Mar Biol (2007) 152:687–695
DOI 10.1007/s00227-007-0725-x

RESEARCH ARTICLE

Local adaptation of immunity against a trematode parasite in marine amphipod populations

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Received: 7 February 2007 / Accepted: 2 May 2007 / Published online: 6 June 2007

Abstract Resources allocated to defence against parasites are not available for investment in other functions such as growth or reproduction, resulting in trade-offs between different components of an organism's fitness. In balancing the cost of infection and the cost of immunity, selection should only favour individuals that allocate more energy to resistance and immune responses in populations regularly exposed to debilitating parasites. Here, we compare the ability of amphipods, *Paracalliope novizealandiae*, to (1) avoid becoming infected and (2) to respond to infection by encapsulating and melanizing parasites, between two natural populations exposed to different risk of parasitism. One population faces high levels of infection by the debilitating trematode parasite *Maritrema novaezealandensis*, whereas the other population is not parasitised by this trematode nor by any other parasite. Under controlled experimental conditions, with exposure to a standardized dose of parasites, amphipods from the parasite-free population acquired significantly more parasites than those from the population regularly experiencing infection. Furthermore, a lower frequency of amphipods from the parasite-free population succeeded at melanizing (and thus killing) parasites, and they melanized a lower percentage of parasites on average, than amphipods from the parasitised population. These differences persist when individual factors, such as amphipod sex or body length, are taken into account as potential confounding variables. These results support the existence of local adaptation against parasites: an amphipod population that never experiences trematode infections is less capable of resisting infection, both in terms of its first line of defence (avoiding infection) and a later line of defence (fighting parasites following infection), than a population regularly exposed to infection.

Communicated by S. D. Connell.

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Introduction

Life history theory is based on the premise that an organism's resources are limited, such that trade-offs occur between costly fitness components like reproduction, predator avoidance and immunity (Stearns 1992; Lochmiller and Deerenberg 2000; Rigby and Moret 2000; Rigby et al. 2002). The resources channelled by an organism into one fitness component are consequently not available for use in other functions. In other words, investment in one fitness component is made at the expense of another, hence creating allocation costs. Selection will favour the optimal allocation of resources between fitness components that maximises the overall fitness of the organism (Sheldon and Verhulst 1996; Lochmiller and Deerenberg 2000; Zuk and Stoehr 2002).

In all organisms, protection against parasites and disease is essential for survival, and thus reproduction. Therefore, an efficient immune system providing resistance against parasites is a key fitness component (Rigby and Moret 2000; Ricklefs and Wikelski 2002; Zuk and Stoehr 2002). In invertebrates, the immune response is relatively simple, though it remains costly (Loker 1994; Gillespie et al. 1997; Schmid-Hempel 2003; Schwarzenbach and Ward 2006). The main defence cells in the invertebrate immune response are various types of haemocytes, many of which play a role in the centrepiece of invertebrate immunity, the

phenoloxidase cascade (Loker 1994; Gillespie et al. 1997; Soderhall and Cerenius 1998; Johansson et al. 2000). This enzyme cascade, initially activated by exposure to foreign material, eventually results in large invaders such as metazoan parasites becoming encapsulated in melanin by haemocytes. During encapsulation, layers of cells deposited around the parasite die and are melanized to form a capsule. Inside the case, the parasite is exposed to toxic substances produced by the host (e.g., enzymes), until the parasite is killed (Soderhall and Cerenius 1998; Cerenius and Soderhall 2004). The main enzyme involved in the melanization process is phenoloxidase, which increases the oxidation of phenols to quinones. The quinones then polymerise into melanin (Gillespie et al. 1997; Soderhall and Cerenius 1998).

Resistance against parasites can take other forms, too, such as behavioural avoidance of infective stages (Hart 1997). There is now considerable empirical evidence that parasite avoidance, as well as having the potential to initiate an immune response and the activation of that response, are associated with significant physiological costs resulting in trade-offs with other fitness components (Lochmiller and Deerenberg 2000; Rigby and Moret 2000; Kraaijeveld et al. 2002; Zuk and Stoehr 2002; Rolff and Siva-Jothy 2003; Schmid-Hempel 2003). For many invertebrates, the activation of immune responses can have costs in terms of reduced survival or lower reproductive success (e.g., Langand et al. 1998; Moret and Schmid-Hempel 2000; Jacot et al. 2004). Given these costs of immunity on the one hand, and the cost of being infected by parasites on the other hand, natural selection should quickly optimise investments into immunity based on the local prevalence and severity of parasitic infections. Selection should only favour host individuals that allocate much energy to resistance and immune responses in populations regularly exposed to debilitating parasites (Rigby and Moret 2000; Lochmiller and Deerenberg 2000; Tschirren and Richner 2006). There are only few examples of local adaptation of immune responses across different geographical populations of the same host (e.g., Kalbe and Kurtz 2006; Lindstrom et al. 2004). For example, Thomas et al. (2000) found that amphipods (*Gammarus* sp.) in a coastal population in southern France regularly encapsulated and melanized microphallid trematode parasites. However, the amphipods targeted their immune responses only toward trematodes of one species encysting around their cerebral ganglia; trematodes of the same or other species encysting in the amphipods' abdomen were left untouched (Thomas et al. 2000). In contrast, in a population from the Black Sea where overall infection levels are about five times higher than in southern France, all trematode species were attacked by immune responses, whatever their location within the host body (Kostadinova and Mavrodieva 2005).

Here, we investigate local adaptation of immune responses in a trematode-amphipod system similar to the one above, but using instead an experimental approach to assess immune potential under controlled conditions. The microphallid trematode *Maritrema novaezealandensis* has a three-host life cycle typical of most trematodes (Martorelli et al. 2004). Free-swimming cercariae are produced asexually in sporocysts within the gonads of the first intermediate host, the mud snail *Zeacumantus subcarinatus*. Once shed into the environment, *M. novaezealandensis* cercariae penetrate second intermediate hosts, which can be small crabs or the benthic amphipod *Paracalliope novizealandiae*, and encyst within the body cavity as metacercariae. The parasite then completes its life cycle via transmission by predation to a seabird definitive host, usually the red-billed gull *Larus novaehollandiae*. Sexual reproduction occurs in the bird host, eggs are released in the faeces and the cycle begins anew when an egg is accidentally ingested by a snail (Martorelli et al. 2004). Because it is dispersed by birds, the parasite itself is unlikely to show local adaptation to host populations. Infection has highly detrimental consequences for the amphipod hosts. As a pathological side-effect of infection, amphipods harbouring *M. novaezealandensis* metacercariae have an aberrant swimming behaviour, potentially leading to increased predation risk in the field (Leung and Poulin 2006). More importantly, infected amphipods incur higher mortality rates, even at moderate levels of infection (Fredensborg et al. 2004). Not surprisingly, encapsulation and melanization of metacercariae by *P. novizealandiae* has been observed in the field, a response that invariably kills the parasite (T. Leung, unpublished). Around Otago Peninsula, South Island, New Zealand, there are populations of the amphipod *P. novizealandiae* that experience high levels of infection by the trematode *M. novaezealandensis*, such as that in Lower Portobello Bay (50–65% prevalence, 1–25 metacercariae per infected host; Fredensborg et al. 2004). In contrast, in one unusual locality, Hoopers Inlet, the snail first intermediate host of the parasite is absent, and thus amphipods are never parasitised by *M. novaezealandensis* (see Fredensborg et al. 2004; Leung and Poulin 2006). Hoopers Inlet and lower Portobello Bay are separated by 35 km of coastline, but are only about 2 km apart, being on opposite sides of Otago Peninsula. This small-scale geographical variation in infection levels, coupled with the amphipod's lack of a planktonic dispersal stage, sets up the possibility of local adaptation of immune responses.

In the present study, we test whether *P. novizealandiae* amphipods from Hoopers Inlet have both (1) lower resistance to parasitic infection and (2) weaker immune responses and encapsulation abilities following infection, compared with amphipods from Lower Portobello Bay. We use a standardised experimental infection procedure to

provide a rigorous evaluation of the hypothesis that local natural levels of exposure to parasitism have driven the evolution of immune abilities.

Materials and methods

Large samples of *P. novizealandiae* amphipods were collected from Lower Portobello Bay (LPB) and Hoopers Inlet (HI), Otago Peninsula, South Island, New Zealand, during March (to quantify natural infections) and May-June (for experimental infections). Samples were collected from tide pools during low tide by sweeping plankton-nets through the vegetation (e.g., sea lettuce *Ulva lactuca*).

A sample of *Z. subcarinatus* snails was haphazardly collected by hand during low tide from Company Bay (Otago Peninsula). This location was chosen for snail collection because it is physically isolated (approx. 7 km linear distance from both LPB and HI) from the two localities from which amphipods were collected, thus providing a 'neutral' source of parasites to infect amphipods from the other two localities. To determine their infection status, snails were incubated for 60 min at 25°C in individual 10 ml plastic Petri dishes with approx. 8 ml of seawater. Only those snails infected with *M. novaezealandensis* (i.e., those that released cercariae during incubation, $n = 30$) were kept for the experiments.

Animals from different localities were housed in separate plastic tanks (330 mm × 165 mm × 120 mm high), half-filled with seawater, and containing sediment (snail tanks only) and vegetation (e.g., sea lettuce) from the original collection site. Tanks were aerated using airstones and kept under natural light and temperature regimes (approx. 12L/12D, 15–20°C). Amphipods and snails were given one week to acclimatize to laboratory conditions before experimental infections were performed.

Natural infections

Ninety-nine *P. novizealandiae* from LPB and 100 from HI were dissected under a dissection microscope (20× magnification). The variables recorded were amphipod length (from rostrum to telson), sex (identified by the presence or absence of enlarged male gnathopods), and the numbers and developmental stage of parasites found. *M. novaezealandensis* metacercariae found in LPB amphipods were classified into one of four groups: (1) recent infection, which resemble cercariae without tail; (2) later infection, with a larger, opaque, flat, and circular body; (3) encysted metacercariae, within a thick clear cyst; and (4) encapsulated metacercariae, including individuals at any developmental stage encased within a dark orange melanin envelope (see Fig. 1).

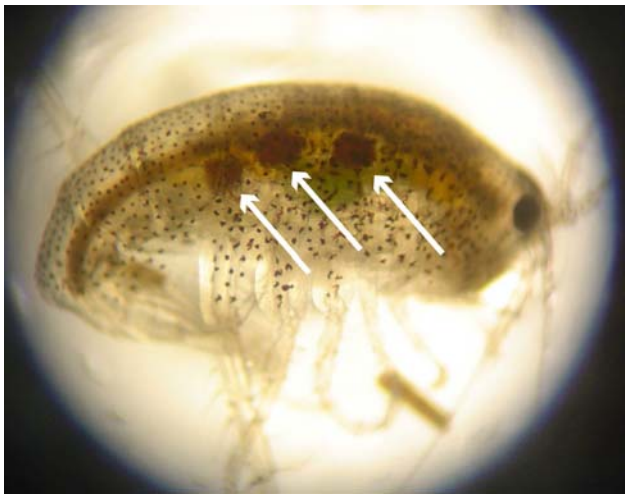

Fig. 1 Three melanized and encapsulated metacercariae (indicated by *arrows*) inside the amphipod *Paracalliope novizealandiae*

Amphipods from HI were dissected to confirm the absence of parasitic infections. As in earlier studies of amphipods from this locality (Fredensborg et al. 2004; Leung and Poulin 2006), all amphipods were found to be uninfected, and so individuals from this site are indeed 'naïve' to natural infection by the trematode *M. novaezealandensis*.

Data were analyzed using the statistical package SPSS 11.0 for Windows. Immune response was measured as the percent melanization (the number of melanized metacercariae divided by the total number of metacercariae present, multiplied by 100) of *M. novaezealandensis* metacercariae in each amphipod. Numbers of parasites per amphipod were $\log_{10}$ transformed to meet the assumptions of parametric tests. Percent melanization data were $\log_{10}(n + 1)$ transformed for the same reason. The sex ratios of *P. novizealandiae* amphipods from HI and LPB were compared using a Fisher's Exact Test. Amphipod lengths from the two sites were compared using an independent sample *t* test. The relationships between log percent melanization and length, and that between log total parasites per host and length for LPB amphipods were analyzed using Pearson's correlation coefficient; these and the following analyses excluded uninfected amphipods ($n = 39$). Log percent melanization and log total number of metacercariae for LPB amphipods were also analyzed using Generalized Linear Models, with log percent melanization or log total parasites as the dependent variables, sex as the fixed factor and length as a covariate.

Experimental infections

A total of 385 amphipods from HI and 480 from LPB were used for experimental infection. Approximately equal numbers of male and female amphipods of various sizes

(minimum size 2.5 mm) were used. LPB amphipods were inspected under a microscope prior to being used in the experiment. Those amphipods with visible infections (e.g., cysts or melanized metacercariae visible through the exoskeleton; see Fig. 1) were excluded from the experiments to minimize any potential bias in immune competence caused by the presence of prior infections or melanizations. Parasites that escaped melanization and could not be seen through the exoskeleton, and that were present prior to experimental infection, could still be distinguished at dissection from those acquired during the experiment, as they were at a later developmental stage; amphipods with prior infections were excluded from the analyses. This ensured that all metacercariae found during the dissections were the result of the experimental infections and were not natural infections already present prior to sampling.

Infected *Z. subcarinatus* snails were placed in plastic Petri dishes (85 mm diameter, 10 mm depth) partially filled with seawater in groups of 2–3 snails per dish (to ensure a genetic mix of parasite cercariae). The snails were incubated at 25°C under constant illumination for 60–120 min to trigger cercarial emergence. The amphipods were placed in individual seawater-filled wells (7 mm diameter, 10 mm depth) of a 96 well microtiter plate. Fifteen *M. novaezealandensis* cercariae were added to each well using a 200 µl Eppendorf pipette. The microtiter plates were then incubated at 25°C under constant illumination for five hours to provide optimal conditions for penetration of cercariae into the amphipods (Fredensborg et al. 2004). The use of 15 cercariae per amphipod in the experimental infections was chosen based on expected intensity-dependent mortality, which is low in amphipods infected with 5 *M. novaezealandensis* cercariae but high in amphipods infected with 25 cercariae (Fredensborg et al. 2004). An intermediate value of 15 cercariae was chosen to maximize infection (and therefore potential immune response) while minimizing mortality.

After infection, amphipods were removed from the microtiter plates and placed in small groups into several housing tanks (as described above). The amphipods were left for at least 7 days post-infection (maximum 21) before dissection, to allow for the completion of an immune response (i.e., melanization of the parasites). Melanization of an incoming parasite can be completed within a few days, but can also only be initiated several days after infection. Many amphipods died during that period, which was expected as their lifespan is limited to a few months and our selection of larger-sized amphipods meant that older individuals were also included. Surviving amphipods (approx. 38% of LPB and 30% of HI amphipods exposed to infection) were then sexed, measured and dissected as described above, with the numbers and developmental stage of parasites found being recorded. Immune response was assessed by recording the number of live metacercariae and the number of metacercariae that were encapsulated and melanized.

Here too, immune response was measured as the percent melanization (the number of melanized metacercariae divided by the total number of metacercariae present, multiplied by 100) of *M. novaezealandensis* metacercariae in each amphipod. Numbers of parasites per amphipod ($\log_{10}$) and percent melanization [$\log_{10}(n + 1)$] were transformed to meet the assumptions of parametric tests. The sex ratios of *P. novizealandiae* amphipods from HI and LPB were compared using a Fisher's exact test. Amphipod length, log total parasite metacercariae, log percent melanization of parasite metacercariae, and number of days between infection and dissection for HI and LPB amphipods were compared using independent sample *t* tests. The relationships between log percent melanization and length, and that between log total parasites per host and length for both HI and LPB amphipods were analyzed using Pearson's correlation coefficient; these and the following analyses excluded uninfected amphipods ($n = 8$ for HI, $n = 6$ for LPB).

The frequency of infected versus uninfected amphipods relative to site of origin was analysed with a Fisher's exact test. Log total parasite metacercariae data were analysed using a generalized linear model, with log total parasite metacercariae as the dependent variable, site of amphipod origin (HI or LPB) and amphipod sex as fixed factors, and amphipod length as the covariate. The frequency of amphipods that successfully melanized at least one metacercaria versus those that did not, relative to site of origin was analysed with a Fisher's exact test. Log percent melanization data were analyzed with a generalized linear model, with log percent melanization as the dependent variable, amphipod site of origin and sex as fixed factors, and amphipod length, days post-infection and log total parasite metacercariae as covariates.

Results

Natural infections

There was no significant difference in the sex ratio of amphipods between the sites of origin (Fisher's exact test, $P = 0.383$) but there was a difference in length, with amphipods from HI being slightly but significantly longer (average 3.5 mm) than those from LPB (average 3.3 mm) ($t = 2.500$, $df = 197$, $P < 0.05$); the same difference remains when only uninfected amphipods are used for the comparison.

Sixty (60.6%) of the 99 dissected amphipods from LPB were parasitised. The number of *M. novaezealandensis* metacercariae per infected amphipod from LPB ranged

from 1 to 35, with the majority harbouring fewer than 5 metacercariae (average 4.85). Most amphipods harboured parasites at different developmental stages, with the average number of metacercarial stages per infected amphipods being 1.8 stages. There was a significant positive relationship between log total parasite metacercariae and amphipod length ($r = 0.350$, $P < 0.01$), with larger amphipods having significantly more parasites than smaller amphipods (Fig. 2). With length taken into account, there was a significant difference in log total parasite metacercariae between male (average 6.2 metacercariae) and female (average 4.1) amphipods ($F_{1,57} = 4.001$, $P < 0.05$).

Of the 60 infected LPB amphipods, 19 (31.7%) had melanized parasite cysts or metacercariae in their body. The average percent metacercariae melanization among all 60 infected amphipods was 17.6%. Log percent parasite melanization did not differ between male and female amphipods (with length taken into account) ($F_{1,57} = 1.688$, $P = 0.199$). In addition, there was no significant relationship between amphipod length and log percent parasite melanization ($r = -0.071$, $P = 0.592$).

Experimental infections

The overall amphipod mortality rate from infection to dissection was 66.5%. Of the amphipods exposed to infection, 144 (30%) out of 480 from LPB and 146 (37.9%) out of 385 from HI survived to dissection. Of these survivors, 6 from LPB and 8 from HI were uninfected, and 17 from LPB were found to harbour one or two metacercariae acquired prior to the experiments; all these amphipods were excluded from analysis, leaving 121 amphipods from LPB and 138 from HI.

There was no significant difference in sex ratio between these remaining LPB and HI amphipods (Fisher's exact test, $P = 0.470$) but there was a significant difference in lengths, with amphipods from LPB being slightly longer (average 3.9 mm) than those from HI (average 3.7 mm) ($t = 4.937$, $df = 257$, $P < 0.001$). There was a significant difference in the number of days from infection to dissection between amphipods from the different sites ($t = 2.847$, $df = 257$, $P = 0.005$), with LPB amphipods being left for, on average, 12.1 days and HI amphipods for 10.8 days.

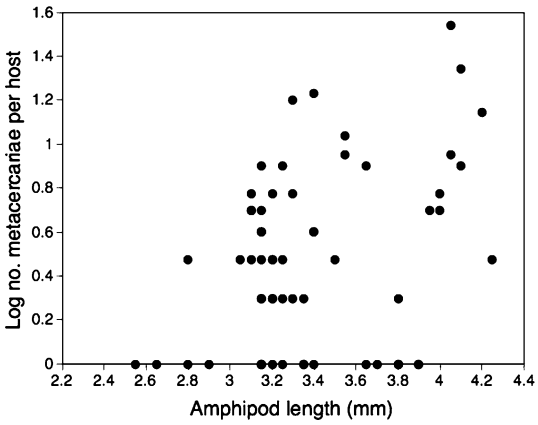

Fig. 2 The relationship between the total number of metacercariae and amphipod length among naturally infected amphipods, *P. novizealandiae*, from Lower Portobello Bay ($n = 60$)

There was no significant relationship between amphipod length and log total parasite metacercariae for the HI amphipods ($r = 0.003$, $P = 0.504$) but there was a significant positive relationship for LPB amphipods ($r = 0.249$, $P < 0.005$). However, there was no significant relationship between log percent melanization and amphipod length for either LPB ($r = 0.081$, $P = 0.317$) or HI ($r = 0.014$, $P = 0.868$) amphipods.

There was no significant difference in the frequency of surviving amphipods that were infected between the two sites (Fisher's Exact Test, $P = 0.785$), but there was a significant difference in the intensity of infection, with HI amphipods infected with significantly more parasites (average 6.9 metacercariae) than LPB amphipods (average 5.3 metacercariae) ($t = 4.267$, $df = 257$, $P < 0.001$). This difference is still significant when amphipod length and sex are taken into account ($F_{1,254} = 19.371$, $P < 0.001$), with none of these other variables having any effect on the number of metacercariae per amphipods (Table 1).

There was a significant difference between sites in the frequency of amphipods that successfully melanized parasite metacercariae, with significantly more amphipods from LPB (17 out of 121 infected amphipods) than from HI (6 out of 138 infected amphipods) melanizing parasites (Fisher's exact test, $P < 0.05$). Also, a significantly higher percentage of metacercariae were melanized by LPB amphipods than by HI amphipods ($t = 2.469$, $df = 257$, $P < 0.01$) (Fig. 3). This difference remained significant when amphipod lengths, sex, prior infections, days between infection and dissection and total number of parasites per amphipod are taken into account ($F_{1,252} = 4.548$, $P < 0.05$). Although none of these other variables had a significant effect on the percentage of metacercariae that were melanized, there was a tendency for a higher melanization rate in

Table 1 Summary of the Generalized Linear Model testing for a difference in (log-transformed) number of parasite metacercariae between amphipods from Lower Portobello Bay and Hoopers Inlet, as well as for the effects of other variables (see text; non-significant interactions omitted from the model)

Variable	*df*	MS	*F*	*P*
Length	1	0.008	0.959	0.328
Sex	1	0.098	1.138	0.287
Site of Origin	1	1.676	19.371	<0.001
Error	254	0.086		

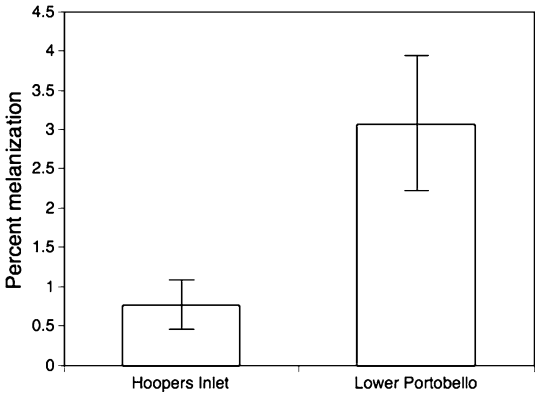

Fig. 3 Average (±1 standard error, from back-transformed data) percentage of metacercariae that were melanized by amphipods, *P. novizealandiae*, from the two sites of origin (Lower Portobello Bay, $n = 121$; Hoopers inlet, $n = 138$), in experimental infections

amphipods with many metacercariae and those dissected a longer time post-infection (Table 2).

Discussion

This study has shown that an amphipod population that never experiences trematode infections is less capable of resisting infection, both in terms of its first line of defence (avoiding infection) and a later line of defence (fighting parasites following infection), than a population regularly exposed to infection. In this and previous studies (see Fredensborg et al. 2004; Leung and Poulin 2006), we have never found other parasites of any kind in these amphipods, making the trematode *M. novaezealandensis* the sole parasitic agent of selection in this system. Under laboratory conditions, the trematode is (1) more likely to infect, and (2) less likely to be encapsulated and melanized, by amphipods from a naïve, parasite-free population. The Hooper inlet amphipod population is the only one we could find where the trematode does not occur, as a result of the chance absence of snail first intermediate hosts. Although it was not possible to find other unexposed populations to assess the generality of our findings, the proximity between the two localities compared here and their similarity in all respects other than parasitism points toward a cost of resistance as the most likely explanation.

Table 2 Summary of the generalized linear model testing for a difference in (log-transformed) percent melanization of parasite metacercariae between amphipods from Lower Portobello Bay and Hoopers inlet, as well as for the effects of other variables (see text; non-significant interactions omitted from the model)

Variable	*df*	MS	*F*	*P*
Length	1	0.002	0.089	0.766
Days post-infection	1	0.437	3.596	0.059
Log total parasites	1	0.461	3.825	0.053
Sex	1	0.007	0.060	0.807
Site of Origin	1	0.552	4.548	0.034
Error	252	0.121		

The cost of immunity, resulting in a trade-off between investments in immunity and investments in other functions, is well documented (Langand et al. 1998; Rigby et al. 2002; Kraaijeveld et al. 2002; Jacot et al. 2004). The proteins, enzymes and melanin required in the phenoloxidase cascade in invertebrate immunity are energetically expensive for the host to produce (Lochmiller and Deerenberg 2000; Rolff and Siva-Jothy 2003). We therefore expect natural selection to favour lower investments in immunity in populations not exposed to high levels of parasitism, i.e., in populations not paying a high cost of infection (see Kalbe and Kurtz 2006). Accordingly, we found that experimentally infected *P. novizealandiae* amphipods from Lower Portobello bay, where natural trematode prevalence is ~60%, have significantly higher encapsulation and melanization abilities than amphipods from Hooper Inlet, where the trematode *M. novaezealandensis* is naturally absent. Under experimental conditions, 13% of infected Lower Portobello Bay amphipods successfully melanized *M. novaezealandensis* metacercariae, while only 4.4% of infected Hoopers Inlet amphipods did so. Also, a significantly higher percentage of metacercariae were melanized by Lower Portobello Bay amphipods (average 3.1% of metacercariae) than by Hoopers Inlet amphipods (average 0.8%). In addition, amphipods from Lower Portobello Bay were more resistant to parasitic infection than amphipods from Hoopers Inlet. From equal exposure to 15 cercariae, amphipods from Hoopers Inlet acquired almost two more metacercariae than Lower Portobello Bay amphipods, an extra load that can have negative impact on survival (Fredensborg et al. 2004). The results suggest that amphipods from a population naturally exposed to a high trematode prevalence have more efficient immune responses and are more resistant to parasitic infection than amphipods from a population with no prior exposure to the parasite. The apparently lower investment of Hoopers Inlet amphipods in resistance against trematodes is fully compatible with the existence of costs of immunity.

There is an interesting difference in the melanization rates of Lower Portobello Bay amphipods between natural and experimental infections. The values obtained in the experimental infection study (13.0% of infected amphipods melanized metacercariae, average percent melanization 3.1%) were much lower than those seen in naturally infected amphipods (31.7% of infected amphipods melanized metacercariae, average percent melanization 17.6%). This could reflect differences in food quality between the

field and the laboratory. Alternatively, there may be seasonal differences in the amphipods' immune ability: amphipods with natural infections were collected in March, while those used for experimental infections were collected in May and June. Another possible explanation for the higher percent melanization in naturally infected amphipods compared with experimentally infected ones is that natural infections are older and the amphipods have had a longer time to encapsulate parasites. Finally, this discrepancy may reflect the memory component of invertebrate immunity. Naturally infected amphipods have been exposed to several waves of infection, as indicated by the fact that they typically harbour parasites at many different developmental stages. In the experimental infections, however, we tried to exclude all amphipods with prior infections. Since an initial infection can cause later immune responses to be stronger (Kurtz and Franz 2003), it may be that the previously unexposed amphipods used in the experiments could not achieve the higher levels of melanization observed in nature, where many hosts have had their immunity primed by previous exposure to trematodes.

Sex differences in infection levels have been reported in many invertebrate populations (Sheridan et al. 2000), with males often more heavily infected than females (e.g., Wedekind and Jakobsen 1998). This pattern was observed here for natural infections, with males from Lower Portobello Bay harbouring on average two more metacercariae than females. However, no sex difference was detected in experimental infections, which suggests that these differences may only develop over longer periods, involving repeated exposures to parasites.

Animals have three major lines of defence against parasitic infection aimed at either preventing the entry of parasites into the host or at reducing the harm caused by parasites once inside the host (see Gross 1993; Sheldon and Verhulst 1996; Rigby and Moret 2000; Rigby et al. 2002; Schmid-Hempel 2003). First, behavioural responses can minimise the risk of infection by leading to the avoidance of microhabitats with high parasite prevalence (Gross 1993; Rigby and Moret 2000; Rigby et al. 2002). In this study, amphipods from both populations were observed to perform bursts of frantic swimming activity during incubation with cercariae in the experimental infection procedure, as though they were attempting to swim away from the cercariae to which they were exposed. Similarly, fish exposed to cercariae of the trematode *Diplostomum spathaceum* quickly swim away from the source of cercariae (Karvonen et al. 2004). Here, amphipods were exposed to cercariae for a long period of time (5 h) in a small volume of water, so it is unlikely that this avoidance behaviour was effective. Another behavioural response observed during and directly after exposure was grooming, akin to the anti-parasite behaviour widely seen in the animal kingdom (e.g., Hart 1997; Mooring et al. 2004). Crustaceans are known to use grooming to avoid parasite infection. For example, the porcellanid crab *Petrolisthes cabrilloi* is commonly infected by a castrating rhizocephalan parasite. Within seconds after infective larvae contact its gills, the crab initiates vigorous gill-grooming with its limbs, continuing until the larvae have been removed (Fleischer et al. 1992). Related crab species that are not naturally infected by rhizocephalans, but that can be infected in the laboratory, react very slowly or not at all when exposed to infective larvae, despite having limbs morphologically identical to those of *P. cabrilloi*. These non-host species lack the appropriate behavioural adaptations to effectively remove the settling larvae (Fleischer et al. 1992). In our experimental study, amphipods were seen to rub their bodies with their legs, as if trying to remove penetrating cercariae. Lower Portobello Bay amphipods may have been better at correctly recognising the perceived irritation as cercarial penetration, thus grooming more efficiently and successfully removing more of the penetrating cercariae than Hoopers Inlet amphipods.

The second line of defence consists of passive mechanical barriers, such as the cuticle (against parasites which directly penetrate) or the gut wall (against parasites which are ingested), which prevent the parasite from penetrating into the host body cavity (Rigby et al. 2002). The cercariae of *M. novaezealandensis* infect their host by direct penetration of the cuticle using a sharp-pointed stylet (Martorelli et al. 2004). It may be that amphipods from Lower Portobello Bay have thicker cuticles, and thus more effective barriers against penetration as an adaptation to natural exposure, than amphipods from Hoopers Inlet.

The third line of defence is the immune response, which acts to eliminate the infection or minimise the harm it causes once the parasite has broken through the first two lines of defence (Sheldon and Verhulst 1996; Rigby et al. 2002). In the present study, amphipods from the population where natural parasite infection levels are high (Lower Portobello Bay) were significantly more likely to initiate an effective encapsulation and melanization immune response than amphipods from the population where natural parasite infection is absent. The first two lines of defence may act to decrease the resource costs of mounting an immune response (Rigby and Moret 2000). Presumably, preventing a parasitic infection by altering behaviour is much 'cheaper' (i.e., requires less resources) than mounting a full-scale encapsulation and melanization response to kill the parasite after infection. It is interesting to note that amphipods from the parasite-free Hoopers Inlet population have not completely lost the ability to melanize parasites. The phenoloxidase cascade used in the encapsulation and melanization response plays roles in other aspects of physiology, such as tanning of eggs, hardening of the cuticle, and wound healing (Zuk and Stoehr 2002; Rolff and Siva-Jothy

2003; Schwarzenbach and Ward 2006). Wounds healed with melanin (e.g., damage to thoracic plates, fractured appendages) were frequently observed in amphipods from both populations. Plaistow et al. (2003) suggested that wounding can often be much more prevalent than infection in natural populations of amphipods. Selection may thus favour the retention of phenoloxidase cascades and some melanization abilities in parasite-free populations.

Taken together, the results suggest that amphipods from Lower Portobello Bay may have behavioural (e.g., grooming), morphological (e.g., strong cuticle) and/or physiological (e.g., melanization response) adaptations that allow them to survive, grow and reproduce successfully in an environment with a high risk of infection. Amphipods from an environment where the risk of infection is zero, due to the natural absence of parasites, appear to have partially lost these abilities. The two amphipod sites of origin are separated by approximately 35 km of coastline, much of which consisting of wave-exposed habitat unsuitable for amphipods. In addition, the amphipod lacks a planktonic dispersal stage, and so migration between these sites appears unlikely. Our results suggest limited gene flow between the Lower Portobello Bay and Hoopers Inlet *P. novizealandiae* amphipod populations.

Greater investments in immunity by amphipods from Lower Portobello Bay should be accompanied by trade-offs with other key fitness components. For instance, female isopods from a population exposed to acanthocephalan parasites produce fewer offspring, under identical laboratory conditions, than females from a parasite-free population (Hasu et al. 2006). To accurately assess trade-offs between immunity and other life history traits, laboratory strains of the two amphipod populations would have to be established. We would predict that, in the absence of parasites, laboratory-reared *P. novizealandiae* from Hoopers Inlet would have higher growth rates, fecundity and/or longevity than those from Lower Portobello Bay because they do not pay the cost of maintaining an effective immune response.

The results of the present study provide evidence for the local adaptation of immune response against the trematode *M. novaezealandensis*, relative to the risk of infection experienced, in the amphipod *P. novizealandiae*. Remarkably, the same parasite has also been implicated as a cause of local adaptation in its first intermediate host, the snail *Z. subcarinatus*. The trematode is much more virulent in its snail host than in its amphipod host: invariably, infected snails are permanently and totally castrated. Populations of snails exposed to high prevalences of infections are characterised by lower age at maturity and higher juvenile growth rates than those in which infections are rare (Fredensborg and Poulin 2006). Overall, these findings are consistent with a role for parasitism as a selective force producing differences among host populations.

Acknowledgments We thank the Ecological Parasitology Group of the University of Otago for commenting on an earlier version of this manuscript. The experimental work described in this paper complies with the current laws and animal ethics regulations of New Zealand.

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