Variation of parasite load and immune parameters in two species of New Zealand shore crabs

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Abstract While parasites are likely to encounter several potential intermediate hosts in natural communities, a parasite's actual range of compatible hosts is limited by numerous biological factors ranging from behaviour to immunology. In crustaceans, two major components of immunity are haemocytes and the prophenoloxidase system involved in the melanisation of foreign particles. Here, we analysed metazoan parasite prevalence and loads in the two sympatric crab species *Hemigrapsus crenulatus* and *Macrophthalmus hirtipes* at two sites. In parallel, we analysed the variation in haemocyte concentration and amount of circulating phenoloxidase (PO) in the haemolymph of the same individuals in an attempt to (a) explain differences in parasite prevalence and loads in the two species at two sites and (b) assess the impact of parasites on these immune parameters. *M. hirtipes* harboured more parasites but also exhibited higher haemocyte concentrations than *H. crenulatus* independent of the study site. Thus, higher investment in haemocyte production for *M. hirtipes* does not seem to result in higher resistance to parasites. Analyses of variation in immune parameters for the two crab species between the two sites that differed in parasite prevalence showed common trends. (a) In general, haemocyte concentrations were higher at the site experiencing higher parasitic pressure while circulating PO activity was lower and (b) haemocyte concentrations were influenced by microphallid trematode metacercariae in individuals from the site with higher parasitic pressure. We suggest that the higher haemocyte concentrations observed in both crab species exposed to higher parasitic pressure may represent an adaptive response to the impact of parasites on this immune parameter.

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Introduction

Parasites are by definition costly for their hosts since they reduce host survival and reproduction, thereby acting as agents of natural selection (Fredensborg and Poulin 2006; Lochmiller and Deerenberg 2000). Furthermore, they represent important factors regulating host populations and structuring entire communities (Poulin and Mouritsen 2006; Thomas et al. 1998, 1999). Host–parasite relationships are essentially driven by the ongoing co-evolution of the interacting species. Thus, the host establishes several lines of defence to avoid being parasitised, while parasites have to be able to circumvent these defences in order to complete their life cycles (Damian 1997; Schmid-Hempel 2008). Therefore, the capacity to escape from parasitism due to elaborate defence mechanisms against initial parasite infection and subsequent proliferation is an important determinant of a host's lifetime reproductive success (i.e. fitness) (Lochmiller and Deerenberg 2000; Sheldon and Verhulst 1996). Consequently, although parasites are potentially able to use more than one species of the same ecological guild as intermediate host in natural communities (Koehler and Poulin 2010), a parasite's actual compatible host range is further limited by biological factors such as physiology, morphology, behaviour and

immunology (Adamson and Caira 1994; Combes 2001; Euzet and Combes 1980).

Apart from physical and chemical barriers (e.g. the cuticle and the gut epithelium) and behaviours reducing exposure to parasites, invertebrates possess innate immune effectors consisting of both cellular and humoral components (reviewed by Jiravanichpaisal et al. 2006), which are activated by the recognition of non-self particles (Cerenius et al. 2008; Jiravanichpaisal et al. 2006; Vazquez et al. 2009). In crustaceans, there are usually three types of circulating haemocytes, cells that have phagocytic activity and the capacity to encapsulate foreign particles (Hose et al. 1990; Matozzo and Marin 2010). These cells constitute the cornerstones of the innate immune system and are also responsible for humoral immune responses, such as the production of antimicrobial peptides (Chisholm and Smith 1992; Destoumieux et al. 1997, 2000; Schnapp et al. 1996) and melanisation as a result of the prophenoloxidase (proPO) enzyme cascade (Söderhäll 1983). According to the classical concept, the precursors that lead to phenoloxidase (PO) activity are already present in the haemolymph before the occurrence of any parasitic challenge and can be further activated to increase PO activity in the case of an immune challenge. Such activation is generally due to serine proteases controlled by inhibitors which provoke the conversion of the precursor into PO and eventually lead to the production of melanin and toxic humoral compounds such as quinone (Cerenius and Söderhäll 2004; Söderhäll and Cerenius 1998). As a consequence, the amount of both the inactive proenzyme as well as the naturally active protein responsible for PO activity in the haemolymph provides a good measure of the level of this system (Cornet et al. 2009). Although the importance of PO activity to fight against parasites is still subject to debate (Cerenius et al. 2008), it has been demonstrated that this immune component provides an efficient protection particularly against microbial parasites in several crustaceans, namely freshwater crayfish (Cerenius et al. 2010; Liu et al. 2007) and shrimps (Fagutao et al. 2009). Nevertheless, knowledge of the immunocompetence of crustacean populations in the wild is still scarce, especially in cases where they are exposed to more than one parasite.

Here, we focus on two sympatric species of New Zealand shore crabs, the hairy-handed crab *Hemigrapsus crenulatus* (Grapsidae) and the stalk-eyed mud crab *Macrophthalmus hirtipes* (Ocypodidae). Both species are common in the intertidal zone of sheltered mudflats throughout New Zealand and confirmed intermediate hosts of numerous macroparasites (Brockerhoff and Smales 2002; Koehler and Poulin 2010; Latham and Poulin 2002a). Despite being closely related (Kitaura et al. 2002), a previous survey of the entire metazoan parasite community infecting these crabs which was done at one site in New Zealand (Lower Portobello Bay) indicates that the two species may differ in their susceptibility to infection with acanthocephalans, microphallid trematodes and nematodes (Koehler and Poulin 2010). The first objective of the present study was to investigate the variation in metazoan parasite prevalence and loads between the two species at two sites. The chosen locations (Lower Portobello Bay and the estuary Taieri Mouth) were expected to exhibit different levels of parasite diversity due to the absence of the mud snail *Zeacumantus subcarinatus*, an important intermediate host for trematodes, from Taieri Mouth (Koehler, unpublished observation). In order to interpret the potential variation in parasite prevalence and loads between the two crabs as well as to assess the effects of the different parasites on their immune system, we also analysed the variation in two essential immune parameters, i.e. haemocyte concentration and PO activity in the haemolymph.

Materials and methods

Animal sampling and handling

Individuals of the hairy-handed crab *H. crenulatus* and the stalk-eyed mud crab *M. hirtipes* were randomly collected during low tide from intertidal mud flats at Taieri Mouth, South Island, New Zealand (46°04′ S, 170°11′ E) and Lower Portobello Bay (LPB), South Island, New Zealand (45°52′ S, 107°42′ E) in March 2010. In the laboratory, all crabs were kept at 20°C in groups of approximately 15 individuals (to avoid crowding conditions) in clear plastic containers (30 cm long×12 cm wide×17 cm high) filled with both 2.5 l of seawater and a thin layer of mud from the study sites as well as four plastic tubes (10 cm long with 4 cm diameter) to provide shelter. Each container was aerated with an airstone and the animals were not fed. All specimens were left in these conditions for 2 days to allow recovery from handling stress. Then, all crabs from the same study site were further analysed within 14 days. No mortality was observed during the entire study period. The gender of each individual was determined and size (carapace width) was measured to the nearest 0.1 mm using vernier callipers.

Haemolymph sampling

One or more walking legs of each crab were cut with scissors at the joint between merus and carpus and four haemolymph samples of 4 μl each were drawn from the resulting hole using a 10-μl micropipette. This amount was immediately redistributed into four tubes containing different solutions for the different analyses that were to follow [i.e. haemocyte concentration (1), PO activity with (2) or without trypsin (3) and with phenylthiourea (PTU) (4)].

Haemocyte concentration

Four microliters of haemolymph from each individual were added to 8 μl of MAS (Modified Anticoagulant Solution: 366 mM NaCl, 9 mM EDTA, 115 mM glucose, 27 mM sodium citrate, pH 7.4) in order to prevent cell coagulation and 4 μl of trypan blue dye were added in order to stain dead cells. The total number of live haemocytes present in 1 ml of haemolymph was determined using a cell counting chamber (Hawksley, Lancing, England).

Analysis of phenoloxidase activity

Two 4-μl haemolymph samples from each individual crab were added to two tubes, each containing 20 μl of sodium cacodylate buffer [10 mM $Na(CH_3)_2AsO_2$ (3 H_2O), 10 mM calcium chloride (2 H_2O), pH 7.4]. Another 4 μl of haemolymph were added to 20 μl of sodium cacodylate buffer complemented with PTU (1 mg ml^{-1} of phenylthiourea in sodium cacodylate buffer, pH 7.4). The samples were immediately frozen in liquid nitrogen and stored at −70°C before being used at a later stage to analyse the enzyme activity of the proPo system.

For each individual, the naturally activated PO enzymes in the haemolymph (hereafter referred to as "Circulating PO") were measured. In this case, no further activation was needed. However, "total PO activity" (i.e. circulating PO plus PO activity resulting from the activation of precursors) was also measured and this step required further activation of the precursor of PO activity into PO. In order to achieve this, 20 μl of trypsin (1 mg ml^{-1} in distilled water) were added to one of the tubes containing sodium cacodylate buffer as well as to the one containing PTU. PTU is a specific inhibitor of both tyrosinase and catecholoxidase reactions, which are inherent to the PO system. Hence, the use of PTU helps to confirm that the reaction measured in its absence is actually due to proper PO activity.

All samples were incubated at 37°C for 45 min to allow activation of the precursors of PO activity in the samples containing trypsin. At the end of that period, their contents were deposited into 96-well microplates and 60 μl of L-DOPA solution (4 mg ml^{-1} in distilled water, prepared previously) were added to each well. The plates were immediately analysed in a microplate reader (Omega Series, BMG Labtech) at 37°C for 3 h. The enzymatic conversion of L-DOPA into dopachrome was determined by measuring the optical density at 490 nm. The resulting absorbance values were used as measures of enzyme activity and those obtained from the last cycle were retained for further data analysis.

Parasite identification and counting

Following the haemolymph sampling, each crab was dissected and all metazoan parasites were identified and counted. Despite the fact that the two closely related acanthocephalan parasites *Profilicollis novaezelandensis* and *Profilicollis antarcticus* have been found to co-occur in both crab species, previous studies indicate that *P. antarcticus* only accounts for about 1% of the total number of acanthocephalans and is not easily distinguishable from *P. novaezelandensis* (Brockerhoff and Smales 2002; Latham and Poulin 2002a, b). Consequently, both acanthocephalan species were pooled for the purpose of this study and will hereafter be referred to as *Profilicollis* spp.

Similarly, the metacercariae of the two prevailing microphallid trematode species *Maritrema novaezealandensis* and *Microphallus* sp. were counted together. However, the stage of trematode development was recorded based on the classification established by Keeney et al. (2007). This differentiation was made since the metacercariae undergo important changes during their development, including a 200-fold increase in size in the case of *M. novaezealandensis* (Fredensborg et al. 2004), and it was thus hypothesised that the different developmental stages might have different effects on the host's immune response. According to this classification, stage 1 microphallid trematodes are early immature and unencysted metacercariae, stage 2 are late immature and unencysted metacercariae, while stage 3 refers to early single-walled cysts and stage 4 to mature double-walled cysts (Keeney et al. 2007). The other parasites encountered were larval stages of the nematode *Ascarophis* sp. as well as those of an acuariid nematode (Moravec et al. 2003).

Data analysis

All statistical tests and models were performed using the R software (R Project 2.8.0). When necessary, the data were Box-Cox transformed to meet the assumptions of normality (Shapiro–Wilk test $p>0.05$) and homoscedasticity (Breusch–Pagan test $p>0.05$) of the residuals. For the parasite counts, transformation did not result in normally distributed data. Hence, the non-parametric Mann–Whitney test was used to compare (a) parasite loads, i.e. numbers of parasites per host, between host species and (b) acanthocephalan loads between sites. Independent *t* tests were used to compare (a) immune parameters between host species, (b) total and circulating PO, (c) PO activity with and without PTU and (4) host size between sites. In all cases, differences were considered significant if $p\leq0.05$.

General linear models (GLM) were then used to determine which factors had an influence on the amount of haemocytes and PO activity in the haemolymph. All analyses started with a full model including all explanatory variables (see below for details) and were then modified using backward stepwise elimination of non-significant ($p>0.05$) factors until only significant terms were retained in the final model. Initial GLMs were done for each species separately and included

four explanatory variables: gender, site of origin, size (carapace width) and the time (number of days) the crabs had spent in the laboratory before being dissected. The latter variable was included in order to check for any potential effect caused by the maintenance conditions in the laboratory. Additionally, since origin turned out to be a highly influential factor, further GLMs were run incorporating only the data from the highly parasitized site, LPB, with the different parasite taxa as explanatory variables. Finally, linear regressions were used to analyse the relationship between immune parameters and the factors that had been identified as being influential by the GLMs.

Results

Parasite distribution

Parasite prevalence per host species and site are represented in Table 1. The specimens from LPB harboured a more diverse range of parasites, including the acanthocephalans *Profilicollis* spp., metacercariae of the two microphallid trematodes *M. novaezealandensis* and *Microphallus* sp., the nematode *Ascarophis* sp. and an acuariid nematode. Furthermore, acanthocephalans, the only parasites encountered at both sites, occurred in significantly higher numbers in hosts from LPB than in hosts from Taieri Mouth (U=65.5, p<0.0001). Overall, *M. hirtipes* was found to carry higher loads of all identified metazoan parasites than *H. crenulatus* (all $U \leq 270$, all $p \leq 0.014$; Fig. 1) except for stage 2 microphallids, which were more abundant in *H. crenulatus* (U=205, p<0.001; Fig. 1).

Variation in immune parameters

Characterisation of PO activity

Trypsin was found to enhance PO activity in *H. crenulatus* (t=−2.295, df=112, p=0.02) but not in *M. hirtipes* (t=−0.571, df=116, p=0.57). However, even the activation in *H. crenulatus* was weak compared to previous results in other crustaceans (e.g. Cornet et al. 2009). Hence, it was assumed that trypsin was not efficient to activate the precursor of PO activity in these crabs. Therefore, we did not obtain a reliable measure of "total PO activity" and only the basal PO activity ("circulating PO") was considered for further analyses. On the other hand, the addition of PTU effectively inhibited the enzymatic reaction in both species (t=2.879, df=107, p=0.005 in *H. crenulatus* and t=2.014, df=115, p=0.046 in *M. hirtipes*), thereby proving that the measured reaction is actually due to a proper PO activity.

Table 1 Gender, size, haemocyte concentration, circulating PO activity and parasite prevalence in *M. hirtipes* and *H. crenulatus* from Taieri Mouth and Lower Portobello Bay

	Species	*N*	Males	Females	Size (mm), mean ± SD	Haemocytes (no/ml×10^6), mean ± SD	Circulating PO (OD), mean ± SD	Number of infected individuals			
								Acanthocephalans	Microphallids stage 2/3/4	*Ascarophis* sp.	Acuariid nematode
Taieri mouth	*M. hirtipes*	32	13	19	17.2±1.5	13.02±9.86	0.536±0.398	12	0/0/0	0	0
	H. crenulatus	26	19	7	14.5±1.6	5.95±3.82	0.468±0.197	0	0/0/0	0	0
LPB	*M. hirtipes*	27	18	9	20.8±2.1	23.34±13.75	0.379±0.563	27	8/27/27	6	24
	H. crenulatus	31	15	16	19.5±1.7	8.13±4.83	0.385±0.185	30	27/31/31	4	3

LPB Lower Portobello Bay, *PO* phenoloxidase

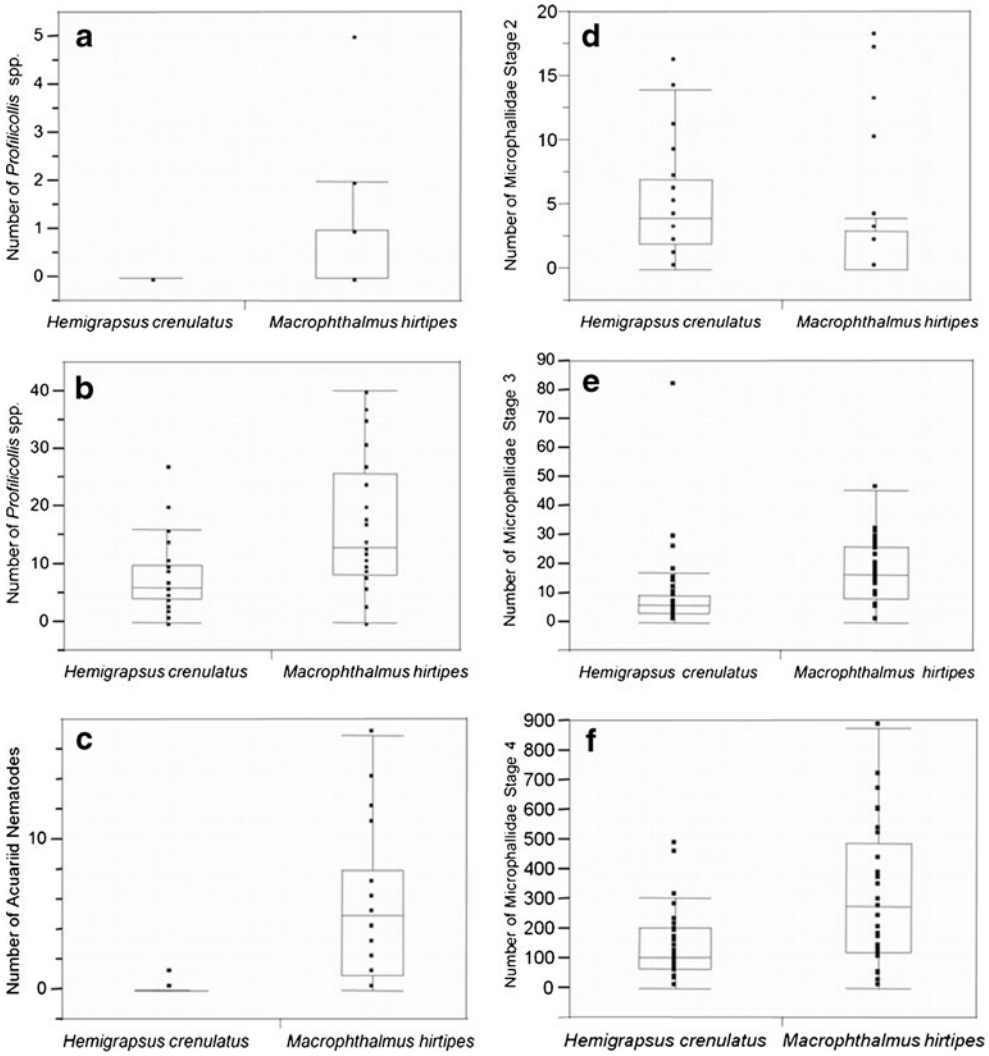

Fig. 1 Parasite loads, i.e. numbers of parasites per individual host, in *H. crenulatus* and *M. hirtipes*: **a** Acanthocephalans at Taieri Mouth (U=270, p=0.014) and **b** at Lower Portobello Bay (U=173.5, p=0.0001), **c** acuariid nematodes at Lower Portobello Bay (U=57, p<0.0001) and **d**, **e**, **f** the different developmental stages of microphallid trematodes at Lower Portobello Bay (stage 2: U=205, p=0.0009; stage 3: U=183.5, p=0.0002; stage 4: U=214.5, p=0.0015). *Black dots* represent data distribution; *boxes* represent medians, 25th and 75th percentiles, whiskers show 10th and 90th percentiles

Variation in haemocyte concentrations

1. Between crab species

   Overall, haemocyte concentrations were found to be higher in *M. hirtipes* than in *H. crenulatus* at both sites (t=−3.759, df=56, p<0.001 for Taieri Mouth and t=−6.292, df=56, p<0.0001 for LPB; Table 1).
2. Between sites

   For both *M. hirtipes* and *H. crenulatus,* the GLMs showed that part of the variation in haemocyte concentrations was explained by the origin (i.e. site) of the crabs (F=11.81, df=1, p=0.001 and F=4.26, df=1, p=0.036, respectively; Table 2). The data further indicate that individuals of both species had higher haemocyte concentrations at LPB than at Taieri Mouth (Table 1). In addition, the GLMs showed that body size influenced haemocyte concentration in *H. crenulatus* (F=5.46, df=1, p=0.023; Table 2) but not in *M. hirtipes*. Hence, the factor "origin" might potentially be confounded with the factor "size" in *H. crenulatus*, since specimens from LPB were larger than those from Taieri Mouth (t=11.151, df=55, p<0.0001; Table 1), with a mean difference in size of 5 mm (Table 1).
3. Effect of metazoan parasite loads on haemocyte concentration

   For both species, haemocyte concentrations at LPB were found to be significantly influenced by only one type of parasite, namely the microphallid trematode metacercariae (F=5.53, df=1, p=0.038 for *M. hirtipes* and F=8.34, df=1, p=0.0094 for *H. crenulatus*; Table 3). However, the variation in haemocyte concentrations was caused by different developmental stages of the metacercariae depending on the crab species. In this context, it has to be pointed out that stage 2 microphallids, i.e. the stage that initiates the infection, occurred in higher numbers in *H. crenulatus* than in *M. hirtipes*, as opposed to other microphallid developmental stages (Fig. 1). Although further linear regression analyses of the relationship between haemocyte concentrations and metacercarial loads turned out to be not

Table 2 Results of the general linear model (GLM) of the factors influencing haemocyte concentration and circulating PO activity in *M. hirtipes* and *H. crenulatus* at both sites

		Macrophthalmus hirtipes			*Hemigrapsus crenulatus*		
	Factor	*Df*	*F*	*p*	*Df*	*F*	*p*
Haemocytes	Gender			n. s.			n. s.
	Origin	1	11.8095	0.001181	1	4.6207	0.03655
	Size			n. s.	1	5.4607	0.02326
	Time lapse			n. s.			n. s.
Circulating PO	Gender			n. s.	1	8.1569	0.006151
	Origin	1	9.7985	0.00284	1	4.3949	0.040930
	Size			n. s.			n. s.
	Time lapse			n. s.			n. s.

(*n. s.* not significant)

significant in both species, there was a tendency towards a negative correlation of haemocyte concentration and the number of stage 3 metacercariae in *M. hirtipes* (R^2=0.11, F=3.19, p=0.086; Fig. 2).

Variation in circulating PO activity

1. Between crab species

   The two species showed similar circulating PO activities independent of the site (t=−0.414, df=55 p=0.68 for Taieri Mouth and t=1.27, df=56, p=0.21 for LPB; Table 1).
2. Between sites

   As for haemocyte concentrations, part of the variation in circulating PO activity could be explained by the origin of the crabs for both species (F=9.8, df=1, p=0.003 for *M. hirtipes* and F=4.39, df=1, p=0.04 for *H. crenulatus*; Table 2). In addition, circulating PO activity also varied significantly with the gender in *H. crenulatus* (F=8.16, df=1, p=0.006; Table 2). The distribution of the data further indicates that enzyme activity was lower at LPB than at Taieri Mouth, although this difference between sites was again more pronounced in the case of *M. hirtipes* (Table 1).
3. Effect of metazoan parasite loads on circulating PO

   None of the various parasite taxa had a significant influence on circulating PO activity in either crab species (Table 3).

Discussion

Immunocompetence is a key component of fitness (Lochmiller and Deerenberg 2000; Sheldon and Verhulst 1996) as well as a major determinant of host–parasite compatibility (Adamson and Caira 1994; Combes 2001; Euzet and Combes 1980). Regarding the two crab species examined here, a previous study investigated the diversity of the metazoan parasite community infecting these hosts at LPB (Koehler and Poulin 2010). However, nothing was known regarding the immune defences of these hosts and their role in shaping the various host–parasite interactions.

Table 3 Results of the general linear model (GLM) of the different parasite taxa influencing haemocyte concentration and circulating PO activity in *M. hirtipes* and *H. crenulatus* at Lower Portobello Bay only

		Macrophthalmus hirtipes			*Hemigrapsus crenulatus*		
	Factor	*Df*	*F*	*p*	*Df*	*F*	*p*
Haemocytes	Acanthocephalans			n. s.			n. s.
	Microphallids stage 2			n. s.	1	8.3423	0.009417
	Microphallids stage 3	1	5.5304	0.03837			n. s.
	Microphallids stage 4			n. s.			n. s.
	Acuariid nematodes			n. s.			n. s.
	Ascarophis			n. s.			n. s.
Circulating PO	Acanthocephalans			n. s.			n. s.
	Microphallids stage 2			n. s.			n. s.
	Microphallids stage 3			n. s.			n. s.
	Microphallids stage 4			n. s.			n. s.
	Acuariid nematodes			n. s.			n. s.
	Ascarophis			n. s.			n. s.

(*n. s.* not significant)

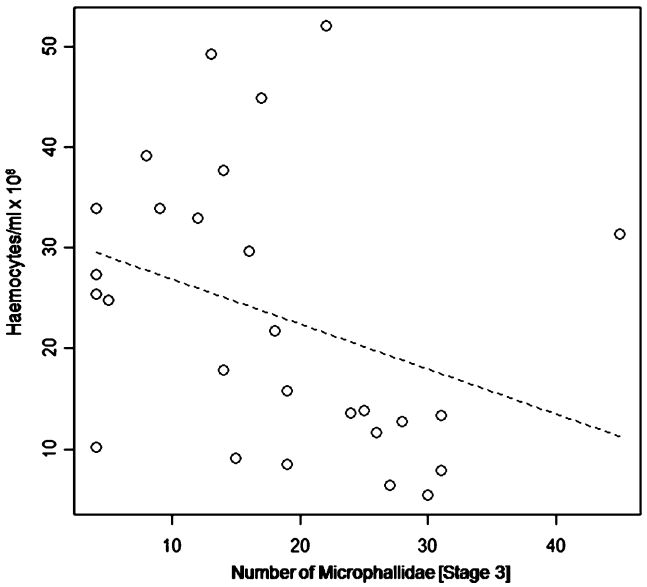

Fig. 2 Linear regression showing the relationship of haemocyte concentration and infection with stage 3 microphallid trematode metacercariae in *M. hirtipes* (N=27; R^2=0.11, p=0.086)

By studying the parasite loads of the two species *M. hirtipes* and *H. crenulatus* at two different sites, we strengthened the observation that *M. hirtipes* carries higher numbers of all parasite taxa than *H. crenulatus* of similar body size, even in populations facing lower parasitic pressure. However, since body size may not correspond to exactly the same age in different species, it cannot be entirely excluded that age differences play a role in the different infection rates observed between the two species. We also analysed the variation in essential immune parameters in order to determine if the lower parasite loads in *H. crenulatus* might be due to a particular adaptation of this species, e.g. on the part of its immune defences. However, the results presented here provide no indication regarding the precise nature of any immunological basis for the different infection levels between the two crab species since *H. crenulatus* exhibits lower haemocyte concentrations than *M. hirtipes*, independent of the study site. Moreover, circulating PO activity of the two species was similar at each site. Instead, such differences in susceptibility between species might also derive from behavioural differences (*H. crenulatus* usually shelters under rocks on the upper shoreline, whereas *M. hirtipes* rests in water-logged burrows in the mud) or from morphological features. On the other hand, the fact that *M. hirtipes* appears to be more susceptible to parasitic infection could also be the result of long-term adaptations on the part of the parasites. In this context, Latham and Poulin (2002a) suggested that at least the acanthocephalans might preferentially manipulate the hiding behaviour of *M. hirtipes* since it is the more abundant species in this region, thus providing more opportunities for the parasite to be passed on to its final avian host. Such fine-tuned adaptations aiming at manipulating one particular host species could also exist in the other parasite taxa considered here, since all of them have to reach their definitive host via predation on their crab intermediate host.

Regardless of this difference between the two species, a common immune feature is that the origin of the studied individuals partially explained the variation in haemocyte concentrations and PO activity, with haemocyte concentrations being higher at LPB than at Taieri Mouth, along with a lower PO activity at LPB. This observation could be highly relevant, given that the studied crab populations from two different intertidal mudflats were markedly different in terms of diversity, prevalence and load of macroparasites. While only a small number of acanthocephalans were found in *M. hirtipes* from Taieri Mouth and *H. crenulatus* was virtually parasite-free, individuals of both species originating from LPB harboured several acanthocephalans (*Profilicollis* spp.), metacercariae of the trematodes *M. novaezealandensis* and *Microphallus* sp., the nematode *Ascarophis* sp. and an acuariid nematode. Hence, it could be argued that the enhanced haemocyte production at the parasite-rich site (LPB) results from the potentially greater selective pressure exerted by the higher level of parasitism in this area. Since both the maintenance of the immune system as well as its use when actually responding to a challenge are considered energetically costly for the individual (Lochmiller and Deerenberg 2000; Schmid-Hempel 2003), it has been proposed that investment in immunity should be optimised according to the local intensity of parasitic infections (Bryan-Walker et al. 2007). This could be the case here, since only individuals able to invest more in their immune response can be expected to persist and to successfully reproduce in an environment with a high parasitic pressure such as LPB.

However, both higher haemocyte concentrations and circulating PO activity have previously been related to a greater resistance against infectious pathogens in crustaceans (e.g. Cornet et al. 2009; Fagutao et al. 2009; Liu et al. 2007). Here, we found an enhanced haemocyte production along with a decrease in circulating PO activity. This could indicate a trade-off between these two immune components, favouring the production of haemocytes over an enhanced PO activity. Since the crabs were facing infections with macroparasites, such a biased investment in haemocytes could be advantageous as these cells are capable of encapsulating even relatively large parasites (Bryan-Walker et al. 2007; Kostadinova and Mavrodieva 2005; Thomas et al. 2000) that cannot be repelled by humoral mechanisms alone. Thus, layers of haemocytes are deposited around the invader and then the cells are melanised to form a capsule, thereby restricting any further activity of the parasite or even killing it with toxic components (Cerenius and Söderhäll 2004;

Söderhäll and Cerenius 1998). Although the process of melanisation eventually relies on the proPO cascade, the presence of large numbers of haemocytes might be the foremost requirement for immune responses particularly directed against large intruders. In contrast, the effectiveness of humoral defence mechanisms alone, such as the activity of the PO enzyme in the haemolymph, has mainly been demonstrated against microbial pathogens in crustaceans (Cerenius et al. 2010; Fagutao et al. 2009; Liu et al. 2007). Moreover, there is evidence that, at least in some crustaceans, what is measured as PO activity could in fact be derived from haemocyanin acting as "functional phenoloxidase" in the plasma fraction of the haemolymph (Cerenius et al. 2008; Jaenicke et al. 2009; Perdomo-Morales et al. 2008). Therefore, there is no clear evidence that the PO activity measured here is actually linked to the phenoloxidase enzyme.

Another aspect of our study was to assess how the different parasite taxa impact host immune parameters. The only parasites found to be influential in this respect were the microphallid trematode metacercariae, which affected haemocyte concentrations in both crab species. Although the results presented here do not justify any further conclusions regarding a direct, potentially immunosuppressive, impact of these parasites in these hosts, it is interesting that this influence was detected for different developmental stages of the metacercariae depending on the crab species. In *M. hirtipes*, it was observed for early single-walled cysts (stage 3), which was not the most abundant stage since stage 4 metacercariae (mature double-walled cysts) occurred in much higher numbers. However, as the latter have reached a more mature stage enclosed in a cyst with no further growth, they may be able to escape from the immune response of their host and probably have stopped any direct manipulations of the host's immune system (Galaktionov et al. 1996). In *H. crenulatus*, the late immature and unencysted second stage metacercariae (stage 2) turned out to be influential. Again, this stage occurred in lower numbers than stage 3 and, even more so, stage 4 metacercariae. However, this stage occurred much more frequently in *H. crenulatus* than in *M. hirtipes*. Since the second stage larvae are not yet encysted and still migrate through the host's body (Galaktionov et al. 1996), probably disrupting tissue on their way, it can be argued that, although less abundant than encysted stages, metacercariae at this developmental stage may be more damaging to the host, particularly when their density is relatively high. Therefore, both species may face the same number of colonisation attempts by microphallid trematodes but these early stages may encounter a higher level of resistance in *H. crenulatus* and consequently, fewer early stage metacercariae would manage to encyst and develop into mature stages in this host.

In conclusion, our observations from two sites suggest that the higher haemocyte concentrations observed in both crab species exposed to higher numbers and diversity of parasites may represent an adaptive response to the higher level of parasitism in their environment. The observed influence of microphallid trematode metacercariae on haemocyte concentrations indicates that these parasites might interact more closely with the host's immune response, possibly even causing an immunodepressive effect. If so, such selective pressure should favour the evolution of more efficient host defences over generations, in relation to the parasitic pressure prevailing in the respective habitat.

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