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Intra- and interclonal phenotypic and genetic variability of the trematode *Maritrema novaezealandensis*

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Explaining the origin and maintenance of phenotypic variation remains a central challenge in evolutionary biology. Using the trematode parasite *Maritrema novaezealandensis*, we examined variability in several morphological, behavioural, and physiological phenotypic traits at the same time as controlling for genotype by using genetically identical parasite clonal lineages. We measured several morphological traits, photoreactive responses, and survivorship to quantify the amount of phenotypic variation within and among 42 clonal parasite lines. Additionally, we tested Lerner's hypothesis that homozygotes are more variable than heterozygotes and assessed correlations between heterozygosity and phenotypic variation among clones. We found substantial differences among clones in morphology, photoreactive behaviour, and survivorship, yet no significant differences among clones in levels of intraclonal phenotypic variability were seen. Although the results demonstrate that conspecific trematode clones have significantly different levels of phenotypic variability, consistent differences over time were not always apparent. Finally, no correlation was found between heterozygosity and phenotypic variation among clones and the pattern of highly variable homozygotes, as observed by Lerner, was not evident in the present study. *Biological Journal of the Linnean Society*, 2011, **103**, 106–116.

ADDITIONAL KEYWORDS: behaviour – developmental noise – heterozygosity – microsatellites – morphology – photoreaction – survival.

INTRODUCTION

Generalists, by definition, have the ability to utilize a wide range of resources relative to specialists. Ultimately, this range may be constrained by genetic and/or phenotypic variation (Futuyma & Moreno, 1988). For example, a species whose individuals collectively possess limited morphological or behavioural variability may be constrained to exploit a relatively narrow subset of available resources. When attempting to relate the phenotypic variability of an organism to the range of resources it can exploit, it can be useful to minimize or control for genotypic effects by studying closely related or clonal individuals. However, even clonal individuals can vary phenotypically as a result of environmental influences (phenotypic plasticity) or random internal events (possibly the result of epigenetic mechanisms) known as developmental noise (Hallgrímsson & Hall, 2005). Disentangling the genetic and environmental causes of phenotypic variability, and characterizing its consequence for resource use, remain important challenges for evolutionary biology.

Lerner (1954) proposed that there should be a negative relationship between phenotypic variability and heterozygosity. He argued that heterozygotes, owing to their more stable development, should have significantly lower levels of morphological variation than homozygotes, which should be associated with a relatively greater frequency of phenotypically 'extreme'

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individuals (Lerner, 1954; Zink, Smith & Patton, 1985; Debat & David, 2001; Hall, 2005). Subsequent tests of this hypothesis have yielded mixed results in terms of support for Lerner's hypothesis (reviewed by Mitton, 1993; Yampolsky & Scheiner, 1994; Deng, 1997; Vøllestad, Hindar & Møller, 1999), with several aspects of the study design influencing their outcomes: method of heterozygosity measurement (pedigree, allozymes, microsatellites), study organism (poikilotherms, homeotherms), and index of developmental noise (fluctuating asymmetry, residual variance).

Studies of developmental noise often involve the measurement of fluctuating asymmetry (differences in bilateral symmetry) in sexually reproducing organisms (Debat & David, 2001; Lens *et al*., 2002; Hallgrímsson & Hall, 2005), whereas others examined the phenotypes of parthenogenetic organisms (Yampolsky & Scheiner, 1994; Vogt *et al*., 2008). In the present study, we selected trematode parasites as ideal candidates for studying phenotypic variation, and subsequently their impact on resource (host) exploitation. Trematodes multiply asexually within a snail intermediate host, producing huge numbers of dispersal stages (known as cercariae), all genetically identical replicates of the original larva that infected the snail. Cercariae play a key role in the trematode life cycle, allowing transmission from the snail host to the next host. Phenotypic characteristics of cercariae, including both morphological and behavioural traits, determine which hosts are encountered and how successful cercariae are at infecting them (Galaktionov & Dobrovolskij, 2003). By studying this clonal stage of the parasite, it is possible to control for genotype and measure the intraclonal variability of the cercarial phenotype. Environmental variability can be reduced by maintaining host snails under constant environmental conditions, whereas genetic variability can be controlled by using genetically identical cercariae shed from the same snail; the remaining phenotypic intraclonal variation observed can then be considered developmental noise. The existence of significantly different phenotypes among cercarial clones would lend evidence to the bet-hedging infection strategy (Fenton & Hudson, 2002; Donaldson-Matasci, Lachmann & Bergstrom, 2008), which suggests that parasites can increase their infection success through phenotypic variation amongst closely related individuals. This could also support the 'adaptive coin-flipping' strategy proposed by Kaplan & Cooper (1984) where variation in propagule size, produced by an individual, and influenced by developmental noise, contributes to increased fitness advantage in unpredictable environments. Such variation in a parasite could be the basis for the maintenance of a generalist strategy, whereas differentiation could ultimately serve as a mechanism for specialization or speciation (Futuyma & Moreno, 1988).

Maritrema novaezealandensis Martorelli *et al*., 2004 (Digenea: Microphallidae) is an intertidal marine trematode that uses the New Zealand mud snail *Zeacumantus subcarinatus* Sowerby, 1855 (Prosobranchia: Batillariidae) as its first intermediate host, various crustaceans as second intermediate hosts, and seabirds as definitive hosts (Martorelli *et al*., 2004). The parasite is transmitted from snail to crustacean hosts via cercariae. These free-living infective stages are genetically identical dispersal/transmission propagules shed from infected snails in batches of tens to hundreds on most days. Once shed into the environment, cercariae swim until they encounter a crustacean, at which point they use their stylet and secretions from special glands (Ginetsinskaya, 1988) to penetrate the cuticle of some of the smaller crustacean hosts (e.g. amphipods) or soft tissues of larger crustacean hosts (e.g. crabs) and encyst in their body cavity (Koehler & Poulin, 2010). This developmental phase of the trematode can be described as generalist as a result of the wide range of crustaceans it can infect (Koehler & Poulin, 2010) and subsequent references to a generalist lifestyle in the present study will refer to this stage of the parasite's life cycle. Cercariae are fueled by glycogen, most of which is stored in their tail (Erasmus, 1958; Lawson & Wilson, 1980), suggesting that tail size may be important for both swimming ability and stamina. Presumably, highly active cercariae use up their energy stores faster and die sooner than less active cercariae. Increased temperature can heighten cercarial activity at the same time as decreasing survivability, as demonstrated by comparisons of survivability at different temperatures (Studer, Thieltges & Poulin, 2010). It has been shown that survival times can differ substantially among individual cercariae (Karvonen *et al*., 2003) and, the present study tests whether variability in survival rates may also differ among clones depending on their size. Finally, cercariae in general are known to have a range of behavioural responses to stimuli (Combes *et al*., 1994; Haas, 1994) and variability among cercariae in a photoreactive behavioural response may also be related to infection success. Other microphallid species display varying degrees of photophobic responses to light (McCarthy, Fitzpatrick & Irwin, 2002), and these may determine the probability of encountering crustacean hosts, as well as the identity of those hosts.

In the present study, we examined a number of morphological, physiological, and behavioural traits in cercariae of *M. novaezealandensis* that likely make important contributions to parasite fitness. These included cercarial body and tail size, stylet length,

survivability, and photoreactive response. Using these traits, we tested Lerner's (1954) hypothesis that homozygotes are more phenotypically variable than heterozygotes. To assess this relationship, we first determined whether both the average value of phenotypic traits and their variability differed to a significant degree at both intra- and interclonal levels. Finally, we use a correlation matrix as an exploratory tool to determine whether the mean value as well as the variability of given phenotypic traits covary with those of other traits.

MATERIAL AND METHODS

Host collection

Zeacumantus subcarinatus snails were collected from Company Bay, Otago Harbour, Dunedin, New Zealand (45°51′23.8″S, 170°35′54.3″E) on 20 August 2008. To screen for infection by *M. novaezealandensis*, we placed individual snails into 12-well polystyrene plates filled with 5 mL of seawater, exposed the snails to conditions that promote cercarial shedding (25 °C under bright light; Fredensborg, Mouritsen & Poulin, 2004) and identified 77 snails from which microphallid cercariae were released. Snails were measured to the nearest 0.1 mm with Vernier calipers from the base of the aperture to the apex and individually marked using cyanoacrylate glue and numbered plastic tags (Bee Works). Snails were maintained in a 40-L aquarium for approximately 13 months under constant conditions before measurements of parasite phenotypic traits were first taken. The aquarium contained an autoclaved mixture of sand and mud and was filled with seawater from Otago Harbour, which was changed monthly and held at 19 °C using an aquarium heater. Captive snails were fed sea lettuce (*Ulva* spp.) *ad libitum*. Light was supplied through full ultraviolet spectrum bulbs set on a 12 : 12 h light/dark cycle (Sylvania GroLux F36W/T8). Snails were remeasured in June 2010 to assess growth.

Heterozygosity

To verify that each experimental snail was only infected by a single clone of *M. novaezealandensis*, we collected pools of approximately 200 parasite cercariae from each snail using the aforementioned shedding method and obtained DNA from them using a Chelex extraction (Walsh, Metzger & Higuchi, 1991). In case multiple parasite species and/or *M. novaezealandensis* clones were present in an individual snail but did not release cercariae during a particular shedding event, cercariae were collected on three occasions at 7–10 days intervals then pooled for genetic analyses. First, to verify that *M. novaezealandensis* was the only microphallid parasite infecting experimental snails, we used the polymerase chain reaction (PCR) to amplify the mitochondrial cytochrome oxidase I gene (COI) using a modified protocol from Miura *et al*. (2005) and the primers JB3: TTTTTTGGGCATCCTGAGGTTT (forward) and COI-R trema: CAACAAATCATGATGCAAAAGG (reverse) with an annealing temperature of 53 °C. The resulting sequences were compared with those from other species of trematodes known to infect *Z. subcarinatus*. No parasites other than *M. novaezealandensis* were detected. We then estimated genome-wide multilocus heterozygosity (MLH; proportion of heterozygous loci among all loci typed) of each of the 77 *M. novaezealandensis* infections by genotyping them at 32 microsatellite loci. Details on primer sequences and PCR conditions are provided in Keeney, Waters & Poulin (2006) and Molecular Ecology Resources Primer Development Consortium (2009). PCR reactions were performed on an Eppendorf Mastercycler thermal cycler and resulting products resolved by eye on 7–9% nondenaturing polyacrylamide gels with a 10-bp DNA ladder. Snails with cercariae having more than two alleles at any locus were considered to be infected by multiple clone and were removed from the study, leaving 42 snails each harbouring a single *M. novaezealandensis* clone. Towards the end of the study, three snails failed to produce adequate numbers of cercariae and were excluded from the second photoreaction trial.

At the conclusion of the study, a subset of 27 clones, haphazardly chosen, were regenotyped to verify that the snails had not become infected by additional *M. novaezealandensis* clones during the course of the study by consuming parasite eggs possibly present in their field-collected food supply. A subsample of eight microsatellite loci was chosen to verify single infections because of their relatively high levels of polymorphism [Mar22 ($N = 8$ alleles), Mar39 ($N = 11$), Mar70 ($N = 7$), Mar74 ($N = 9$), Mar88 ($N = 10$), Mar111 ($N = 6$), Mno28 ($N = 8$), and Mno47 ($N = 7$)].

Morphology

In September 2009, snails were left to shed cercariae in 12-well plates filled with seawater for 1 h at 25 °C, after which the snails were removed and cercariae were allowed to equilibrate for 1 h to ensure that all were at least 1 h old. A mixture of cercariae and seawater (80 μL) was placed on a 26×76 mm glass microscope slide and covered with a 22×22 mm cover slip ringed with a petroleum jelly barrier to prevent distortion and desiccation of the parasites. Slides were then placed in an incubator at 55 °C for 10 min to kill and straighten the cercariae in a consistent and repeatable manner. The morphological

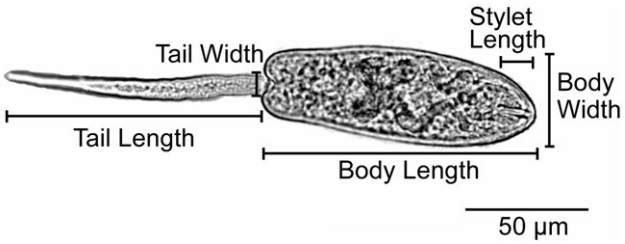

Figure 1. Photograph of *Maritrema novaezealandensis* cercaria indicating morphological measurements. The stylet is visible through the cercaria's anterior body.

measurements taken were: body length, maximum body width, tail length, maximum tail width, and stylet length (Fig. 1). All trait measurements were taken by one observer (A. Koehler) to reduce observer error. Measurements were made on a compound microscope at ×400 using an eyepiece reticle. Care was taken to ensure that only those cercariae whose bodies and tails lay along a flat plane were measured; approximately 40 haphazardly-chosen cercariae were measured for each clone. To determine whether morphology of cercariae produced within a snail varied over time, measurements were made on cercariae released in two different shedding bouts separated from each other by six months (September 2009 and March 2010) and a total of approximately 3450 cercariae were measured. Environmental conditions within the snail-holding tank remained constant during this period. In addition to calculating a mean value for each trait and for each clone at both time periods, phenotypic (or 'trait'), variability was estimated as the coefficient of variation (CV, the ratio of the standard deviation to the mean) for each morphological trait.

Photoreaction

The reaction of cercariae to light was measured using ten replicate, seawater-filled glass troughs (length 92 mm, width 9 mm, height 6 mm high, volume 2 mL) with one-half of each trough painted black and the other half left transparent. For each clone, a 20 µL aliquot of cercariae (approximately 30 individuals) and seawater taken from the same batch shed for morphological measurements was added to the middle of each trough. The black half of each trough was then covered with an opaque plate of glass. The troughs were aligned in a haphazard order (for each trial) beneath cold light source lamps and positioned beneath a dissection microscope for counting purposes. After 20 min, the glass plate was removed and the number of cercariae were counted on the dark and light sides of each trough. The proportion of cercariae in the light versus dark sides, was averaged across all ten replicates for each clone. As for morphological measurements, this experiment was conducted in September 2009 and repeated in March 2010.

Survival

For these trials, a blocking design was applied by using two identical 96-well round-bottom plates. Each clone was allotted one row (ten wells) per plate for a total of 20 wells per clone (all wells on the outer edges of the plate were avoided). Clones were assigned haphazardly to each row. Snails were shed for 1 h and equal volumes of cercariae (approximately 50 individuals) and seawater mixture were added to each well. A total of six clones were tested per day over a period of 10 days in November 2009. The plates were placed in an incubator at 25 °C with a fluorescent bulb light source. Cessation of movement was considered functionally equivalent to death because cercariae that can no longer move about freely are incapable of infection (i.e. their primary function). After 6 h, the numbers of cercariae that were mobile (fully active) or immobile (those dead or unable to freely swim around the well) were recorded. This was repeated each hour until over 50% of the cercariae in each well were immobile. The time (in minutes) at which 50% of the cercariae reached immobility was approximated by plotting the number of immobile cercariae over time. This method is similar to that of Pechenik & Fried (1995), where the time until 50% mortality rather than 50% immobility was used. Survivability measurements were only taken once.

Statistical analysis

Statistics were performed in JMP, version 7.0 (SAS Institute) and R, version 2.11.1 (R Development Core Team, 2010). When appropriate, data were Box-Cox transformed to meet assumptions of normality and homogeneity of variances. Analyses were grouped into three comparison categories:

Intraclonal phenotypic variability

Correlation coefficients (r) were used to assess repeatability for morphological and photoreaction phenotypic traits (both average trait values and CV) between the two trials separated by 6 months; there are other ways of measuring repeatability, although a correlation coefficient provides a simple quantitative estimate of the tendency for a measurement taken at one time to match that taken at a later time. Morphological measurements taken at time 1 and time 2 were also compared using a two-sample Student's t-test.

Interclonal phenotypic variability

Two-way analysis of variance (ANOVA) was used to assess phenotypic differences in morphology, with

clone identity and time (first and second round of measurements separated by 6 months) as factors. Because the response variable was a proportion, it was necessary to use a generalized linear model (GLM) with a quasi-binomial distribution to detect clonal differences in photoreaction, again with clone identity and time as factors. A linear mixed model (LMM) was used to detect differences in survival (i.e. the time needed for half the cercariae to stop moving); it included the terms clonal identity, initial number of cercariae per well, and column of wells (nested within a well plate), all classified as random factors. The number of cercariae per well was included to account for any positive or negative density-dependent effects. A 95% highest posterior density confidence interval of coefficient estimates, for each random effect in the LMM, was calculated based on Markov chain Monte Carlo simulations (10 000 samples) using the 'pvals-.fnc' function in the software package 'languageR' (Baayen, Davidson & Bates, 2008). As suggested by Baayen *et al*. (2008), likelihood ratio tests (LR χ^2) were used to compare models with the random effects present and removed to infer statistical significance. A test of overall phenotypic variation was performed using a Kruskal–Wallis test to compare the CV values of all traits among the different clones.

Heterozygosity and correlation matrix

A matrix of correlation coefficients among all pairs of traits, and between heterozygosity values (based on 32 microsatellite loci) and each trait, was constructed using both mean and CV values in an effort to identify any patterns of covariation among clones. We also included snail shell length in this matrix, aiming to determine how the only host variable available affected the phenotypic measures obtained. Because the use of this approach is associated with an increased risk of type I error, we used it for qualitative/exploratory purposes only. Additionally, the relationships between heterozygosity and both change in mean trait value through time and change in magnitude of trait variation through time were examined for morphological and photoreactive traits. Trait change through time was calculated by dividing the absolute value of change (i.e. difference between CV at time 1 and at time 2) by the mean of the initial and final values.

RESULTS

Intraclonal variability

Measurement of average body length, body width and tail length were all significantly correlated between the two time periods, suggesting that these traits remained consistent over time (Fig. 2), although it is notable that correlation coefficients are not very high and thus the repeatability is relatively weak. Correlation coefficients for the mean and CV of each trait, grouped by time period, are provided in Table 1. None of the other trait means or CVs were repeatable. On average, based on Student's *t*-test, morphological trait values decreased slightly during the period between measurements (Table 2).

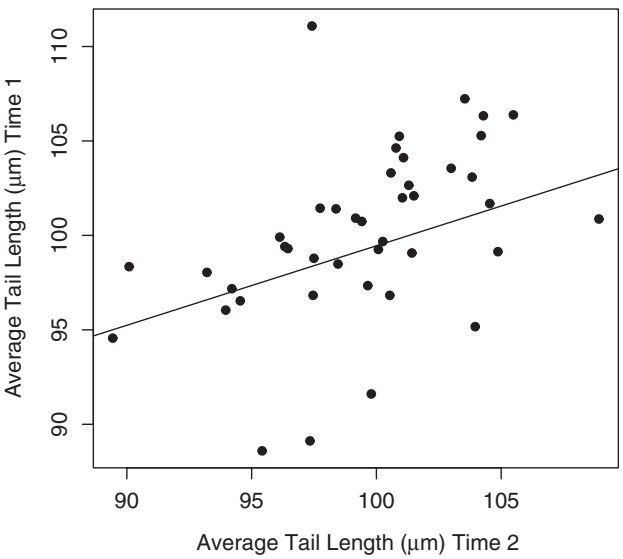

Figure 2. Relationship between mean cercarial tail lengths from 42 clones measured at one point in time and the same measurement taken 6 months later ($R^2 = 0.22$, $F_{1,40} = 11.1$, $P = 0.0019$). A relatively high R^2 value is evidence of high repeatability in trait value between time periods.

Interclonal variability

The two-way ANOVAs to detect differences between the morphological traits of different clones showed that the interaction and clone effect were significant for all traits, and the time effect was significant for all traits except body width (Table 3).

Photoreaction measured the proportion of cercariae found on the light side of a choice chamber. On average, the percentage of positively photoreactive cercariae was 10.8% (±0.005% SE), with clonal values ranging from 0.7% ± 0.005% to 43.8% ± 0.082%. On the basis of the GLM, all main factors including the interaction had significant effects (Table 3) (i.e. attraction to light varied among clones, and over time within given clones).

The LMM detected differences in the effects of the various factors when average time taken for 50% of the cercariae to reach immobility in each well was the dependent variable. Here, the clonal factor was

Table 1. Correlations of trait values between two points in time using mean trait values and coefficients of variation (CV) among 42 clones of the trematode *Maritrema novaezealandensis*

Trait	*r* (mean)	*P*	*r* (CV)	*P*
Body length	0.4451	0.0031**	–0.0814	0.6083
Body width	0.3189	0.0395*	0.0276	0.8621
Tail length	0.4662	0.0019**	0.3013	0.0525
Tail width	0.1237	0.4349	0.1822	0.2480
Stylet length	0.2937	0.0590	0.0567	0.7215
Photoreaction	0.1304	0.4232	0.1153	0.4785

*$P < 0.05$; **$P < 0.005$.

Table 2. Mean ± SE morphological trait values over time for 42 clones of the trematode *Maritrema novaezealandensis*. Significance based on Student's *t*-test

Trait	Time 1 (*N*)	Time 2 (*N*)	*P*
Body length (μm)	102.27 ± 0.13 (2132)	101.53 ± 0.17 (1288)	0.0005
Body width (μm)	34.78 ± 0.05 (1444)	34.73 ± 0.06 (1288)	0.3988
Tail length (μm)	100.30 ± 0.19 (1559)	99.48 ± 0.17 (1288)	0.0014
Tail width (μm)	9.58 ± 0.02 (1933)	9.23 ± 0.02 (1288)	< 0.0001
Stylet length (μm)	12.23 ± 0.02 (1729)	12.03 ± 0.03 (1288)	< 0.0001

Table 3. Two-way analyses of variance or generalized linear models on variation in morphological and photoreactive traits among 42 clones of the trematode *Maritrema novaezealandensis*

Trait	Clone effect	Time effect	Interaction
Body length	$F_{42,3335} = 32.80$**	$F_{1,3335} = 18.33$**	$F_{41,3335} = 12.53$**
Body width	$F_{42,2647} = 21.68$**	$F_{1,2647} = 3.21$	$F_{41,2647} = 11.86$**
Tail length	$F_{42,2762} = 37.04$**	$F_{1,2762} = 6.48$*	$F_{41,2762} = 12.34$**
Tail width	$F_{42,3136} = 14.34$**	$F_{1,3136} = 192.78$**	$F_{41,3136} = 11.46$**
Stylet length	$F_{42,2762} = 15.74$**	$F_{1,2762} = 29.88$*	$F_{41,2762} = 8.75$**
Photoreaction	$F_{42,788} = 7.60$**	$F_{1,787} = 10.94$**	$F_{39,748} = 5.33$**

*$P < 0.05$; **$P < 0.001$.

responsible for most of the variance (86.4%), whereas the effects of row, plate, and the initial number of cercariae accounted for less than 1% of the variance (Table 4). The range of survival times varied greatly among the clones with the longest surviving clone reaching 50% immobility at 956.4 min (±10.6 min SE) and the shortest surviving clone reaching 50% immobility at 507.7 min (±2.8 min SE), an almost two-fold difference.

To test whether some clones exhibited more overall phenotypic variability than others, a Kruskal–Wallis test was performed using the coefficients of variation generated from each of the phenotypic traits examined (six CV values per clone). The mean ranks of the CVs were not significantly different among the clones ($H = 11.23$, d.f. = 41, $P = 1.00$).

Heterozygosity and the phenotypic correlation matrix

The 42 single *M. novaezealandensis* infections ranged in heterozygosity (MLH) from 31.3% to 71.9%. The only trait correlated with heterozygosity among all clones was average stylet length (Table 5). Additionally, no relationship between heterozygosity and both change in mean trait value through time and change in magnitude of trait variation through time for morphological and photoreactive traits was found. One exception was a slight correlation ($P = 0.011$, $R^2 = 0.159$) between change in the magnitude of variation in photoreaction through time and heterozygosity; however, the removal of an outlier greatly reduced the correlation ($P = 0.052$, $R^2 = 0.098$).

Table 4. Results of the linear mixed model on the factors influencing cercarial survival (time until 50% immobility) among 42 clones of the trematode *Maritrema novaezealandensis*

Factor	Variance	SD	Coefficient estimate	95% HPD interval	LR χ^2	*P*
Clone	11832.6	109.1	44.59	40.20–48.61	1363.70	< 0.001
Column (plate)	35.6	5.9	3.82	0.00–8.94	3.64	0.056
Plate	32.0	5.9	121.52	0.00–420.09	1.96	0.162
Number of cercariae	51.2	7.2	2.83	0.00–7.63	2.71	0.100
Residual	1750.6	42.4	48.82	46.07–51.60		

Highest posterior density (HPD) intervals were obtained by using Markov chain Monte Carlo sampling. *P*-values are based on likelihood-ratio (LR) tests.

For the remaining mean trait values, stylet length was positively correlated with body length, body width, and tail width (Table 5). There was no correlation between tail size and survival time. The average CV for survival correlated with snail length and average CV for photoreaction. Change in snail length correlated with initial snail length and CV for tail length. None of the remaining correlations in the matrix were significant.

DISCUSSION

Understanding the origin and maintenance of phenotypic variation remains a central question in evolutionary biology (Hallgrímsson & Hall, 2005). In the present study, we examined phenotypic variation in multiple clonal lineages of a parasitic trematode and found evidence of the sort of extensive variability that may be essential for a generalist parasite. We observed: (1) limited and very weak repeatability of morphological and behavioural traits over time within given clones; (2) substantial differences among clones in morphology, behaviour, and survivorship; (3) no differences among clones in levels of intraclonal phenotypic variability; and (4) no correlation between heterozygosity and phenotypic variation among clones.

MORPHOLOGY

Measurements for three of the five morphological traits (average body length, body width, and tail length) were statistically repeatable over time (Fig. 2, Table 1). Given that environmental and genetic factors were controlled in our experimental design, this result may suggest that the variation we detected was the result of developmental noise. Potential environmental factors that might have influenced the cercarial phenotype throughout the duration of this experiment include biotic factors within the snail where cercariae are formed, and abiotic factors such as water temperature and light; however, all these remained constant for the duration of the experiment. Cercariae develop within sporocysts, which are germinal sacs that replace the snail's digestive gland and gonads (Galaktionov & Dobrovolskij, 2003). It is possible that some of the morphological variability arises from differences in the nutrient supply to the sporocyst. If sporocysts have a major influence on variable cercarial morphology, then snail size, and consequently the amount of nutrients available to the sporocysts, could be correlated with cercarial morphology. Previous studies have found a positive relationship between snail size and cercarial production (Loker, 1983; Niemann & Lewis, 1990; McCarthy *et al*., 2002; Graham, 2003). Loker (1983) examined eight species of schistosomes and found a positive correlation between cercarial length and snail shell size, suggesting that, at the species level, larger snails produce larger cercariae. Gérard, Moné & Théron (1993) studied the *Schistosoma mansoni–Biomphalaria glabrata* system and found that cercariae produced by larger snails were significantly larger than those produced by smaller snails. The prediction that larger snails would have more available resources devoted to cercarial production was not supported by the results of the present study. Neither snail shell length nor snail growth were correlated with any of the cercarial phenotypic traits measured in the present study, with the exception of snail growth and CV of tail length (Table 5). We were unable to measure the density of sporocysts per snail, and this too may influence cercarial phenotype. Overall cercarial size tended to slightly, yet significantly, decrease over time (Table 2). This may be the result of a general decline in snail health, a product of having the snails in captivity for almost 2 years; nevertheless, greater phenotypic changes over time in one clone than in another can still reflect genetic differences among clones independently of snail health.

Table 5. Matrix of correlation coefficients (r) across mean trait values (above the diagonal) and coefficient of variation (below the diagonal) among 42 clones of the trematode *Maritrema novaezealandensis*

	Snail L	ΔSnail L	Body L	Body W	Tail L	Tail W	Stylet L	Survival	Photoreaction	HZ
Snail L		−0.54*	−0.08	−0.15	0.03	−0.01	−0.26	0.00	0.00	−0.15
ΔSnail L	−0.54*		0.07	0.08	−0.03	0.12	−0.01	−0.05	0.04	0.24
Body L	−0.18	0.05		−0.24	0.17	0.26	0.36*	0.07	−0.04	0.15
Body W	0.14	−0.13	−0.04		0.19	0.11	0.36*	0.15	0.15	−0.18
Tail L	−0.18	0.39*	0.09	−0.02		−0.28	−0.01	0.01	−0.05	−0.23
Tail W	0.04	0.04	0.01	0.11	0.16		0.34*	0.23	−0.20	0.18
Stylet L	0.02	−0.02	0.28	0.16	−0.28	0.12		0.13	0.14	0.37*
Survival	0.32*	−0.22	−0.03	0.07	−0.26	0.04	0.06		0.07	0.03
Photoreaction	−0.20	−0.02	0.09	0.28	0.00	−0.08	0.00	−0.33*		−0.02
HZ	−0.15	0.22	−0.08	0.00	0.18	−0.08	−0.10	−0.02	0.09	

L, length; W, width; ΔSnail L, change in snail length; HZ, heterozygosity.
*$P < 0.05$.

On the whole, we were able to detect significant morphological differences among some of the clones. However, these differences among clones were dependent on the time at which measurements were taken, as indicated by the significant interaction factor (Table 3). One interpretation of this result is that some clones showed greater temporal variability in phenotype than others, causing the magnitude of the differences between clones to change as a function of when they were measured. If there were perfect repeatability of phenotypic measurements, then the interaction would not be significant. Nevertheless, overall, there was substantial variability in morphology and behaviour among clones.

The coefficients of variation in morphology were also not repeatable, meaning that the variation taken at time 1 was different from that taken at time 2, yet, for some traits, the resulting phenotypes were similar under controlled environmental conditions. This could be explained by developmental canalization, or the ability of an organism to produce a consistent phenotype regardless of differences in genotype or environment (Waddington, 1942). The differences in coefficients of variation may simply be the result of variation resulting from different levels of developmental noise.

In the correlation matrix of trait averages, stylet length was the only variable to correlate with any of the other variables (body length, body width, tail width, and heterozygosity). This makes biological sense because larger cercariae would have longer stylets. However, measurements of other traits expected to covary, such as body length and tail length, were not correlated. Stylet length may be an important component of cercarial infection/transmission success if cercariae equipped with larger stylets are better able to penetrate hosts than those with smaller stylets. The positive correlation with heterozygosity might thus be in agreement with the heterozygosity–fitness correlation hypothesis stating that heterozygotes are generally more fit than homozygotes (Hansson & Westerberg, 2002; Chapman *et al*., 2009). This finding could be explored in future studies linking stylet length to cercarial infection performance.

PHOTOREACTION

By contrast to the cercariae of other parasitic trematode species that demonstrate highly stereotypical behaviours including specialized swimming/crawling patterns, geotaxis, and photoreactive behaviours (Combes *et al*., 1994), *M. novaezealandensis* cercariae exhibit little quantifiable behaviour. Our experiment tested their preference for light or dark habitat and most cercariae, with some exceptions, preferred the

dark half of the test chamber. Differences among clones were observed (Table 3); however, the ratio of cercariae preferring dark to light was not repeatable between the two trials (Table 1), suggesting either that the behaviour is extremely variable or that its measurement in the present study was subject to error. McCarthy *et al*. (2002) used a similar method to measure photoreactive responses of the microphallids *Microphallus similis* and *Maritrema arenaria*. Microphallids are not equipped with photoreceptors, yet they sense light gradients and swim away from light towards the dark. This behaviour may increase the cercaria's chances of reaching a crustacean host, many of which, similar to the crabs preferred by *M. novaezealandensis* (Koehler & Poulin, 2010), live in the darkness beneath rocks (McCarthy *et al*., 2002). Future experiments should use a more sensitive method capable of distinguishing more subtle behavioural differences.

Survival

We were able to detect significant differences in survival times among clones (Table 4). We did not find evidence for our prediction that cercariae with larger tails would have longer survival times. Ideally, morphological and survival measurements would have been conducted simultaneously on the same cercariae to eliminate any effect of cercarial size variation as a result of time between measurements; however, this was logistically unachievable. Although glycogen is the energy source for cercariae and most of it is stored in the cercaria's tail (Erasmus, 1958), the size of the tail does not appear to be a good indicator of survival time. Lawson & Wilson (1980) found that some batches of *Schistosoma mansoni* cercariae from different snails had three times the level of glycogen stores relative to others. This variation in glycogen levels could explain the great variability (almost twofold difference) that we recorded in survival times. The mean coefficient of variation for survival correlated with both the mean coefficient of variation for behaviour and snail length. These correlations are difficult to interpret biologically and we suspect they are spurious results common to such analyses.

Heterozygosity

By contrast to the prediction first made by Lerner (1954), no correlation was found between heterozygosity and morphological variation. Many of the previous studies that examined the relationship between heterozygosity and variation used allozymes to calculate heterozygosity and fluctuating asymmetry (a common measure of developmental noise) to measure variability (Mitton, 1993; Vøllestad *et al*., 1999). On the basis of these earlier findings, it appears that ectotherms generally display a negative correlation between heterozygosity and variation, whereas that relationship tends to be positive for endotherms; nevertheless, there is no definitive consensus on this topic. A comparative study that investigated heterozygosity measures derived from both allozymes and microsatellites found that heterozygosities from allozymes may not be neutral (Borrell *et al*., 2004). Zachos, Hartl & Suchentrunk (2007) suggest that data from microsatellites allow an assessment of overall genomic heterozygosity compared to allozymes. Their study found a negative correlation between heterozygosity and morphological variability in mammals. In the present study, our estimation of heterozygosity is based on 32 microsatellite loci and, given the number of loci, we are confident that our values reflect a robust estimate of genome-wide heterozygosity.

In summary, we were able to demonstrate that conspecific trematode clones have significantly different levels of phenotypic variability, although these differences do not always remain consistent over time. We were unable to find a relationship between heterozygosity and either the magnitude or variability in the expression of the phenotypic traits examined in this study. Nevertheless, our ability to detect phenotypic differences between and within clones is valuable for future research. In particular, relating the infection success of clones with specific phenotypic attributes could lead to a better understanding of the constraints acting on generalist parasites.

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