections could be an important factor influencing host fitness, parasite growth and infectivity.

In addition to the influence of the genetic diversity of infections, density-dependent reductions in parasite growth can occur from host resource limitation or immune responses (Keymer, 1982; Shostak and Scott, 1993). Reductions in trematode growth have been documented when increasing numbers of metacercariae share the same intermediate host (Sandland and Goater, 2000; Brown *et al.* 2003). Theoretically, the optimal size that a parasite should reach within its intermediate host also depends on its relatedness to other parasites in the same host (Parker *et al.* 2003). Resource monopoly is not necessarily the best strategy among identical genotypes sharing a host and in these circumstances an individual parasite may be less aggressive in its exploitation of host resources.

In this study we use an experimental approach to examine the influence of both genetic relatedness and density-dependent factors on host mortality, parasite infectivity, and within-host parasite growth in the marine trematode *Maritrema novaezealandensis* (Microphallidae) and its amphipod second intermediate host *Paracalliope novizealandiae*. In nature, metacercariae of this trematode species generally occur in mixed-clone infections inside second intermediate hosts (Keeney *et al.* 2007*a*, *b*), but the significance of this observation remains unknown. We determine (1) if host mortality differs following exposure to single- *vs* double-genotype parasite infections; (2) if the number of parasites successfully infecting hosts differs between single- *vs* double-genotype parasite infections; (3) if individual parasites grow faster or show more variation in their growth within hosts when they share a host with different conspecific genotypes *vs* identical genotypes; and (4) if growth rates or variation show density-dependent effects and if these are influenced by the genetic identity of the parasites.

MATERIALS AND METHODS

Study system

The marine trematode *M. novaezealandensis* is common within shallow coastal bays throughout the South Island of New Zealand. It utilizes the mud snail *Zeacumantus subcarinatus* as a first intermediate host, several crustaceans, including the crab *Macrophthalmus hirtipes* and amphipod *P. novizealandiae*, as second intermediate hosts, and shorebirds as definitive hosts (Martorelli *et al.* 2004). Once cercariae are shed from snails and penetrate a second intermediate host, the larval parasite increases in size and encysts, forming fully developed metacercariae in approximately 4–5 weeks (Martorelli *et al.* 2004). The life cycle is completed when a bird consumes the parasite along with its crustacean host, with sexual reproduction then taking place within the bird's gut.

Snail screening

Approximately 200 *Z. subcarinatus* snails were collected from Lower Portobello Bay, South Island, New Zealand on 4 November 2006. Fifty snails were haphazardly selected and screened to identify single-genotype *M. novaezealandensis* infections. Individual snails were screened by being placed into 60 mm Petri dishes containing 0·22 μm filtered seawater at 25 °C for 6 h under constant illumination. Cercariae shed from infected snails were collected and placed into 1·5 ml tubes for DNA extraction. Due to difficulties with genotyping individual *M. novaezealandensis* cercariae, approximately 50–100 cercariae were pooled from each snail for genetic analysis. Two samples were initially collected from each snail and 2 additional samples were collected from each snail 7 days later. The genotypes of pooled cercariae were determined for 5 microsatellite loci (Mno-1, Mno-2, Mno-28, Mno-30, and Mno-47) as described by Keeney *et al.* (2006), providing sufficient resolution for the identification of distinct genetic clones (Keeney *et al.* 2007*a*). Snails whose pooled cercariae DNA samples did not possess more than 2 alleles at any locus were treated as having single-genotype infections (corroborated by post-infection genotyping).

Amphipod infections

Two pairs of parasite genotypes were combined from specific snails harbouring single-genotype infections for experimental infections (genotype A paired with genotype B, genotype C paired with genotype D). In addition, 2 snails infected with 2 *M. novaezealandensis* genotypes each were left unpaired (1 snail shedding genotypes E and F and 1 snail shedding genotypes G and H) and used to infect amphipods. This was done to increase the number of mixed genotype infections for growth analyses as the requirement of 2 specific snails shedding sufficient cercariae on the same day could have limited our success in setting up double infections. All mixed infections consisted of genetic clones that differed by

Table 1. List of experimental treatment combinations

Series	Tank	Genotype(s)	Dosage
1	1	G,H*	Low
1	2	G,H*	Low
1	3	C	Low
1	4	C	Low
1	5	A	Low
1	6	A	Low
1	7	C,D	Low
1	8	C,D	Low
1	9	B	Low
1	10	B	Low
1	11	A	High
1	12	A	High
1	13	D	Low
1	14	A,B	Low
1	15	A,B	Low
2	16	E,F*	High
2	17	G,H*	High
2	18	A,B	High
2	19	B	High
2	20	E,F*	High
2	21	E,F*	Low
2	22	E,F*	Low
2	23	A,B	High
2	24	B	High

* Denotes infections involving snails with double-genotype infections that were not included in analyses of cercariae infection success or amphipod mortality.

at least 50% of their alleles; this is consistent with patterns of mixed infections seen in naturally infected amphipods (Keeney *et al.* 2007*b*). Data generated from amphipods infected by snails harbouring 2 *M. novaezealandensis* genotypes (genotypes E, F, G, H) were not included in analyses of cercariae success or amphipod mortality as the exact number of each parasite genotype used during infection of amphipods was not quantified.

For each pair of parasite genotypes, amphipods were infected with either a low (6 total cercariae/amphipod) or high (12 total cercariae/amphipod) infection dose, and either cercariae from one genotype in the pair, the other genotype in the pair, or equal numbers (3 and 3, or 6 and 6) of cercariae from both genotypes (Table 1). High and low doses were used to produce a range of parasite infection intensities within hosts that reflect natural intensities (Fredensborg *et al.* 2004; Keeney *et al.* 2007*b*). Due to insufficient cercariae production, we were not able to infect amphipods with high doses of genotypes C and D.

For each treatment combination, we attempted to infect 2 sets of 96 amphipods. Uninfected amphipods were collected from Hooper's Inlet, South Island, New Zealand where the snail first intermediate host of *M. novaezealandensis* does not occur; no infected amphipods have ever been found from that locality (Fredensborg *et al.* 2004). For infections, snails were

placed into 60 mm Petri dishes containing 0·22 μm filtered seawater at 25 °C for approximately 1 h under constant illumination. Cercariae obtained during that time were transferred with a 200 μl pipette to individual wells in 96-well plates (250 μl volume) each containing an uninfected amphipod in 75 μl of seawater. Amphipods were then incubated at 25 °C for 5 h under constant illumination. After incubation, each set of 96 amphipods was placed into a 1 litre tank of seawater containing sea lettuce (*Ulva lactuca*) and an airstone. Tanks were arranged randomly on a laboratory bench and left at room temperature throughout the study. Two tanks of 96 control amphipods were manipulated in the same manner with water pipetted into their wells instead of cercariae. On each day of infection, 30 cercariae were measured under a binocular microscope (length and width of body) from each snail used to estimate initial cercariae sizes. Experimental infections were conducted in 2 series with series 1 running from 15 November 2006 to 11 December 2006 and series 2 running from 17 January 2007 to 23 February 2007. Because of the large numbers of cercariae required for infections, the selection of specific snails each day was often dictated by the ability of the snails to produce sufficient cercariae.

Fourteen days after infection (a time-period sufficient for substantial parasite growth, but not complete encystment and development (Martorelli *et al.* 2004)), the number of amphipods alive in each tank was counted and the sex and length of each amphipod was recorded. Each amphipod was dissected and the number of *M. novaezealandensis* metacercariae was recorded. The maximum length and width of each metacercaria was measured and individual metacercariae were placed in 1·5 ml tubes for DNA extraction. For DNA extraction, individual metacercariae were placed in 400 μl of 5% chelex containing 0·1 mg/ml proteinase K, incubated at 60 °C for 2–8 h and boiled at 100 °C for 8 min. To identify specific genotypes, the genotypes at 2 microsatellite loci (combinations of Mno-1, Mno-28, Mno-30, and Mno-47) were determined for all individual *M. novaezealandensis* metacercariae collected from mixed-genotype infections and for a subset of 25 metacercariae per single-genotype infection tank (all individual metacercariae matched the expected cercariae genotypes) as described in Keeney *et al.* (2006).

Data analysis

For host mortality and infectivity analyses, parasites were grouped according to whether they came from amphipods that were exposed to single- or double-genotype infection. For within-host growth analyses, parasites were grouped according to the genetic diversity (single- or double-genotype infection) of metacercariae sharing amphipod hosts, and only

amphipods with 2 or more metacercariae were included. Parasites from amphipods in the initial double-genotype treatments that were successfully infected by a single genotype were included in the single-genotype group.

All statistical analyses were performed using SPSS 16.0 for Windows. Two-way analysis of variance (ANOVA) was used to examine the influence of infection treatment (single- vs double-genotype) and cercariae dose (low vs high) on the percentage of dead amphipods per tank. A *t*-test was used to compare amphipod mortality between treatments and controls. Mann-Whitney U (M-W) tests were used to examine the influence of infection dose (high vs low) on the number and percentage of cercariae successfully infecting amphipods in single, double, and combined (single and double infection results pooled) infections. To examine the effects of genetic diversity on cercariae infection success, the percentage of cercariae of each genotype and of all genotypes infecting amphipods was compared between amphipods exposed to single- and double-genotype cercariae with M-W tests.

Two measurements were used to examine the influence of several factors, including genetic diversity, on within-host parasite growth. First, we used parasite size as an estimate of parasite growth and compared growth rates within amphipods infected with either 1 or 2 parasite genotypes. Parasite size was calculated as the area of an ellipse $(\pi LW)/4$, with L = parasite length and W = parasite width. Because this growth estimate could be biased if parasite size at the time of infection was not consistent, we calculated the size (not including tail) of 30 cercariae shed by each snail each day they were used for infections and examined differences in cercariae size based on genotype and date of shedding independently with Kruskal-Wallis (K-W) tests. After detecting significant differences in cercariae size among genotypes and within genotypes from different days, the mean size of cercariae shed by the snail on the day of infection was subtracted from each post-infection metacercariae body size. These corrected sizes (parasite growth) were used in our models. Second, we calculated the coefficient of variation (CV) in parasite growth (CV = 100 * standard deviation/ mean) within each amphipod and used these in our models to determine what factors cause greater variation in parasite utilization of host resources.

Two sets of generalized linear models were used to examine the effects of several variables on the response variables parasite growth and CV. Models incorporated gamma distributions, log links, and maximum likelihood estimated scale parameters, and the following potential explanatory variables were examined: single- or double-genotype infection (infection), first or second infection series (series), number of parasites within amphipods (# para), amphipod sex (sex), and amphipod length (length).

For each response variable, all possible models involving at least one of the explanatory variables were analysed for main effects. Model selection for each response variable was based on the lowest Akaike Information Criterion corrected for finite sample sizes (AICc) and facilitated by calculating differences in AICc values between each model and the model with the lowest AICc score (Δ_i AICc). Models with Δ_i AICc scores $\geqslant 4$ have considerably less empirical support (Burnham and Anderson, 2002). Akaike weights (w_i) were calculated to examine the weight of evidence supporting each model as the actual best model in our set of candidates and the evidence in favour of our selected model(s) was calculated as the ratio of Akaike weights ($w_{\text{selected model}}/w_{\text{alternative model}}$ or w_i/w_j), which provides the relative likelihood of model i being the actual best model versus model j (Burnham and Anderson, 2002). Interactions between pairs of main effects were examined in a subset of best models (models with Δ_i AICc scores <4 or $w_i/w_j < 10$). All interactions were initially included and sequentially removed if the interaction was not significant and its removal did not raise the AICc value. Significance of main effects and interactions was determined from the best model(s) using $P < 0.05$ as the criterion for significance.

RESULTS

In total, 307 amphipods (148 male, 159 female) survived out of 1632 initially exposed to parasites; mortality analyses are based on these data. No effect on amphipod mortality (mean survival = $18.8 \pm 8.8\%$ S.D.) was detected for the main effects infection treatment ($F_{1,13} = 0.343$, $P = 0.568$) and cercariae dosage ($F_{1,13} = 0.052$, $P = 0.824$), and there was no significant interaction effect ($F_{1,13} = 0.001$, $P = 0.979$). A significant difference in amphipod mortality was not detected between treatments and controls (*t*-test $t_{17} = -1.169$, $P = 0.259$). A significantly higher number of parasites were recovered from amphipods exposed to high versus low parasite doses with single- (M-W test, $Z = -5.692$, $P < 0.001$), double- ($Z = -4.740$, $P < 0.001$), and combined ($Z = -7.328$, $P < 0.001$) genotype infections. No difference in the percentage of cercariae successfully infecting amphipods was detected between high and low dose treatments with single- ($Z = -0.886$, $P = 0.375$), double- ($Z = -0.937$, $P = 0.349$), or combined ($Z = -1.231$, $P = 0.218$) genotype infections. Genotype A showed a significant decrease in cercariae success in double- versus single-genotype infections ($Z = -2.319$, $P = 0.020$) (Fig. 1). Genotype B also displayed a decrease in cercariae success in double- versus single-genotype infections, but this was not statistically significant ($Z = -1.453$, $P = 0.146$). Genotypes C and D did not show significant differences in cercariae success between treatments (genotype C: $Z = -0.070$,
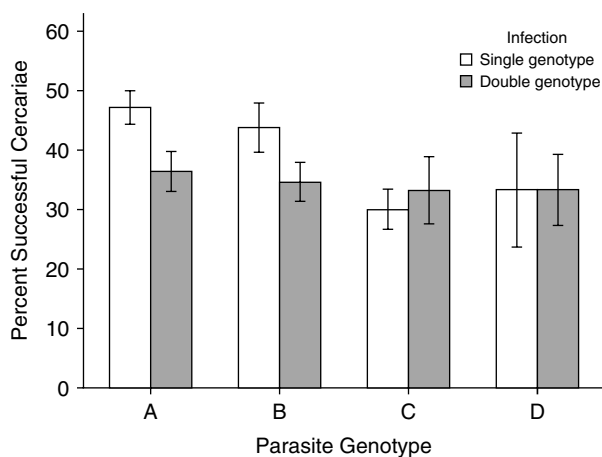
Fig. 1. Percentage of *Maritrema novaezealandensis* cercariae successfully infecting amphipods in single- and double-genotype infections. Bars represent mean values ± standard error.

$P = 0.944$; genotype D: $Z = -0.244$, $P = 0.807$. When all genotypes were pooled, an overall decrease in infection success in double-genotype infections was detected, but it was not quite statistically significant ($Z = -1.785$, $P = 0.074$). Significant differences in the mean size of cercariae were detected among different genotypes (K-W test, $H = 74.791$, $P < 0.001$) as well as within genotypes shed on different days ($H \geqslant 54.029$, $P < 0.001$).

A total of 1200 *M. novaezealandensis* metacercariae from 265 amphipods (127 male, 138 female) were used in growth analyses. On average, metacercariae were 4–5 times larger at the end of the experiment than they were as cercariae pre-infection. The single best main effects model for parasite growth within amphipods included infection series, single- or double-genotype infection, amphipod sex, and amphipod length as explanatory variables (Table 2). Based on Akaike weight ratios, there was no strong evidence that this model was better than the models with infection series, amphipod sex, and amphipod length ($w_i/w_j = 2.273$), infection series, number of parasites, amphipod sex, and amphipod length ($w_i/w_j = 2.386$), or infection series, single- or double-genotype infection, number of parasites, amphipod sex, and amphipod length ($w_i/w_j = 1.037$) as effects. Interaction effects were examined between all 5 main effects and the inclusion of several interactions significantly improved our final model (AICc = 2662·210, Δ_i AICc = -12.973 from best model without interactions). The final model included infection series, amphipod sex, number of parasites, and amphipod length as main effects and infection series ∗ amphipod sex, infection series ∗ number of parasites, number of parasites ∗ amphipod sex, and number of parasites ∗ amphipod length as interaction effects. In this model 2 main effects were significant, with parasites from the second infection series being larger ($\chi^2 = 19.599$, $P < 0.001$) and number of parasites within amphipods being negatively associated with parasite growth ($\chi^2 = 5.738$, $P = 0.017$). Amphipod sex ($\chi^2 = 0.220$, $P = 0.639$) and amphipod length ($\chi^2 = 0.435$, $P = 0.509$) were not significant as main effects. Several interactions were also significant. Infection series ∗ amphipod sex indicated parasites in female amphipods from infection series two had disproportionately more growth ($\chi^2 = 4.616$, $P = 0.032$). Infection series ∗ number of parasites indicated that parasites in the second infection series showed less of a decrease in growth as the number of parasites increased ($\chi^2 = 8.323$, $P = 0.004$). Number of parasites ∗ sex revealed that a smaller decrease in growth with increasing number of parasites occurred in female amphipods ($\chi^2 = 4.369$, $P = 0.037$). Lastly, number of parasites ∗ length indicated that a smaller decrease in growth occurred with increasing number of parasites as amphipod size increased ($\chi^2 = 4.481$, $P = 0.034$). Inclusion of single- or double-genotype infection had minimal influence on the model (AICc = 2662·612), and it did not have a significant effect on parasite growth ($\chi^2 = 1.773$, $P = 0.183$) or have any significant interactions with other effects.

The single best main effects model for parasite CV included amphipod sex as the only effect (Table 3). This model was only weakly supported as the best model versus numerous other candidate models, including all single effect models, with which it had low Akaike weight ratios. With all of these models, a significant effect on CV was not detected for any variable (infection series $\chi^2 = 0.327$, $P = 0.568$; single- or double-genotype infection $\chi^2 = 0.223$, $P = 0.637$; number of parasites $\chi^2 = 0.983$, $P = 0.321$; amphipod sex $\chi^2 = 2.497$, $P = 0.114$; amphipod length $\chi^2 = 1.800$, $P = 0.180$). For variables included in multiple models with similar support, the highest χ^2 and lowest P-value have been presented. Inclusion of interactions between main effects did not improve any models and no interactions were significant.

DISCUSSION

Host mortality and cercariae infection success

The genetic makeup of parasite infections can have significant effects on the fitness of their hosts. Competition between different parasite genotypes can favour faster utilization of host resources and/or reproduction rates leading to increased virulence (Frank, 1992, 1996). In addition, differences in the cost to the host or ability of the host to defend against multiple parasite genotypes versus single genotypes can reduce host fitness (Read and Taylor, 2001). In this study, we examined fitness effects in terms of host mortality and did not detect any difference in *P. novizealandiae* mortality between those exposed to single- and double-genotype infections. It is

Table 2. *Generalized linear models examining main effects on growth of Maritrema novaezealandensis metacercariae recovered from amphipods*

(Dashes (—) represent ratios with numerators of approximately zero.)

Model effects	AICc	Δ_i AICc	w_i	w_i/w_j
Series	2683·428	8·245	0·005	61·713
Infection	2891·459	216·276	0·000	—
# Para	2898·071	222·888	0·000	—
Sex	2922·548	247·365	0·000	—
Length	2929·868	254·685	0·000	—
Series, Infection	2681·930	6·747	0·011	29·180
Series, # Para	2682·058	6·875	0·010	31·109
Series, Sex	2684·376	9·193	0·003	99·137
Series, Length	2684·298	9·115	0·003	95·345
Infection, # Para	2868·573	193·390	0·000	—
Infection, Sex	2884·429	209·246	0·000	—
Infection, Length	2891·026	215·843	0·000	—
# Para, Sex	2893·135	217·952	0·000	—
# Para, Length	2897·252	222·069	0·000	—
Sex, Length	2921·566	246·383	0·000	—
Series, Infection, # Para	2680·524	5·341	0·022	14·447
Series, Infection, Sex	2682·656	7·473	0·008	41·951
Series, Infection, Length	2682·992	7·809	0·006	49·625
Series, # Para, Sex	2683·012	7·829	0·006	50·124
Series, # Para, Length	2683·274	8·091	0·006	57·140
Series, Sex, Length	2676·825	1·642	0·139	2·273
Infection, # Para, Sex	2862·960	187·777	0·000	—
Infection, # Para, Length	2866·850	191·667	0·000	—
Infection, Sex, Length	2885·047	209·864	0·000	—
# Para, Sex, Length	2894·865	219·682	0·000	—
Series, Infection, # Para, Length	2681·253	6·070	0·015	20·801
Series, Infection, # Para, Sex	2681·909	6·726	0·011	28·876
Series, Infection, Sex, Length	2675·183	0·000	0·316	1·000
Series, # Para, Sex, Length	2676·922	1·739	0·133	2·386
Infection, # Para, Sex, Length	2864·947	189·764	0·000	—
Series, Infection, # Para, Sex, Length	2675·256	0·073	0·305	1·037

possible that our selection of experimental clones did not cover much of the genetic variation within the source population. Nonetheless, the results at least suggest that if adaptations to the presence of genetically different conspecifics exist in the trematode *M. novaezealandensis*, such as altering host resource utilization, they are not intense enough to create a detectable difference in host mortality.

Parasite transmission success depends on rates of within-host reproduction, which in turn affect virulence (Davies *et al*. 2002). This can compensate for the detrimental effects to the host, although increased reproduction does not always affect host fitness (Vizoso and Ebert, 2005). The present study examined mortality of second intermediate amphipod hosts; individual *M. novaezealandensis* do not reproduce in this host, but instead grow, encyst, and await predation by a definitive host. Death of the amphipod host before consumption by the definitive host also means death for the parasites. Selection should therefore work against the evolution of strategies causing increased host mortality without compensatory transmission to the final hosts. It is possible that multiple genotype infections influence

other aspects of host fitness that are not detectable as differences in survival. Also, our results do not rule out the possibility that the genetic diversity of *M. novaezealandensis* within snail first intermediate hosts, where parasite replication occurs, has a greater effect on host mortality, as in other snail-trematode systems (Davies *et al*. 2002). It is also possible that increased genetic diversity does influence host mortality when larger numbers of parasite genotypes (>2) infect individual amphipods.

The genetic diversity of parasite infections can also influence the number of parasites that successfully infect a host during exposure, producing variation in parasite load. Total parasite load can decrease as genetic diversity increases (Wedekind and Ruetschi, 2000; Jager and Schjorring, 2006) through direct competition for entry sites or direct and indirect use of the host's immune system against other genotypes (Rauch *et al*. 2008). In contrast, genetically diverse parasite infections can also lead to increased parasite loads within hosts if the hosts are unable to respond as effectively to diverse infections (Read and Taylor, 2001). In our study, only 1 of the 4 genotypes (genotype A) displayed a significant decrease in

Table 3. Generalized linear models examining main effects on coefficient of variation in growth of *Maritrema novaezealandensis* metacercariae recovered from amphipods

Model effects	AICc	Δ_i AICc	w_i	w_i/w_j
Series	2230·528	2·310	0·042	3·174
Infection	2230·607	2·389	0·040	3·302
# Para	2229·911	1·693	0·057	2·331
Sex	2228·218	0·000	0·133	1·000
Length	2228·901	0·683	0·094	1·407
Series, Infection	2232·577	4·359	0·015	8·842
Series, # Para	2231·512	3·294	0·026	5·191
Series, Sex	2230·198	1·980	0·049	2·691
Series, Length	2230·908	2·690	0·035	3·838
Infection, # Para	2231·793	3·575	0·022	5·974
Infection, Sex	2230·179	1·961	0·050	2·666
Infection, Length	2230·862	2·644	0·035	3·751
# Para, Sex	2229·525	1·307	0·069	1·922
# Para, Length	2230·347	2·129	0·046	2·899
Sex, Length	2230·254	2·036	0·048	2·768
Series, Infection, # Para	2233·562	5·344	0·009	14·469
Series, Infection, Sex	2232·231	4·013	0·018	7·437
Series, Infection, Length	2232·928	4·710	0·013	10·538
Series, # Para, Sex	2231·277	3·059	0·029	4·616
Series, # Para, Length	2232·183	3·965	0·018	7·261
Series, Sex, Length	2232·259	4·041	0·018	7·542
Infection, # Para, Sex	2231·381	3·163	0·027	4·862
Infection, # Para, Length	2232·220	4·002	0·018	7·396
Infection, Sex, Length	2232·230	4·012	0·018	7·434
# Para, Sex, Length	2231·598	3·380	0·025	5·419
Series, Infection, # Para, Length	2234·201	5·983	0·007	19·916
Series, Infection, # Para, Sex	2233·303	5·085	0·010	12·711
Series, Infection, Sex, Length	2234·303	6·085	0·006	20·958
Series, # Para, Sex, Length	2233·371	5·153	0·010	13·151
Infection, # Para, Sex, Length	2233·470	5·252	0·010	13·818
Series, Infection, # Para, Sex, Length	2235·414	7·196	0·004	36·525

infection success in double- versus single-genotype infections; other genotypes (B, C and D) showed no statistical difference in infection success. As the number of parasites recovered from amphipods increased proportionately with the number of cercariae to which they were exposed, host response factors and/or competition among parasites did not affect infection success regardless of the genetic identity of cercariae. It is possible that competitive effects, either between parasite genotypes or individuals of the same genotype, could become evident if amphipods were exposed to larger numbers of cercariae, but our goal was to mimic natural infection intensities within amphipods which range between 1 and 20 parasites, with mean intensities of approximately 3–6 (Fredensborg *et al.* 2004; Keeney *et al.* 2007*b*). Our results suggest that the diversity of parasite genotypes has a slight influence on infection success in our system but only for specific parasite genotypes. As natural infections of *M. novaezealandensis* in amphipods are characterized by high genetic diversity with only occasional pairs of identical genotypes infecting the same amphipod (Keeney *et al.* 2007*b*), strong selective pressures may not exist for differential host responses or interactions between self and non-self genotypes.

Growth within the host

We did not detect any effect of genetic diversity of infection on growth or variation in growth among *M. novaezealandensis* within *P. novizealandiae* amphipod intermediate hosts. Therefore, the parasites are not altering their growth rates in response to the presence of non-self genotypes within hosts. Although competition between closely related parasites can theoretically lead to decreased host exploitation and final parasite size (Parker *et al.* 2003), it may have less of an influence on initial parasite growth rates as studied here. It is also possible that *M. novaezealandensis* is utilizing its host resources at an optimal level irrespective of infection diversity and that increases in growth rate would lead to subsequent host death (while decreases would have minimal influence on host mortality) and therefore parasite death. This would remove the selective advantage of increasing growth when in the presence of other genotypes, assuming that the parasites are able to identify self and non-self genotypes within amphipod hosts. Selection for differential growth in single- versus double-genotype infections could occur if it coincided with increased transmission of related parasites through alterations in host

behaviour or morphology (Brown, 1999), but we do not have evidence that this is occurring in this host-parasite system.

Although the genetic diversity of infections did not influence parasite growth within amphipods, other factors did. Parasites within amphipods in the second infection series grew more than the parasites in the first series. This was most likely due to differences in average room temperature between the two series. Below a certain threshold, the growth rate of metacercariae often increases with increasing temperature (Chubb, 1979; Sandland and Goater, 2000). Mean daily temperatures varied significantly between series (series 1 mean temperature $\pm$ S.D. $= 12 \cdot 0 \pm 2 \cdot 7$ °C, series 2 mean temperature $\pm$ S.D. $= 14 \cdot 4 \pm 2 \cdot 6$ °C; t-test $t_{63} = -3 \cdot 646$, $P = 0 \cdot 001$) causing greater growth in series two parasites. This result was not unexpected, and the inclusion of 'series' in our models allowed us to compensate for this difference.

Competition for the limited resources available within a host as well as increased host immune responses to larger numbers of parasites can produce density-dependent decreases in parasite growth (Keymer, 1982; Shostak and Scott, 1993; Poulin, 2007). Indeed, reductions in metacercariae size with increasing parasite load have been detected in several trematode species (Sandland and Goater, 2000; Brown *et al.* 2003; Fredensborg and Poulin, 2005). Unlike many trematodes, *M. novaezealandensis* metacercariae grow and develop substantially during the first few weeks after infection (Poulin and Latham, 2003; Fredensborg *et al.* 2004). This is likely to create competition for space as well as nutrients when numerous parasites are present within the host, causing a decrease in the average size of individual parasites. Also, if the degree of exploitation by parasites influences host survival, selection could favour decreased growth with increasing numbers in parasites that require host survival for transmission (Parker *et al.* 2003). Fredensborg and Poulin (2005) found lower individual mean sizes of fully developed *M. novaezealandensis* metacercariae within heavily-infected crabs; they suggested this could result from resource limitations during initial growth when multiple parasites infect the host, as the slight size differences would not impact host fitness. Our results support differences occurring during early growth, but we do not rule out the possibility that parasite growth strategy could also influence *M. novaezealandensis* during this stage as the smaller amphipod hosts may be more sensitive to parasite growth. Because smaller metacercariae tend to produce fewer eggs as adults (Fredensborg and Poulin, 2005), there could be a trade-off between decreasing virulence and individual parasite fecundity.

Several additional factors influenced the observed density-dependent decrease in parasite growth. There was less of a decrease in growth with increasing parasite number within larger amphipods. This result is consistent with competition among parasites within amphipods causing decreased growth as larger amphipods offer more space and nutrients (Poulin, 1996a), lessening competitive effects. If parasites can detect space or food availability and adjust their growth accordingly, this observation is also consistent with an optimal growth scenario (Parker *et al.* 2003) in which the parasites, not just resource availability, are controlling their growth rates. A decrease in the extent of density-dependence was also observed when growth rates were higher. It is therefore possible that under certain conditions, such as increased temperature, parasites in higher intensity infections do not display proportionate decreases in host resource utilization despite limited resource availability or consequences to host fitness.

We also detected less of a decrease in growth with increasing parasite number in female amphipods, whose parasites also displayed more growth when growth rates were higher. Male vertebrates often display a higher susceptibility to parasites than females (Poulin, 1996b; Schalk and Forbes, 1997), potentially resulting from the immunosuppressive effects of testosterone (Zuk and McKean, 1996), as well as increased parasite growth (Poulin, 1996c). Although arthropods lack these hormones, other developmental hormones or sexual dimorphisms including behaviour (Zuk and McKean, 1996) can produce differences in parasite susceptibility or within-host parasite growth between sexes. To date, a general sex bias in parasite infections has not been detected in arthropods (Sheridan *et al.* 2000), though inequality of sizes between sexes often confounds results. It should be stressed that in the present study an increase in overall growth was not detected in female amphipods. There was less of a decrease in growth in females when infection intensity was high than in males, and growth increased disproportionately in females when overall growth rates were higher.

Overall, we did not detect a significant influence of genetic diversity of infection on the host mortality, infectivity, or growth of the marine trematode *M. novaezealandensis* within amphipod hosts. Density-dependent reductions in growth revealed that the number of parasites infecting hosts is more important than their genetic relatedness. Temperature, host size, and host sex also influenced the degree to which density-dependence affects parasite growth. It must be pointed out again that our study included only a small number of clones, and did not necessarily capture the full genetic diversity existing in *M. novaezealandensis*. With this caveat in mind, genetic diversity of infection may play a relatively small role in this host-parasite system compared to the myriad other factors regulating the host-parasite interaction.

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