IDEAS AND PERSPECTIVES

Variation in the risk of being wounded: an overlooked factor in studies of invertebrate immune function?

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Abstract

In invertebrates, wounding can trigger an immune response, and will often expose organisms to parasites and pathogens. Here we show that in the amphipod *Gammarus pulex*, wounding abundance is negatively correlated with PhenolOxidase activity (a major component of the invertebrate immune response), and that the occurrence and abundance of wounding is extremely high and varies significantly between five natural populations. In some populations the prevalence and abundance of wounds also varied between sexes. Given that, using and maintaining an efficient immune system is costly, we suggest that the frequency of wounding may be an important selective pressure influencing an organism's optimal investment in immune defences.

Keywords

Acanthocephalan, costs of immunity, *Gammarus pulex*, parasite resistance, variation, wounding.

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INTRODUCTION

Variable patterns of parasite resistance have been suggested to be shaped, among other things, by a cost-benefit ratio of the immune response (Sheldon & Verhulst 1996; Read & Allen 2000; Schmid-Hempel & Ebert 2003). Whilst evolution toward a higher level of resistance leads to genetic trade-offs with other life history traits (see Kraaijeveld *et al.* 1998), using the immune system is also known to be costly (Råberg *et al.* 1998; Moret & Schmid-Hempel 2000; Tiën *et al.* 2001). In order to minimize costs, an organism's investment in immunity is supposed to be optimized depending upon the risk of exposure to immune challenges and the type of challenge to which they are exposed (Behnke *et al.* 1992; Reeson *et al.* 1998; Barnes & Siva-Jothy 2000; Wilson *et al.* 2001). Variation in the risk of immune challenge is most frequently related to the prevalence and virulence of parasites and pathogens (e.g. Fellowes & Kraaijeveld 1998; Reeson *et al.* 1998). Here, we point out, that variation in another source of immune challenge, the frequency of wounding, has largely been overlooked.

The outer integument of an organism can be viewed as its first line of defence, protecting it from infection or colonization by foreign bodies. When an organism is wounded, this first line of defence is penetrated. Wounds are therefore one of the main points of entry for parasites and pathogens (the other being ingestion). It is therefore intuitive that animals that are exposed to higher levels of wounding will be exposed to more immune challenges. In addition, wounding produces an immune response, even in the absence of parasites or pathogens (Hoffmann 1995; Nayar & Knight 1995; Nagai *et al.* 2001).

Since mounting an immune response is costly and can impose trade-offs with other fitness parameters (Råberg *et al.* 1998; Moret & Schmid-Hempel 2000; Siva-Jothy *et al.* 2001; Tiën *et al.* 2001), variable wounding frequencies, or variation in the risk of being wounded, will potentially have evolutionary consequences for immune defences.

There may be many factors that influence wounding frequency including intra- and inter-specific competition, numbers of predators, frequency of sexual interactions, or the frequency of exposure to extreme environmental perturbations. If the probability of wounding varies between

populations, or between different classes of individuals within populations, then the cost imposed by the immune response involved in wound healing may lead to consistent differences in immune function between these groups. On a long-term basis, this may favour either the selection of cheaper immune mechanisms that provide a similar efficiency, or alternatively it may select for a lower investment in immune function that reduces the costs induced by frequent wounding.

The important point we are trying to establish here is that if wounding is frequent and varies consistently between populations or specific groups of individuals, then it may represent an important selective pressure influencing immune function, either independently, or in synchrony with the risk of exposure to parasites and pathogens. The importance of wounding risk is difficult to assess because no study has, to our knowledge, ever previously quantified the prevalence and abundance of wounding in a natural system, or tested whether levels of wounding vary between different populations or between different classes of individuals within the same population.

Here, we first tested if wounding might influence the level of immune response in the amphipod crustacean *Gammarus pulex*. This system is ideal for studying variation in wounding, because wounds can be clearly observed through the body wall as dark melanised plugs. We then examined whether the risk of wounding varies between populations, by quantifying the prevalence and abundance of wounding in five natural populations of *G. pulex*. The prevalence and abundance of wounding was compared with the prevalence and abundance of infection with acanthocephalan macro-parasites.

MATERIAL AND METHODS

Gammarids used to test the level of immune response were collected in the river Tille at 'Les Maillys' (Burgundy, eastern France). They were sampled using the kick-sampling method (Hynes 1954). Two samples were collected: in the first one, around 50 animals were collected randomly. Then, owing to the low parasite prevalence found in this sample, individuals infected by acanthocephalan parasites were actively searched for, until around the same number of infected vs. uninfected hosts had been found. The animals were kept in pots filled with water from the river and were provided with oxygen, and kept at low temperature during the return to the laboratory (around 30 min). In the laboratory, the animals were kept in an aquarium under standard conditions (15 °C) and were fed with dead leaves before being used for the experiment.

The experiment occurred within the 24 h following collection, and all individuals were treated in one run. Each individual was isolated in a 1.5-ml Eppendorf tube and kept on ice, sexed and measured by linear dimensions (distance from fourth coxal plate basis to individual dorsal limit) using a stereoscopic microscope Nikon SMZ-10A (Nikon Instech Co., Ltd, Kanagawa, Japan) and a video-analysis system VTO 232 from Linkam Scientific Instruments Ltd (Tadworth, UK) (Bollache *et al.* 2000). The number of wounds (i.e. any isolated melanised spot) was counted at the same time. A haemolymph sample was collected from each individual in order to measure PhenolOxidase (PO)-enzyme activity (see below). The animals were then dissected in Ringer's solution under a stereoscopic microscope to search for the presence of acanthocephalan parasites in their body cavity (*Pomphorynchus laevis* cytacanth, larvae that successfully invade the general cavity following ingestion).

Haemolymph extracts were taken by perfusing the haemocoel of chilled gammarids with 250 μl of ice-cold sodium cacodylate buffer (0.01 M Na-cacodylate, 0.005 M $CaCl_2$, pH 6.5). Samples were immediately frozen in liquid nitrogen and then stored in a freezer (−80°C). For the PO assay the samples were thawed on ice and 20 μl were placed into microtiter plate wells containing 140 μl of cold distilled water, 20 μl of cold saline phosphate buffer (PBS: 8.74 g NaCl; 1.78 g Na_2HPO4, $2H_2O$; 1000 ml distilled water; pH 6.5). Then 20 μl of cold L-Dopa solution (4 mg ml^{-1} of distilled water) were added into each well and the reaction was allowed to proceed for 40 min at 30°C in a microtiter plate reader (Versamax, Molecular Devices). Readings were taken at 490 nm and analysed using SOFTmax®PRO 4.0 software (Molecular Devices Corporation, Sunnyvale, CA, USA). Enzyme activity was measured as the slope (V_{max} value) of the reaction curve during the linear phase of the reaction.

Samples of animals used to estimate the frequency of wounding and infection across populations were collected from five different rivers in Burgundy (*Site 1*- River *Val Suzon*, medium-large gravel, depth (cm) ± SE = 20.0 ± 2.2; *Site 2*-River *Saône,* fine-medium gravel, depth ± SE = 12.3 ± 2.8; *Site 3*-River *Norge*, large gravel, depth ± SE = 30.7 ± 3.8; *Site 4*-River *Ouche*, large gravel, depth ± SE = 21.5 ± 2.6; *Site 5*-Stream in *Arquebuse Park*, Dijon, fine gravel, depth ± SE = 26.0 ± 0). Each sample consisted of 10 separate sub-samples that represented the full diversity of habitat present at the sampling site. For each sub-sample, animals were collected until at least 10 males and 10 females had been caught. All sub-samples were then pooled, and all caught animals were immediately fixed in 70% alcohol. At each site we recorded the type and size of the substrate present, and the average water depth and flow rate (measured as the number of seconds taken for a float to travel 5 m). All animals were measured and re-sexed in the laboratory within 2 weeks of their capture using a dissecting microscope, and the number of wounds counted (see before). All animals were dissected to count the number of acanthocephalan parasites present.

Analyses were carried out as follows. First PO activity (square-root transformed) was tested against four effects and their interactions: sex, body size, number of wounds [Ln (wounds + 1) transformed] and parasite infection. Second, the prevalence (frequency of occurrence) and abundance (number per gammarid) of wounds and acanthocephalans were tested against three main effects and their interactions: collection site, sex, and body size. Analyses were carried out by means of generalized linear models assuming either a Normal, Binomial or Poisson error according to the recorded variable and a link function (McCullagh & Nelder 1989). Variables to be included were selected using a mixed stepwise procedure (Montgomery & Peck 1982), adding or removing variables from the model, based on the Akaike's Information Criterion (A.I.C.). Variables considered in the initial models were covariates, factors and second-order interactions. All analyses were made using the S-plus® statistical software (MathSoft, Cambridge, MA, USA).

RESULTS

About 86% animals had wounds in the sample from 'Les Maillys', and the range of wound number was between 1 and 29 (overall mean $\pm$ SE = 4.18 $\pm$ 0.54). In this non-random sample, there was no significant difference between sexes for the average number of wounds, the infection status and average individual size [$t_{70} = 0.10$, $P = 0.32$ (Ln-transformed data); $\chi^2_1 = 0.33$, $P = 0.56$; $t_{70} = 0.14$, $P = 0.89$, respectively], and size was not correlated with the number of wounds ($r^2 = 0.01$; $F_{1,70} = 0.76$; $P = 0.38$). The level of PO activity was negatively correlated with wound abundance, i.e. individuals that had sustained more wounding had lower levels of PO activity (Table 1, Fig. 1a). Large gammarids provided more haemolymph and consequently had higher levels of PO activity compared with small individuals, but this relationship depended upon the host sex (Table 1, Fig. 1b):

Table 1 Generalized linear model (normal error) testing for the effect of size, sex, number of wounds (Ln transformed) and infection status on the level of PhenolOxidase activity (values square-root transformed) in the amphipod *Gammarus pulex*

| Source of variation	d.f.	F	P-value
Size	1	45.16	<0.001
Sex	1	8.10	0.006
Ln (wounds + 1)	1	4.60	0.035
Infection by *Pomphorynchus laevis*	1	23.12	<0.001
Size × sex	1	7.01	0.010
Error	66		
Total	72		

A stepwise procedure was used to build the model and only variables and interactions that had significant effects were considered.

large females had higher PO-enzyme activity than large males. Finally, *G. pulex* that were infected with the acanthocephalan parasite *P. laevis* had lower PO activities compared with uninfected individuals (Fig. 1c, Table 1).

The prevalence and abundance of wounding was also extremely high in all other populations studied (Fig. 2). About 85% of gammarids had wounds and the number of wounds per individual ranged from 1 to 27 (overall mean $\pm$ SE = 3.23 $\pm$ 0.1). However, both the prevalence and the mean abundance of wounds differed significantly
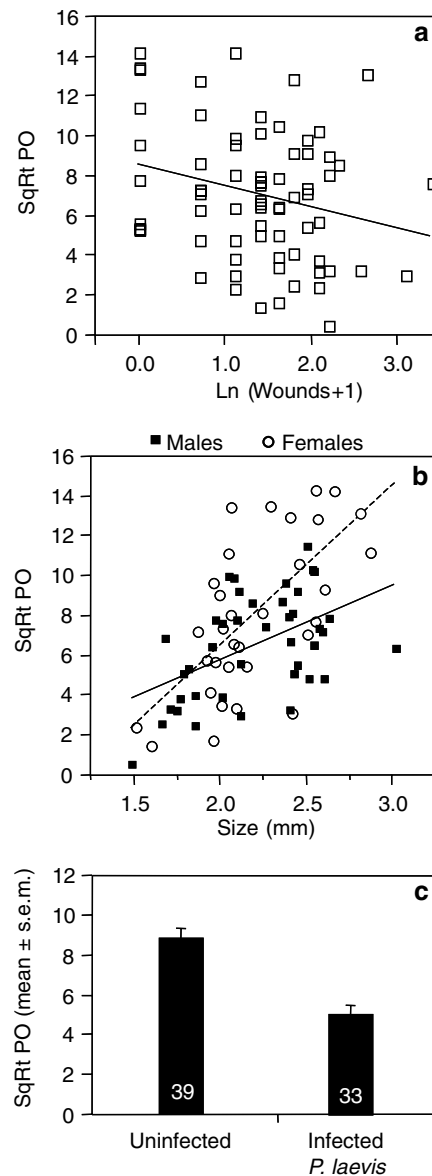
Figure 1 The relationship between PhenolOxidase (PO) activity in *Gammarus pulex* and (a) wound abundance, (b) size and sex, and (c) infection with the acanthocephalan *Pomphorynchus laevis* (sample sizes are given within bars).
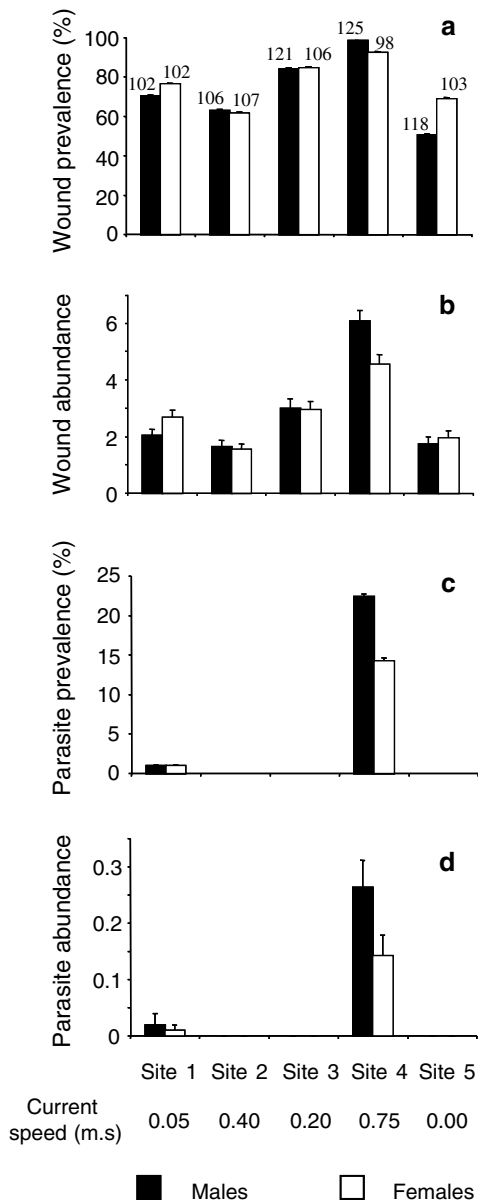
Figure 2 (a) Prevalence ± SE, (b) mean abundance ± SE of wounds, (c) prevalence ± SE and (d) mean abundance ± SE of acanthocephalan parasites in *Gammarus pulex* sampled in five different sites (see text). All numbers above the columns refer to host sample sizes.

between the five populations (Table 2, Fig. 2). Larger gammarids were more likely to be wounded and had a higher abundance of wounds (Table 2). There was a significant interaction between sex and sampling site. At sites with slow currents (sites 1 and 5) females tended to have more wounds whereas at sites with fast currents (sites 2–4) males had more wounds (Fig. 2).

Compared with the risk of being wounded, the prevalence and abundance of acanthocephalan parasites in this study was extremely low, with only 44 of the 1088 gammarids collected being infected with one or more parasites. The prevalence and mean abundance of parasites differed significantly between infected populations (Fig. 2, Table 2). Parasite prevalence did not differ between sexes in these populations, although parasite abundance was significantly higher in males (Fig. 2, Table 2). Our result supports those of a previous, much larger study (see Outreman *et al.* 2002), which showed that acanthocephalans in *G. pulex* are often sporadically distributed and are more likely to be found in males than in females.

DISCUSSION

In invertebrates, wounding is known to induce an immune response that is similar to that used to encapsulate parasites and pathogens (Hoffmann 1995; Nayar & Knight 1995; Nagai *et al.* 2001). As mounting an immune response is costly (Råberg *et al.* 1998; Moret & Schmid-Hempel 2000; Tiën *et al.* 2001), it is expected that wound healing may constrain future resistance to parasites. Here, we show that PO activity in *G. pulex* is negatively correlated with wounding. Our data do not enable us to distinguish whether animals that are wounded have reduced PO activity, or whether animals that have low PO activity are more susceptible to wounding. However, Moreau *et al.* (2000) have shown that experimental wounding of *Drosophila melanogaster* results in a reduction in PO activity, suggesting that the first interpretation of our results is plausible. This might be explained by a higher monopolization of immune defence after wounding, inducing a general decrease in the potential for future defence (see Siva-Jothy *et al.* 2001). In the current study, an alternative, but non-exclusive, explanation could be that wounding can lead to 'shell disease syndrome' in a number of crustaceans, including gammarids (e.g. Dyrynda 1998). This syndrome is supposed to be caused by infection of wounds by a variety of bacteria. Bacterial infection might therefore also influence the level of immune response. However, it is not clear how frequent these bacterial infections are after wounding, and a recent study suggests that these bacterial infections do not influence the level of PO nor antibacterial activity in a crab (Vogan & Rowley 2002). The level of PO activity was also negatively correlated with the infection by an acanthocephalan parasite, which confirms some of our previous results and has been discussed elsewhere. Again, our data do not enable us to distinguish whether animals that are infected with parasites have reduced PO activity, or whether animals that have low PO activity are more susceptible to parasite infection. Experiments which manipulate wounding frequency in the presence or absence of exposure to parasites

Table 2 Generalized linear models showing the effects of sex, size and sampling site on the frequency and the mean abundance of wounds and the infection level by two acanthocephalan parasites in the amphipod *Gammarus pulex*

Source of variation	Variables Wounding						Parasite*					
	Frequency†			Abundance‡			Frequency†			Abundance‡		
	d.f.	F	P-value	d.f.	F	P-value	d.f.	F	P-value	d.f.	F	P-value
Site (1)	4	120.49	<0.001	4	46.29	<0.001	1	37.43	<0.001	1	19.36	<0.001
Host sex (2)	1	5.09	0.021	1	0.65	>0.050	–	–	–	1	3.92	0.048
Host size (3)	1	23.61	<0.001	1	153.63	<0.001	–	–	–	1	0.81	>0.050
Interaction (1) × (2)	4	10.83	0.023	4	2.50	0.020	–	–	–	–	–	–
Interaction (1) × (3)	–	–	–	4	2.92	0.041	–	–	–	1	4.02	0.045
Error	1077			1073			425			422		
Total	1087			1087			426			426		

Only significant variables and interactions in a stepwise model were considered.
*For these models, samples with no parasites were excluded.
†Binomial distribution.
‡Poisson distribution.

will be necessary to separate the specific effect of wounding and parasitic infection.

We have also shown here that in *G. pulex* the risk of being wounded is extremely high in natural populations, and varies significantly between sites along different river courses, and also varies with individual size and, in some cases, between sexes. We do not yet know the cause of wounding in these populations. However, some reasonable hypothesis can be drawn from our results. Larger gammarids (i.e. the oldest as size is correlated with age in most crustaceans) were more likely to be wounded and had a higher abundance of wounds. This may be because larger gammarids also have longer intermoult durations (Sutcliffe & Carrick 1981), and therefore their cuticle will be exposed to the risk of wounding for longer. Individuals that have recently moulted have no melanised wounds (S. Plaistow, pers. obs.). So in all cases, wounding abundance only reflects the 'recent' wounding history of the individual, i.e. within a single moult cycle. The explanation for differences between sexes is less clear, as either males or females were found to be the higher wounded sex, according to population. Higher wounding risk in females could be because of intersexual conflict over copulation (e.g. Crudgington & Siva-Jothy 2000), a reasonable explanation considering that, during pair formation, *G. pulex* females often try to resist to males (see references in Jormalainen 1998), resulting in a fight in which females could be wounded. However, at one of our sample sites, males were more wounded than females, and this corresponded with the river with the higher current speed. In that case, differences in wounding could be due to the fact that male *G. pulex* can be more active than females (e.g. searching for a mate, Lehmann 1967), and could therefore be more exposed to wounding during travel in a high current.

In conclusion, despite being correlative, our study suggests a potential effect of wounding on immune defences. Wounding is the weakest variable influencing PO activity in our statistical model (see Table 1), but it is much more frequent than parasitic infection in natural populations (Fig. 2). A cost of wound healing is therefore likely even in the absence of parasites, and may explain part of the natural variation in immune defence in animals. Our hope is that these results will encourage other studies, to test these ideas in other systems. For example, as wound healing is also a cell-mediated immune response in vertebrates (Martin 1997), we believe that wounding may have similar consequences in this taxa.

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