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Genetic and phenotypic influences on clone-level success and host specialization in a generalist parasite

A. V. KOEHLER*, Y. P. SPRINGER*, H. S. RANDHAWA*, T. L. F. LEUNG*, D. B. KEENEY† & R. POULIN*

*Department of Zoology, University of Otago, Dunedin, New Zealand
†Department of Biological Sciences, Le Moyne College, Syracuse, NY, USA

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Abstract

Studying resource specialization at the individual level can identify factors constraining the evolution of generalism. We quantified genotypic and phenotypic variability among infective stages of 20 clones of the parasitic trematode *Maritrema novaezealandensis* and measured their infection success and post-infection fitness (growth, egg output) in several crabs and amphipods. First, different clones varied in their infection success of different crustaceans. Second, neither genetic nor phenotypic traits had consistent effects on infection success across all host species. Although the results suggest a relationship between infection success and phenotypic variability, phenotypically variable clones were not better at infecting more host species than less variable ones. Third, genetic and phenotypic traits also showed no consistent correlations with post-infection fitness measures. Overall, we found no consistent clone-level specialization, with some clones acting as specialists and others, generalists. The trematode population therefore maintains an overall generalist strategy by comprising a mixture of clone-level specialists and generalists.

Introduction

The degree to which populations of organisms are specialized on their key resources has been an important area of study in ecology and evolution (Futuyma & Moreno, 1988; Ferry-Graham *et al.*, 2002; Devictor *et al.*, 2010). Specialization is the process by which niche partitioning occurs (Van Valen, 1965) and is therefore a critical driver of patterns and changes in biodiversity. The evolution towards specialization in resource use can be influenced by morphological, physiological and behavioural traits that offer a fitness advantage for the organism (Futuyma & Moreno, 1988).

When a generalist population is scrutinized more closely, resource-use specialization by individuals can become apparent. Bolnick *et al.* (2003), Bolnick *et al.*, 2011) have argued that the recognition of individual-level resource specialists can have a substantial effect on the ecological and evolutionary dynamics of the population. For example, when individual specialization is taken into account, a more complete picture of the system is realized, such as more precise estimates of interaction strengths in ecological networks (Bolnick *et al.*, 2011). From an evolutionary perspective, individual specialization can hasten speciation (Bush, 1994; Bolnick *et al.*, 2003) as suspected to be the case with phytophagous insects specializing on plants (Via, 1999; Wood *et al.*, 1999).

For parasites, resource specialization is often equated with host specificity, or the range of host species exploited by a parasite species (Adamson & Caira, 1994; Poulin, 2007). Traditionally, the filter concept (Euzet & Combes, 1980) uses a series of encounter and compatibility filters to illustrate how host range is constrained in parasites. The encounter filter excludes hosts that are never naturally encountered by a parasite due to either allopatric distributions or behaviour. The compatibility

Correspondence: Anson V. Koehler, Department of Zoology, University of Otago, PO Box 56, Dunedin 9054, New Zealand.
Tel.: +64 03 479 5848; fax: +64 03 479 7584;
e-mail: ansonkoehler@gmail.com

filter excludes hosts offering resources on which the parasite is unable to survive, as well as hosts with an immune system preventing parasite establishment. The resulting spectrum of hosts determines whether the parasite is a generalist, accessing a broad range of resources while possibly trading off the ability to maximize fitness on any particular resource, or a specialist, maximizing fitness on a few resources, a strict reliance making them more prone to extinction (Ward, 1992; Poulin, 2007). When considering individual specialization in parasites, there is potential for subsets of specialists within generalist parasite species, especially those containing active dispersal stages in their life cycles (Théron & Combes, 1995), to evolve as distinct host races (Kawecki, 1998; Ward *et al.*, 1998; McCoy *et al.*, 2001; McCoy, 2003; Huyse *et al.*, 2005) via the aid of multiple hosts as allopatric barriers.

Trematode parasites are ideal organisms with which to test ideas concerning resource-use specialization in the form of constraints on host specificity. Their complex life cycles usually include stages at which they can only infect one or very few host species, and other stages during which they can exploit a broad range of hosts (Combes, 2001). Snails typically serve as first intermediate hosts, in which larval transmission/dispersal propagules (cercariae) are produced asexually before being shed into the water. In general, a single initial infection yields large quantities (hundreds to several thousands) of these genetically identical clones that disperse from snails until they come into contact with a second intermediate host. These large numbers of clonal cercariae allow for multiple replicates in experimental infection trials, a key element for rigorous tests of hypotheses regarding within-species specialization.

Keeney *et al.* (2009) found significant differences in cercarial size among and within eight *Maritrema novaezealandensis* trematode clones (distinguished using five microsatellite loci), each from a singly infected snail. Subsequently, Koehler *et al.* (2011b) compared cercariae from 42 unique *M. novaezealandensis* clones and found significant differences among clones in terms of morphology, behaviour and survivability; a subset of those 42 clones is used in the present study. Whether these interclonal differences have any bearing on parasite fitness or host specificity remains to be seen. On the one hand, genetic differences among clones, such as differences in heterozygosity, may translate into some clones performing well in one or very few host species and other clones performing well in a broad range of hosts. Indeed, positive correlations between fitness traits and heterozygosity have been reported for a wide range of organisms (Hansson & Westerberg, 2002; Chapman *et al.*, 2009), but not properly investigated regarding parasite infection success or subsequent growth in different hosts. On the other hand, differences among clones in behavioural or morphological traits can directly affect which hosts they are likely to encounter and how likely they are to penetrate these hosts. In particular, the level of within-clone variation in phenotypic traits could determine encounter and compatibility with different host species, with clones displaying high phenotypic plasticity being more likely to exploit many host species than clones with more constrained phenotypic variation.

Using a marine trematode parasite species that utilizes a taxonomically diverse assemblage of invertebrate species as second intermediate hosts, we ask the following questions: (i) are parasite clones specialized (i.e. better adapted) for different crustacean hosts due to consistent, interclonal differences in phenotype and/or genotype, while still maintaining a generalist strategy at the population level? (ii) do particular phenotypic and genetic factors and their variation affect each clone's infection success in four crustacean hosts? and (iii) are these phenotypic and genetic factors important determinants of post-infection parasite fitness in two of these crustacean hosts? Overall, this study provides an in-depth look at the factors shaping intraspecific variation in host specialization and new insights into its significance.

Materials and methods

Study system

We used the trematode *M. novaezealandensis* (Digenea: Microphallidae) whose first intermediate host is the intertidal mudsnail *Zeacumantus subcarinatus* (Prosobranchia: Batillariidae), found primarily along the eastern coast of the South Island of New Zealand. The cercariae of this parasite can infect a wide range of crustacean hosts by penetrating the cuticle of some of the smaller species (e.g. amphipods) or soft tissues of larger ones (e.g. crabs) (Koehler & Poulin, 2010) using a structure known as a stylet. Once inside a host, the parasite encysts as a metacercaria from which the adult parasite emerges once the crustacean has been eaten by the bird definitive host. Within the intestine of the bird final host, the adult stage then produces eggs by outcrossing or self-fertilization. Eggs are excreted into the environment where they are consumed by snails, completing the parasite's life cycle.

Snails

Zeacumantus subcarinatus snails were collected from Company Bay, Otago Harbor, New Zealand (45°51′24″S, 170°35′54″E), on 20 August 2008. Details on how they were screened for infection by *M. novaezealandensis* can be found in Koehler *et al.* (2011b). Infected snails were maintained in a 40-L aquarium for approximately 22 months throughout the duration of the experiments. The aquarium contained an autoclaved mixture of sand and mud and was filled with seawater from Otago Harbor, which was changed monthly and held at 19 °C using an aquarium heater. Light was supplied through full UV spectrum bulbs set on a 12-h cycle (Sylvania

GroLux F36W/T8, Munich, Germany). Captive snails were fed sea lettuce (*Ulva* spp.) *ad libitum*.

Genotype measurements

The processes of determining whether snails harboured single clone infections and the estimation of genome-wide multilocus heterozygosity (MLH; proportion of heterozygous loci among all 32 loci typed) of the parasites using microsatellites from Keeney *et al.* (2006) and Molecular Ecology Resources Primer Development Consortium (2009) are detailed in Koehler *et al.* (2011b). For this experiment, 20 *M. novaezealandensis* clones that collectively represented a range of MLH values from 31.25% to 71.88% (mean 49.85% ± 2.60) were identified (Table S1).

Phenotype measurements

Morphology

During September 2009 and March 2010, the following morphological measurements were taken from approximately 80 cercariae from each of the 20 clones: body length, maximum body width, tail length, maximum tail width and stylet length. As detailed in Koehler *et al.* (2011b), several steps were taken to ensure that cercariae were killed, straightened and measured in a systematic manner. Measurements from both time periods were combined, as temporal variation was small and our goal was to obtain typical clone-level values. Phenotypic variability was estimated by calculating the coefficient of variation (CV) for each morphological trait individually, and these were summed across traits to produce an overall measure of within-clone morphological variation. Separately, a principal component analysis (PCA) was used to generate a principal component value based on measurements of the five morphological traits for each clone. Highly correlated variables, such as cercarial morphology measurements, can be reduced into one value through the use of a PCA as was done in Altizer & Davis (2010). Here, low PCA values correspond to cercariae with generally small bodies, short tails and short stylets, and high values correspond to large cercariae with big tails and long stylets.

Photoreaction

Concurrent with morphological measurements, the reaction of cercariae to light was quantified using ten replicate, seawater-filled glass troughs (92 mm long by 9 mm wide by 6 mm high, 2 mL volume) with half of each trough painted black and the other half left transparent. Cercariae (approximately 30) were added to the middle of the trough, and after 20 min, the number of cercariae was counted on the dark and light sides of each trough. Further details can be found in Koehler *et al.* (2011b). The proportion of cercariae in the light vs. dark sides was averaged across all ten replicates to obtain mean and CV values for each clone. This experiment was conducted in September 2009 and repeated in March 2010, and the results were combined.

Survival

For each clone, cercariae were added to 20 wells of a 96-well culture plate, incubated at 25 °C, and the numbers of cercariae were recorded as either mobile or immobile (for full details see Koehler *et al.*, 2011b) each hour until over half of the cercariae in each well were immobile. The length of time (in min) at which half of the cercariae reached immobility was calculated and used as the cercariae's survival time (stamina); data for each clone were averaged across replicates to obtain the mean and CV. This trial was run only once in November 2009.

Infection experiments

Selection of crustacean hosts

To span the spectrum of host compatibility and encounter in nature, 4-s intermediate hosts were chosen from four different crustacean families: crabs, *Macrophthalmus hirtipes* (Brachyura: Macrophthalmidae) and *Hemigrapsus crenulatus*, (Brachyura: Varunidae) and amphipods, *Paracalliope novizealandiae* (Gammaridea: Paracalliopiidae) and *Heterophoxus stephenseni* (Gammaridea: Phoxocephalidae). All hosts chosen for this experiment are known to be susceptible to the parasite under natural conditions (Koehler & Poulin, 2010; Koehler *et al.*, 2011a). Both species of crab exhibit a high prevalence of *M. novaezealandensis* (> 95%) in the main study area, but mean parasite abundance within individual hosts is on average four times greater in *M. hirtipes* (Koehler & Poulin, 2010). The amphipods chosen for this study have very different behaviours that lead to drastic differences in natural infections. Koehler *et al.* (2011a) reported 71% prevalence in the algae-living amphipod *P. novizealandiae*, compared to 17% prevalence in the burrowing amphipod *H. stephenseni*. Interclonal differences in infection success were assessed using infection experiments involving all four crustacean species, whereas post-infection fitness measures are obtained using only the two crab species.

To obtain host individuals that were free of *M. novaezealandensis* infections, one or more collection locations were identified for each species from which the parasite was absent. Two methods were used to identify these sites. First, extensive field surveys were conducted to locate *Z. subcarinatus* snails at a potential collection site. As *M. novaezealandensis* must use this snail as its first intermediate host, the absence of *Z. subcarinatus* would automatically preclude infection of any crustacean second intermediate hosts (Fredensborg *et al.*, 2004). Second, for each of the four experimental host species, 20 individuals were collected from each potential source site and dissected to check for *M. novaezealandensis* metacercariae. Sites for which both methods confirmed

the absence of *M. novaezealandensis* were chosen for crustacean collection. Additionally, to minimize potential effects of multiple infections of other parasite species, collection localities were chosen with reduced parasite loads compared to surrounding areas.

Crab infections

On 19 May 2009, 480 *M. hirtipes* were collected from Taieri River Mouth, Otago, New Zealand (46°3′3″S, 170°11′24″ E), and on 10 June 2009, 480 *H. crenulatus* were collected from the same site. In total, each of 20 parasite clones was used to infect 24 individual crabs of each crab species. Infections were conducted in batches over a 5-day period starting 1–3 days after crab capture. During infections with parasite cercariae, crabs were held in plastic Petri dishes (35 mm diameter by 10 mm height) filled with 1 mL of seawater. These small containers prevented any avoidance behaviour by the crabs when exposed to cercariae by confining their movements. Cercariae were shed from snails by placing the latter in a 25 °C incubator with constant light source for an hour. Approximately 100 cercariae were added to each Petri dish through a hole drilled into the lid, and a rubber band was used to secure it to the base dish. Crabs were kept in a dark incubator at 24 °C for 5 h (incubation conditions adapted from Fredensborg *et al.*, 2004) to allow enough time for host penetration to take place. Crabs were then transferred from the Petri dishes to marked 1-L plastic containers (six crabs all infected with the same clone per container) filled with aerated seawater and kept at approximately 18 °C with weekly feedings of frozen brine shrimp and fish food flakes and weekly water changes for 6 weeks until the metacercariae reached maturity. During this period, container positions on the shelving racks were randomly rotated each week. Metacercariae are considered mature when they reach the double-walled cyst stage (Keeney *et al.*, 2007), a process taking approximately 6 weeks based on time estimates from preliminary trials and by killing a few haphazardly selected experimental crabs to gauge the development of the metacercariae.

Crab dissection

After 6 weeks, crabs were killed by severing of the ventral nerve cord. All tissues were thoroughly examined for macroparasites including nematodes, trematodes, acanthocephalans and parasitic isopods. Although crabs should not have been infected with *M. novaezealandensis* prior to the experiment, it was not possible to avoid pre-existing infection by other species of parasites. Parasite prevalence and mean intensity (total number of parasites divided by the total number of infected hosts) were calculated for each pre-existing parasite species except for a highly abundant nematode for which only presence/absence was recorded. Two observers (A. Koehler and T. Leung) performed all dissections. Infection success of *M. novaezealandensis* was calculated by dividing the number of cysts recovered by 100, i.e. the approximate number of cercariae used initially.

Parasite culture/excystment

For all *M. novaezealandensis* recovered by dissection, metacercarial cyst volume was calculated using the volume of an ellipsoid ($V = \pi \times L \times W^2/6$), where L is maximum cyst length and W is maximum width. Once metacercariae were counted and measured, parasite excystment was induced to estimate growth and fecundity within a simulated bird intestine (the parasite's final host). Metacercariae were placed individually into 0.6-mL Eppendorf tubes containing 0.4 mL of culture medium simulating the aqueous environment of the avian intestine. Following Fredensborg & Poulin (2005b), the medium consisted of approximately 80% NCTC-109 solution (Invitrogen, Carlsbad, CA, USA), approximately 20% chicken serum (Invitrogen) that had been inactivated at 56 °C for 30 min prior to medium preparation and an aliquot of a penicillin/streptomycin antibiotic solution yielding a concentration of 100 μg mL^{-1} streptomycin and 100 U mL^{-1} penicillin. Tubes containing the metacercariae were kept under dark conditions in an incubator set at 40 °C for 40 h. Excysted adult parasites were heat-fixed in 10% neutral buffered formalin. Their total length (L) and width (W) at widest point were measured to calculate their area using the formula ($A = L \times W$), and the number of uterine eggs (initial egg output) in each parasite was counted as in Loker (1983).

Amphipod infection

On 12 January 2010 and 14 February 2010, large numbers of *P. novizealandiae* and *H. stephenseni* were collected from Hooper's Inlet, Otago Peninsula, New Zealand (45°51′13″S, 170°40′12″E). Because of snail mortality, only 15 clones for *P. novizealandiae* and 11 for *H. stephenseni* (of the original 20) were available for use in experimental infections at the time of host collection. Infection trials were conducted over 3 days following each collection date using 96-well plates where, for each clone, 30 amphipods were individually placed into separate wells followed by approximately 45 cercariae (dosage based on prior infection trials). The small wells restricted the amphipod's movements, thereby minimizing any natural avoidance behaviours. The plates were placed in a 25 °C incubator for 3 h to optimize infection conditions (Fredensborg *et al.*, 2004; Studer *et al.*, 2010). Because amphipods do not survive several weeks (the time needed for the full development of parasite metacercariae) in captivity, no attempt was made to measure parasite growth to the post-cyst stage and egg production *in vitro*. After exposure to cercariae, amphipods were transferred into 380-mL containers of seawater and incubated at 16 °C overnight. Amphipods that died during this period were excluded from analysis. All remaining amphipods were killed by decapitation the following day and immediately dissected, and the number of cercariae successfully

infecting each host was recorded; no other metazoan parasites were found in any amphipod. Amphipod size was measured by taking the length of the head capsule (a reliable estimator of amphipod body size, Wilhelm & Lasenby, 1998) and grouped into four associated size classes: small: 0.290–0.356 μm; medium: 0.357–0.423 μm; large: 0.424–0.490 μm; and X-large: 0.491–0.556 μm. Three observers (A. Koehler, T. Leung and H. Randhawa) performed all dissections.

Statistics

General statistics

Differences in mean parasite abundance either between crab hosts, or between amphipod hosts, were tested using unpaired *t*-tests or Wilcoxon signed rank tests. Host sexual dimorphism was tested for, in terms of size, using unpaired *t*-tests. These and all other analyses were performed in JMP (Version 7.0; SAS Institute Inc., Cary, NC, USA) and R v2.11.1 (R Development Core Team, 2010).

Clonal specialization

To detect whether there were differences in infection success among clones, generalized linear models (GLMs) with negative binomial error structure were used for each crab species and linear models (LMs) were used for each amphipod species. Given that infection of different crustaceans had to be performed using slightly different methods and at different time periods, it was not possible to include a clone-by-host species interaction in the models described below, to test for specialization as in Gemmill *et al.* (2000). Instead, Spearman's rank correlations were used to determine whether the infection success achieved in one host correlated with that achieved in another host, across all clones, and for all pairs of hosts. Positive correlations would indicate that certain clones tend to perform well in all hosts and that others consistently perform poorly, whereas negative correlations or no correlations might instead indicate that different clones may be specialized for different hosts.

Infection success of clones within host species

A generalized linear mixed-effects model (GLMM) with a quasi-Poisson error structure was used to examine factors associated with infection success in the crab *M. hirtipes*. Metacercarial count per host individual was the response variable, clone was a random factor and the following were fixed-effect predictor variables: crab sex and size, parasite heterozygosity, the principal component of cercarial morphology (from the PCA), summed CV of cercarial morphology, average and CV of cercarial survival time, average and CV of cercarial phototaxic behaviour and observer identity. Additionally, pre-existing acanthocephalan (*Profilicollis* spp.) parasite counts were also included (other pre-existing parasite species were either not found in great enough numbers to necessitate inclusion in the model, or in the case of *Ascarophis* sp. nematodes, occurred in 100% of *M. hirtipes* but individual counts were not made). All continuous response variables were centred (standardized). For each analytical model, variables were checked for multicollinearity using the function 'vif' in the R-package 'car'. All mixed-effects models were performed with the R-package 'lme4'. For infections of the crab *H. crenulatus*, owing to the relatively small number of successful infections, data were treated as presence or absence of cysts, and a binomial error structure was used, thereby avoiding a model with zero-inflated overdispersion. The same predictor variables were used as in the GLMM for *M. hirtipes*.

For both amphipod species, GLMMs with a Poisson error structure were used to assess which factors had significant effects on infection success. The response variable was the number successfully infecting cercariae. The same predictor variables were used as those in the crab GLMM except for pre-existing parasites as no parasites other than *M. novaezealandensis* were encountered. The interaction between amphipod sex and size was included in all models.

Differences in parasite fitness among clones and between crabs

To test for differences in fitness among clones, LMs were performed for each measure of fitness: metacercarial cyst volume, adult worm area and adult egg count, using combined data from both crab host species. Spearman's rank correlations were then used to test for correlations among clones between fitness measures achieved in one crab species vs. those achieved in the other crab, to determine whether some clones outperformed others for each of the fitness measures regardless of host identity. Finally, separate linear mixed-effects models (LLMs) with each fitness measure as a response variable were used to assess which factors had significant effects on parasite performance. Again, clone was the random factor, and predictor variables were the same as in the above GLMM with the addition of host species identity and the exclusion of observer identity (as H. Randhawa took all fitness measurements). Owing to issues of multicollinearity, CV of cercarial morphology was removed from the models of adult egg numbers and adult body size area. Response variables were not transformed as they approximated normality.

Results

General findings

In total, 1427 crustaceans were dissected. Sexual size dimorphism was detected in three of the four crustacean host species, with males being slightly larger than females (Table S2).

In crab hosts, the mean abundance of *M. novaezealandensis* was significantly higher in *M. hirtipes* (Mean ± SE = 1.67 ± 0.13, range 0.35–3.25) compared to *H. crenulatus*

(0.14 ± 0.02, range 0–0.41) (Wilcoxon signed ranks test: $W = 49580.5$, $P < 0.0001$, Table S3). In amphipod hosts, the mean abundance of *M. novaezealandensis* was 14.25 ± 0.50 in *P. novizealandiae* (range 10.83–20.17) and 12.13 ± 0.43 in *H. stephenseni* (range 7.29–17.93), with these values also being significantly different ($t_{573} = -3.19$, $P = 0.0015$) (Table S3).

Clonal specialization

Differences in parasite mean abundance were detected among clones for each crustacean host species: *M. hirtipes* LR $\chi^2_{19} = 71.51$, *H. crenulatus* LR $\chi^2_{19} = 51.01$, *P. novizealandiae* $F_{11,291} = 4.202$ and *H. stephenseni* $F_{10,261} = 6.490$ (all $P < 0.0001$) (Fig. 1). There were no significant correlations among clones between mean parasite abundance in one host species and that in another, for any pair of crustacean species (Table S4).

Infection success of clones within host species

None of the phenotypic or genotypic factors under examination had consistently significant effects on parasite infection success in all four host species (Table 1). Averages (and coefficients of variation) for each cercarial clone's phenotypic trait and genotype are given in Table S1. Infection success decreased with crab size for *M. hirtipes* (Fig. S1). Cercariae with longer survival times had higher infection success in both *H. crenulatus* and *P. novizealandiae*. Clones that preferred darkness over light, and with less variability in photoreaction, had greater infection success in *H. crenulatus*. Higher variability (CV) in both survival time and photoreaction leads to decreased infections in *H. crenulatus*. For *P. novizealandiae*, the negative interaction between size and sex of the amphipod was significant, suggesting that increasing body size affected the susceptibility of males and females differently (Fig. 2). Host size was a significant factor for *H. stephenseni*, with more cercariae successfully infecting larger amphipods. Observer identity was a significant factor for the number of parasites recovered in both amphipods, although removing the data of either observer from the study did not alter the overall results.

Differences in parasite fitness among clones and between crabs

Significant differences were detected among clones for each fitness measure achieved in crab hosts (cyst volume: $F_{18,245} = 7.386$; adult area: $F_{18,176} = 3.537$; and egg

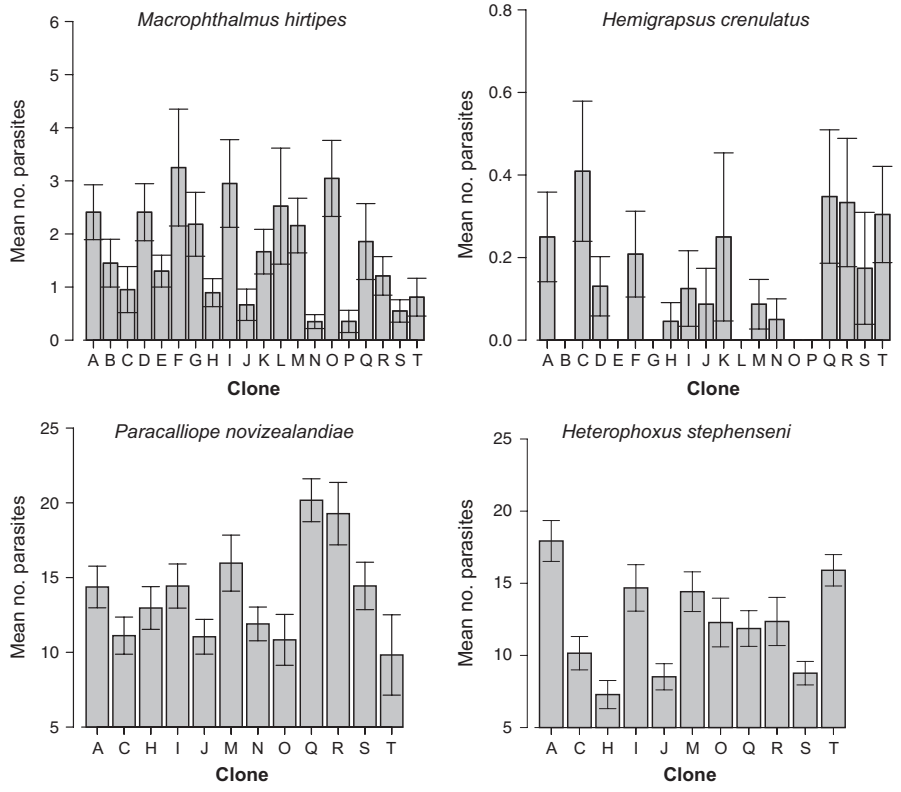

Fig. 1 Mean (±SE) infection success of each *Maritrema novaezealandensis* clone in each of four crustacean host species. Note: fewer clones were available for infection of the two amphipod species. Infection success was measured as number of cysts after 5 weeks in the crabs and number of metacercariae recovered after 24 h in the amphipods.

Table 1 Summary of the GLMMs for each crustacean host with total number of successfully infecting parasites as the response variable and clone as the random variable. For each variable, associated *t* or *z* values and *P*-values are shown. Distributional types (family) and number of observations are included. All models included host sex and size (and their interaction), parasite heterozygosity, the principal component of cercarial morphology (from a PCA), summed CV of cercarial morphology, average and CV of cercarial survival time, average and CV of cercarial phototaxic behaviour and observer identity. Models with crabs also included the number of pre-existing acanthocephalans. Factors with significant parameter estimates (95% confidence interval bounded away from zero) in bold.

Response	Distribution	Factor	Estimate	SE	*z*-value*	*P*-value	95% CI
No. of cysts in	Quasi-Poisson	(Intercept)	−2.780	4.408	−0.63	0.529	−11.4190 to 5.8587
Macrophthalmus hirtipes	(d.f. = 265)	Sex:male	0.350	0.191	1.83	1.931	−0.0245 to 0.7250
		Size	**−0.178**	**0.080**	**−2.24**	**0.026**	**−0.3346 to −0.0221**
		Sex:size	0.033	0.136	0.24	1.191	−0.2333 to 0.2990
		PCA morphology	−0.433	1.293	−0.34	0.738	−2.9675 to 2.1016
		CV morphology	0.083	0.148	0.56	1.426	−0.2067 to 0.3731
		Survival	0.001	0.002	0.62	1.465	−0.0030 to 0.0057
		CV survival	0.118	0.130	0.91	1.636	−0.1361 to 0.3717
		Avg behaviour	0.369	5.321	0.07	1.055	−10.0594 to 10.7973
		CV behaviour	0.001	0.009	0.07	1.056	−0.0174 to 0.0186
		Heterozygosity	−0.013	0.023	−0.57	0.566	−0.0588 to 0.0322
		Acanthocephalan	−0.021	0.034	−0.62	0.534	−0.0890 to 0.0461
		Observer 1	−0.260	0.181	−1.44	0.152	−0.6159 to 0.0949
No. of cysts in	Binomial	(Intercept)	−1.679	3.851	−0.44	0.663	−9.2262 to 5.8686
Hemigrapsus crenulatus	(d.f. = 297)	Sex:male	0.193	0.447	0.43	0.666	−0.6829 to 1.0691
		Size	−0.238	0.240	−0.99	0.322	−0.7091 to 0.2331
		Sex:size	0.291	0.276	1.06	0.291	−0.2498 to 0.8323
		PCA morphology	1.021	1.287	0.79	0.427	−1.5013 to 3.5443
		CV morphology	−0.048	0.137	−0.35	0.727	−0.3168 to 0.2208
		Survival	**0.010**	**0.004**	**2.76**	**0.006**	**0.0028 to 0.0166**
		CV survival	**−0.303**	**0.120**	**−2.52**	**0.012**	**−0.5382 to −0.0670**
		Avg behaviour	**−28.646**	**8.895**	**−3.22**	**0.001**	**−46.0793 to −11.2121**
		CV behaviour	**−0.035**	**0.012**	**−2.97**	**0.003**	**−0.0589 to −0.0121**
		Heterozygosity	0.001	0.018	0.06	0.954	−0.0352 to 0.0373
		Acanthocephalan	−0.718	0.395	−1.82	0.069	−1.4926 to 0.0565
		Observer 1	−0.157	0.408	−0.38	0.701	−0.9568 to 0.6437
No. of cysts in	Poisson	(Intercept)	0.286	1.820	0.16	0.875	−3.2809 to 3.8519
Paracalliope novizealandiae	(d.f. = 303)	**Sex:male**	**0.651**	**0.094**	**6.92**	**< 0.001**	**0.4668 to 0.8357**
		Size Med	**0.342**	**0.068**	**5.04**	**< 0.001**	**0.2091 to 0.4756**
		Size Lg	**0.263**	**0.079**	**3.34**	**0.001**	**0.1087 to 0.4173**
		Size XL	0.062	0.148	0.42	0.673	−0.2272 to 0.3518
		Sex:size Med	**−0.581**	**0.104**	**−5.59**	**< 0.001**	**−0.7849 to −0.3772**
		Sex:size Lg	**−0.597**	**0.111**	**−5.38**	**< 0.001**	**−0.8149 to −0.3798**
		Sex:size XL	−0.196	0.176	−1.11	0.266	−0.5405 to 0.1494
		PCA morphology	−0.029	0.528	−0.06	0.956	−1.0637 to 1.0050
		CV morphology	0.033	0.062	0.53	0.596	−0.0884 to 0.1539
		Survival	**0.002**	**0.001**	**2.43**	**0.015**	**0.0004 to 0.0041**
		CV survival	−0.012	0.054	−0.23	0.818	−0.1176 to 0.0928
		Avg behaviour	−2.204	2.773	−0.80	0.427	−7.6386 to 3.2314
		CV behaviour	0.000	0.004	0.10	0.921	−0.0072 to 0.0080
		Heterozygosity	−0.004	0.010	−0.39	0.696	−0.0236 to 0.0157
		Observer 1	**−0.664**	**0.049**	**−13.56**	**< 0.001**	**−0.7602 to −0.5682**
		Observer 2	**−0.109**	**0.042**	**−2.57**	**0.010**	**−0.1922 to −0.0259**
No. of cysts in	Poisson	(**Intercept**)	**3.735**	**1.050**	**3.56**	**0.000**	**1.6774 to 5.7936**
Heterophoxus stephenseni	(d.f. = 272)	Sex:male	0.086	0.143	0.60	0.549	−0.1947 to 0.3664
		Size Med	**0.145**	**0.073**	**1.97**	**0.049**	**0.0009 to 0.2882**
		Size Lg	**0.162**	**0.075**	**2.15**	**0.031**	**0.0145 to 0.3098**
		Size XL	0.107	0.100	1.07	0.285	−0.0888 to 0.3018
		Sex:size Med	0.104	0.156	0.67	0.503	−0.2012 to 0.4099
		Sex:size Lg	−0.087	0.153	−0.57	0.568	−0.3870 to 0.2125
		Sex:size XL	−0.070	0.188	−0.37	0.709	−0.4379 to 0.2977

Table 1 (*Continued*).

Response	Distribution	Factor	Estimate	SE	*z*-value*	*P*-value	95% CI
		PCA morphology	0.428	0.307	1.39	0.164	−0.1745 to 1.0306
		CV morphology	−0.063	0.036	−1.72	0.086	−0.1339 to 0.0088
		Survival	0.000	0.001	−0.37	0.712	−0.0017 to 0.0011
		CV survival	0.032	0.030	1.05	0.293	−0.0275 to 0.0911
		Avg behaviour	2.816	1.872	1.51	0.132	−0.8522 to 6.4847
		CV behaviour	0.004	0.003	1.23	0.220	−0.0022 to 0.0096
		Heterozygosity	−0.009	0.006	−1.66	0.098	−0.0200 to 0.0017
		Observer 1	**−0.843**	**0.060**	**−13.97**	**< 0.001**	**−0.9609 to −0.7245**
		Observer 2	**−0.161**	**0.039**	**−4.08**	**< 0.001**	**−0.2382 to −0.0836**

GLMM, generalized linear mixed-effects model; PCA, principal component analysis; CV, coefficient of variation; SE, standard error.
*t-values reported for quasi-Poisson model run with *M. hirtipes*.

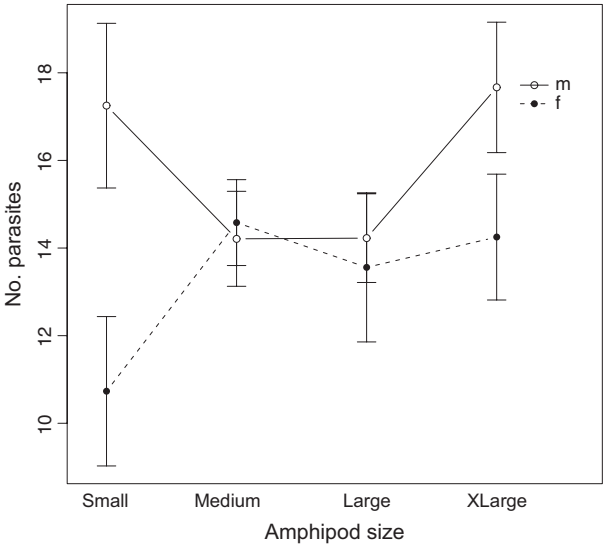

Fig. 2 Interaction between amphipod sex (m = males, f = females) and size and the mean (±SE) number of *Maritrema novaezealandensis* successfully infecting a host for both *Paracalliope novizealandiae* and *Heterophoxus stephenseni* combined.

count: $F_{18,212} = 7.530$; all $P < 0.0001$) (Fig. 3). No significant correlation across all clones was found between average fitness achieved in one crab species and that achieved in the other, for any of the three fitness measures (Table S5). Average fitness measures (metacercarial cyst volume, adult body area and number of eggs *in utero*) achieved by the parasite clones in both crab species are provided in Table S6.

Post-infection fitness parameters were significantly higher in *M. hirtipes* than in *H. crenulatus* (Fig. 4, Table 2). Cercariae with lower variation in survival time produced larger cysts (Table 2). Cysts were larger in male crabs than in female crabs, and conversely, adult trematodes were larger in female hosts than in males (Table 2). Finally, larger cercariae tended to produce more fecund adult trematodes (Fig. S2, Table 2).

Discussion

Although we found evidence that clones of the parasitic trematode *M. novaezealandensis* vary in their abilities to infect different host species, none of the genetic or phenotypic traits we considered as potential causal mechanisms had a consistent effect on cercarial infection success or post-infection fitness measures across all four host species used in our experiment. The hypothesis that clones with high variability (phenotypic and/or genotypic) are more successful at infecting a wider range of hosts was not supported by our results, although some of our models recognized lack of phenotypic variability as being important. Factors other than interclonal differences, however, had significant effects and, when considered in light of the ecological differences among the focal hosts, suggest constraints on host specificity acting at both the individual clonal level and the population level.

Clonal specialization

Overall, infection success of the clones was generally inconsistent among host species. For instance, clone 'A' performed well in all hosts, whereas clone 'J' did below average in all hosts. Meanwhile, clone 'O' did well in *M. hirtipes*, failed to infect any *H. crenulatus* and achieved only average infection success in both amphipods. Additionally, when three fitness measures were compared between the two crab hosts, the lack of significant correlations again demonstrated a range of clonal specialization, with some clones performing well only in one crab species and others achieving average fitness in both hosts. Although based on data from only four of the many crustacean hosts used in nature, these results suggest specialization of some clones for different hosts, supporting the idea that generalist populations are comprised of individual specialists (Bolnick *et al.*, 2003; Bolnick *et al.*, 2011); however, collectively, at the population level, a generalist strategy is maintained. Similarly, clonal specialization for particular host genotypes has been documented in another trematode, with the population

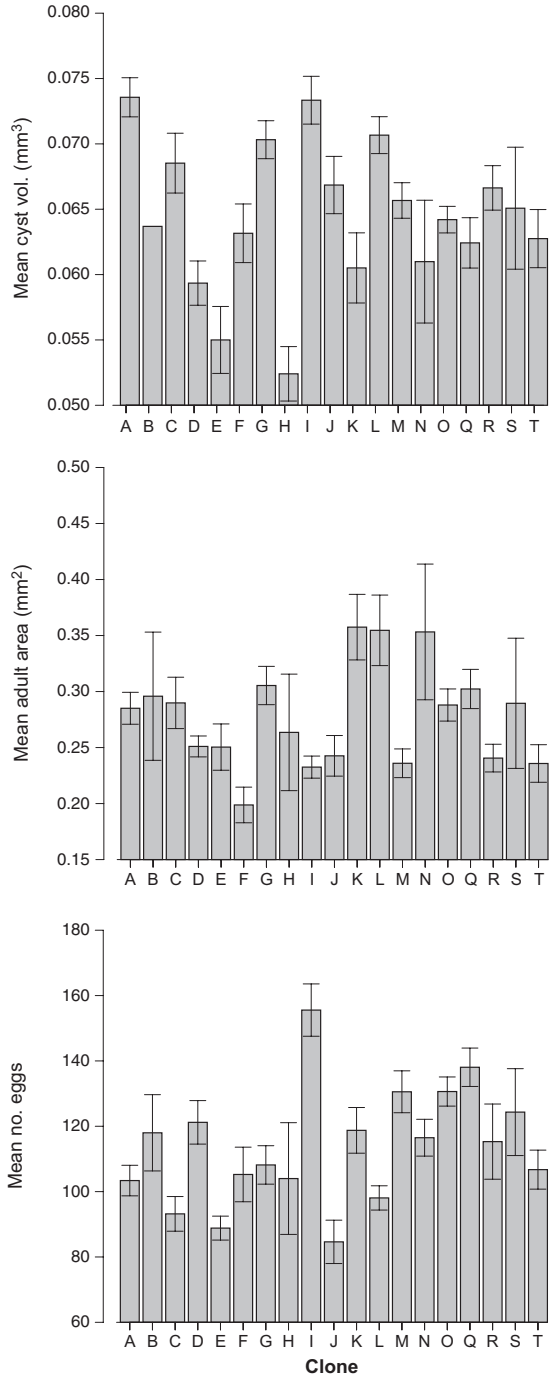

Fig. 3 Mean (±SE) fitness score (metacercarial cyst volume, adult worm body area and number of uterine eggs in the adult worm) of each *Maritrema novaezealandensis* clone infecting the crabs *Macrophthalmus hirtipes* and *Hemigrapsus crenulatus*.

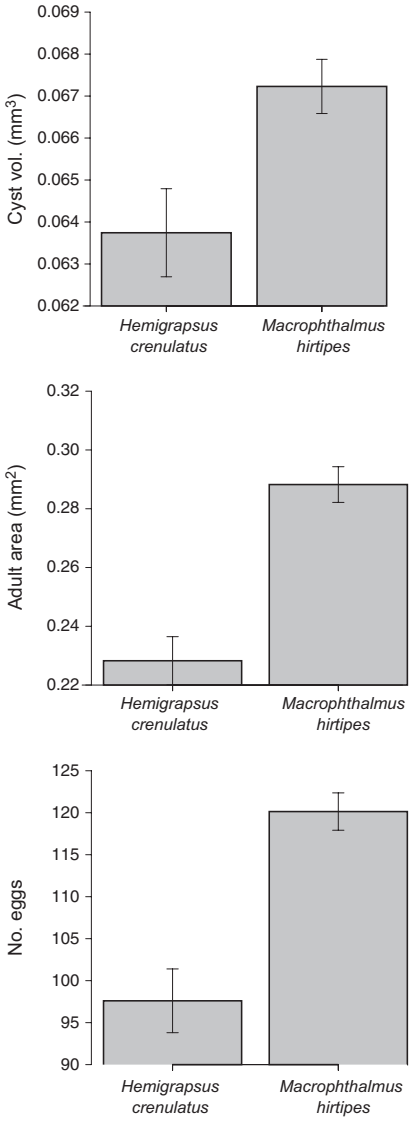

Fig. 4 Mean (±SE) fitness score (metacercarial cyst volume, adult worm body area and number of uterine eggs in the adult worm) of *Maritrema novaezealandensis* in the two host crabs *Macrophthalmus hirtipes* and *Hemigrapsus crenulatus*.

consisting of a mixture of specialized genotypes (Lively & Dybdahl, 2000). An alternative explanation might be that the apparent variation among clones in the degree of specialization is in fact due to snail effects. One clone is inevitably associated with a single snail; this is a characteristic of this system. However, not only did we minimize variation in shell size or housing conditions among snails, but also there is no empirical basis to expect that how trematodes choose their second intermediate hosts is affected by the identity of their previous host.

Other studies investigating infection success of distinct clones have produced varied results. Carius *et al.* (2001) experimentally exposed *Daphnia* to bacteria in a genotype-by-genotype design and found that no parasite isolate was superior in performance to any other isolates. Via (1991) measured success of aphid clones on multiple hosts and found a trade-off in infection success, with

Table 2 Summary of the LMM run for each trematode fitness measure achieved in the crabs *Macrophthalmus hirtipes* and *Hemigrapsus crenulatus*. All models included crab sex and size (and their interaction), parasite heterozygosity, the principal component of cercarial morphology (from a PCA), summed CV of cercarial morphology, average and CV of cercarial survival time, average and CV of cercarial photoreactive behaviour and the number of pre-existing acanthocephalans. Factors with significant parameter estimates (95% confidence interval bounded away from zero) in bold.

Response	Factor	Estimate	SE	*z*-value	95% CI
Cyst vol. (μm^3) (d.f. = 206)	**(Intercept)**	**0.07365**	**0.03467**	**2.12**	0.00570 to 0.14160
	Sex:male	**0.00244**	**0.00115**	**2.12**	**0.00019 to 0.00470**
	Size	−0.00040	0.00060	−0.67	−0.00157 to 0.00077
	Sex:Size	0.00110	0.00083	1.32	−0.00053 to 0.00274
	Species MH	**0.00592**	**0.00168**	**3.53**	**0.00263 to 0.00920**
	PCA morphology	0.01552	0.01030	1.51	−0.00467 to 0.03571
	CV morphology	−0.00087	0.00116	−0.75	−0.00315 to 0.00141
	Survival	0.00001	0.00002	0.79	−0.00002 to 0.00005
	CV survival	**−0.00229**	**0.00103**	**−2.23**	**−0.00431 to −0.00028**
	Avg behaviour	−0.04784	0.04260	−1.12	−0.13134 to 0.03566
	CV behaviour	−0.00010	0.00007	−1.35	−0.00025 to 0.00004
	Heterozygosity	0.00006	0.00018	0.30	−0.00030 to 0.00041
	Acanthocephalan	0.00023	0.00020	1.17	−0.00015 to 0.00061
Adult size (μm^2) (d.f. = 139)	(Intercept)	0.1534	0.1516	1.01	−0.1437 to 0.4505
	Sex:male	**−0.0357**	**0.0104**	**−3.45**	**−0.0560 to −0.0154**
	Size	0.0075	0.0060	1.26	−0.0042 to 0.0192
	Sex:Size	−0.0123	0.0085	−1.45	−0.0288 to 0.0043
	Species MH	**0.0639**	**0.0196**	**3.25**	**0.0254 to 0.1024**
	PCA morphology	0.0483	0.0570	0.85	−0.0635 to 0.1600
	Survival	0.0000	0.0001	−0.07	−0.0003 to 0.0002
	CV survival	−0.0067	0.0067	−1.00	−0.0198 to 0.0065
	Avg behaviour	−0.1324	0.2866	−0.46	−0.6941 to 0.4293
	CV behaviour	−0.0006	0.0006	−1.07	−0.0017 to 0.0005
	Heterozygosity	0.0019	0.0014	1.30	−0.0010 to 0.0047
	Acanthocephalan	−0.0016	0.0020	−0.81	−0.0055 to 0.0023
No. eggs (d.f. = 165)	(Intercept)	−77.89	58.47	−1.33	−192.48 to 36.71
	Sex:male	6.57	4.55	1.44	−2.35 to 15.48
	Size	−0.49	2.26	−0.22	−4.93 to 3.95
	Sex:Size	−2.49	3.33	−0.75	−9.01 to 4.04
	Species MH	**27.25**	**8.43**	**3.23**	**10.73 to 43.76**
	PCA morphology	**51.40**	**21.63**	**2.38**	**9.01 to 93.78**
	Survival	0.01	0.05	0.28	−0.08 to 0.11
	CV survival	1.67	2.54	0.66	−3.30 to 6.65
	Avg behaviour	−11.29	106.24	−0.11	−219.53 to 196.95
	CV behaviour	0.00	0.21	−0.01	−0.41 to 0.41
	Heterozygosity	0.96	0.55	1.75	−0.11 to 2.03
	Acanthocephalan	−1.25	0.89	−1.40	−3.00 to 0.50

CV, coefficient of variation; LMM, linear mixed models; PCA, principal component analysis; MH, *Macrophthalmus hirtipes*; SE, standard error.

clones that were superior on one host being inferior on another host. Seppälä *et al.* (2007) recorded wide-ranging intraindividual variability in infectivity of genetically distinct cercariae when exposed to fish. Finally, Leung & Poulin (2010) exposed distinct clones of a trematode parasite to two species of bivalves and found no evidence of intraspecific variation in infection success.

There is no consensus on whether the evolution of specialization is directional (Thompson, 1994; Poulin, 2007). Generalists can be derived from specialists (Poulin *et al.*, 2006; Johnson *et al.*, 2009), although some suggest that generalist parasites evolve irreversibly towards specialized host races (Kawecki, 1998; Stireman, 2005). McCoy (2003) and Théron & Combes (1995) argued that speciation is more likely in parasites with life cycles involving active dispersal. A generalist like *M. novaezealandensis* might thus speciate into multiple host races through interclonal specialization. However, speciation would only be possible if the definitive bird hosts showed species-specific selectivity in the crustaceans they consumed. To date, only the red-billed gull (*Chroicocephalus scopulinus*) has been recorded as a definitive host for *M. novaezealandensis* (Martorelli *et al.*, 2004), but other waterbirds are likely hosts too. Diet studies and variation in bill morphology suggest that interspecific differences in crustacean prey selection exist among those birds

(Heather & Robertson, 1996). Segregation of cercariae into different second intermediate hosts owing to clonal specialization followed by specialization in bird feeding habits could favour host races and ultimately speciation.

Infection success of clones within host species

Crabs

In nature, *M. novaezealandensis* is more successful in the crab *M. hirtipes* than in *H. crenulatus* (Koehler & Poulin, 2010), although both crabs live in sympatry and display similar behaviour. This difference in natural infections allows for a comparison, in a controlled laboratory setting, of the factors influencing infection of a relatively susceptible host and a more resistant species. Crab size was the only significant factor related to infection success in *M. hirtipes*, with parasites having greater infection success in smaller crabs than large ones. Cercariae penetrate crabs via the gills (Cable & Hunninen, 1940; Saville & Irwin, 2005; Smith *et al.*, 2007); therefore, perhaps smaller crabs have less defensible gills that are easier to penetrate. Naturally infected *M. hirtipes* do not show a correlation between size and infection intensity (Koehler & Poulin, 2010), although Fredensborg & Poulin (2005a) found that infection intensity decreased with increasing crab size. Here, size was not correlated with infection success in *H. crenulatus*, suggesting that its defence mechanisms (physical or immunological), which prevent infection levels comparable to those in *M. hirtipes,* are established early in the crab's life.

Cercariae with greater stamina (survivability) were more successful at infecting the crab *H. crenulatus*, suggesting that host defences withstand short-lived cercariae but eventually succumb to more persistent parasite clones. Penetrating cercariae may weaken gill tissue to gain entry, and thus cercariae with greater stamina may be more likely to complete the infection process. Conversely, survivability is not a significant factor for cercariae infecting *M. hirtipes*, implying that this host is more susceptible to a range of invading *M. novaezealandensis* cercarial phenotypes.

Maritrema novaezealandensis cercariae do not demonstrate the dramatic behaviours (specialized responses to stimuli) seen in other trematodes (Combes *et al.*, 1994). Nevertheless, they are capable of photoreactive behaviour rather than purely random dispersion (Koehler *et al.*, 2011b). In general, *M. novaezealandensis* cercariae are attracted to light for about an hour after emerging from a snail, and then their affinity switches to darkness (A. Koehler, personal observation). Cercarial clonal lines displaying photophobic behaviour had increased infection success in *H. crenulatus*. Presumably the initial attraction to light coaxes cercariae out of the snails, and the subsequent photophobic switch increases their chances of encountering *H. crenulatus*, which inhabit crevices beneath rocks (McLay, 1988), or *M. hirtipes*, which live in burrows (Nye, 1974). This does not explain why the photoreaction factor was only significant for *H. crenulatus* and not for *M. hirtipes*. It may be that the susceptibility of *M. hirtipes* is so great that cercarial behaviour matters little. The use of clear Petri dishes and well plates in the infection trials did not allow us to test the effects of light on infection dynamics.

Parasite clones characterized by less variation in survival time and photoreactive tendencies had higher infection success in *H. crenulatus*. Variability in phenotypic characters may lead to a wider range of infected hosts (Ward, 1992), but consistency in clonal behaviour and survival may benefit clones when infecting a less susceptible host like *H. crenulatus* if average phenotypic attributes are optimal for infection.

Prior infections by acanthocephalans did not affect infection success of *M. novaezealandensis*. Interactions between coinfecting parasites can influence pathogenicity (Petney & Andrews, 1998), alter the epidemiology of each parasite species within the host (Cox, 2001; Pedersen & Fenton, 2007) and change helminth community structure (Poulin, 2001). A field study (Poulin *et al.*, 2003) found reciprocal effects on growth between acanthocephalans (*Profilicollis* sp.) and *M. novaezealandensis* in crabs. Although there may be an effect of acanthocephalans on *M. novaezealandensis* in natural settings, none were detected within the relatively short duration of our experiments.

Amphipods

For both amphipod species, the interaction between host size and sex was significant for infection success by *M. novaezealandensis*, most likely due to host sexual dimorphism (males are larger than females; see Table S2). For *P. novizealandiae*, infection success peaks in both the smallest and largest males, although this pattern was not observed in females. The greater surface area available for cercarial penetration in males may provide an explanation, along with behavioural or immune differences between male and female hosts (Poulin, 1996a; Cowan *et al.*, 2007). Sexual dimorphism in *P. novizealandiae* is much greater than in *H. stephenseni*, and therefore only size was a significant factor for the latter.

Amphipod avoidance behaviour may explain why persistent cercariae were more successful than those with shorter survival times at infecting *P. novizealandiae*. In contrast, survival was not a factor for cercariae infecting *H. stephenseni*. This discrepancy may reflect differences in the life histories of the two amphipods. *Paracalliope novizealandiae* is constantly exposed to *M. novaezealandensis* cercariae (Fredensborg *et al.*, 2004), whereas *H. stephenseni* is a burrower (Oakden, 1984) only occasionally exposed above the sediment. In artificial conditions without the sediment in which *H. stephenseni* burrows, both amphipod hosts are nearly equally infected (Koehler *et al.*, 2011a). However, when natural host behaviours are restricted within a small

plastic well, persistent cercariae can overcome physical barriers that may prevent infection of *P. novizealandiae* by short-lived cercariae. Additionally, the fact that survivability does not influence cercariae infecting *H. stephenseni* could signify that a tougher carapace or other physical defences prevent infection in this amphipod. From an evolutionary viewpoint, the parasite may be better adapted at overcoming *P. novizealandiae*'s defences than those of *H. stephenseni* because it encounters more frequently the former than the latter.

Heterozygosity

Parasite heterozygosity played no clear role in parasite fitness or infection success in this system. Previous studies investigating the relationship between genetic diversity (heterozygosity) and fitness traits typically involve populations that are inbred or recently bottlenecked, as heterozygosity is often used as an indicator of inbreeding depression (Hansson & Westerberg, 2002; Chapman *et al.*, 2009; Szulkin *et al.*, 2010). However, heterozygosity–fitness correlations are also relevant to ecological and evolutionary issues in noninbred populations (see D'Souza & Michiels, 2008). An earlier mesocosm study of host preference, in which *M. novaezealandensis* cercariae were simultaneously exposed to both *P. novizealandiae* and *H. stephenseni,* showed no correlation with heterozygosity (Koehler *et al.*, 2011a). In other trematodes, Beltran *et al.* (2008) found that heterozygosity did not affect pair formation in *Schistosoma mansoni*, yet Prugnolle *et al.* (2004) found a correlation between female *S. mansoni* fitness and heterozygosity. Here, clonal heterozygosity had no measurable impact on the parasite's performance both during and after infection and thus is unrelated to fitness.

Differences in parasite fitness among clones and between crabs

Variation in the infection success achieved by a generalist parasite among sympatric hosts is often associated with interspecific differences in encounter and compatibility. Hosts with low compatibility include those in which parasite transmission reaches a dead-end (Thieltges *et al.*, 2008) as well as those in which the parasite develops poorly (Koehler & Poulin, 2010). Koehler & Poulin (2010) found differences in *M. novaezealandensis* mean abundance among 14 species of intertidal crustaceans that were attributed to external morphology, immunity or microhabitat. Here, the post-infection fitness achieved by *M. novaezealandensis* differed between two crab hosts, one (*M. hirtipes*) that is more heavily infected in nature than the other (*H. crenulatus*). In *M. hirtipes*, cysts and the adult worms they yielded were larger and their egg count was greater than in *H. crenulatus.* Thus, *M. hirtipes* is not only more suitable as a host than *H. crenulatus* in an ecological sense, but it is also physiologically more compatible for parasite development.

Host sex also influenced parasite fitness parameters. Male hosts harbour more parasites than females in both vertebrates (Poulin, 1996a) and arthropods (Sheridan *et al.*, 2000). In addition, helminth parasites often achieve higher growth in male than in female hosts (Poulin, 1996b). Here, cysts attained larger sizes in male crabs than in females, although both sexes are nearly identical in size for *H. crenulatus* and only slightly different for *M. hirtipes*.

More importantly, we found no evidence that interclonal differences in heterozygosity or phenotype influenced post-infection fitness parameters. In interspecific comparisons, trematode species with large cercariae also have large-bodied adults (Poulin & Latham, 2003); however, this pattern was not evident in our interclonal analysis. Nevertheless, cercarial size (based on the principal components of the PCA) correlated with adult worm fecundity, with larger cercariae producing more fecund adults. Thus, phenotypic traits in early life may determine success in later life. In contrast, heterozygosity of cercarial clones had no significant effect on their later fitness, again failing to support any heterozygosity–fitness correlation (Chapman *et al.*, 2009; Szulkin *et al.*, 2010).

In summary, although significant variation among trematode clones in both infection success and post-infection fitness parameters was demonstrated, this variation could not be explained consistently by either the genetic variation or phenotypic traits (or their variance) of the various clones tested. Owing to one or more other factors, however, it appears that some clones act as host specialists and others as generalists. The *M. novaezealandensis* population studied may therefore maintain an overall generalist exploitation of the crustacean community by comprising a mix of clone-level host specialists and generalists.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Number of *Maritrema novaezealandensis* metacercariae as a function of *Macrophthalmus hirtipes* carapace width (mm) in experimental infections, with data from all clones combined.

Figure S2 Number of uterine eggs per *Maritrema novaezealandensis* metacercaria grown *in vitro* after removal from crabs, as a function of principal component values (from a PCA) of cercarial morphological measurements.

Table S1 Average (±standard error) cercarial traits of the trematode *Maritrema novaezealandensis* sorted by clone.

Table S2 Comparisons of average crustacean sizes between sexes using an unpaired *t*-test.

Table S3 Summary of crustacean infections with 20 distinct clones of the trematode *Maritrema novaezealandensis*.

Table S4 Spearman's rank correlation coefficient (ρ) between mean parasite abundance in each of two host species, across all *Maritrema novaezealandensis* clones.

Table S5 Spearman's rank correlation between average fitness measures achieved in the crabs *Macrophthalmus hirtipes* and *Hemigrapsus crenulatus*, across all clones.

Table S6 Averaged fitness measures of the trematode *Maritrema novaezealandensis* sorted by clone in the host crabs *Macrophthalmus hirtipes* and *Hemigrapsus crenulatus*.

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