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Immune depression induced by acanthocephalan parasites in their intermediate crustacean host: Consequences for the risk of super-infection and links with host behavioural manipulation

Stéphane Cornet *, Nathalie Franceschi, Alexandre Bauer, Thierry Rigaud, Yannick Moret

Université de Bourgogne, UMR CNRS 5561 Biogéosciences, Équipe Écologie Évolutive, 6 Boulevard Gabriel, 21000 Dijon, France

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ABSTRACT

Parasite survival in hosts mainly depends on the capacity to circumvent the host immune response. Acanthocephalan infections in gammarids are linked with decreased activity of the prophenoloxidase (ProPO) system, suggesting an active immunosuppression process. Nevertheless, experimental evidence for this hypothesis is lacking: whether these parasites affect several immune pathways is unknown and the consequences of such immune change have not been investigated. In particular, the consequences for other pathogens are not known; neither are the links with other parasite-induced manipulations of the host. Firstly, using experimental infections of *Pomphorhynchus laevis* we confirmed that the lower immune activity in parasitised *Gammarus pulex* is induced by the parasite infection. Second, using natural infections of three different parasites, *P. laevis*, *Pomphorhynchus tereticollis* and *Polymorphus minutus*, we showed that acanthocephalan infection was associated with reduction of the activity of the ProPO system and the haemocyte concentration (two major parameters of crustacean immunity) suggesting that immune depression is a phenomenon affecting several immunological activities. This was confirmed by the fact that acanthocephalan infection (whatever the parasite species) was linked to a lower efficiency to eliminate a bacterial infection. The result suggests a cost of parasite immune depression. Finally, acanthocephalans are also known to induce behavioural alterations in the intermediate host which favour their transmission to definitive hosts. We did not find any correlation between behavioural and immunological alterations in both experimentally and naturally-infected gammarids. Overall, this study suggests that whilst immune depression might be beneficial to acanthocephalan survival within the intermediate gammarid host, it might also be costly if it increases host mortality to additional infections before transmission of the parasite.

1. Introduction

A critical condition for internal parasites to successfully accomplish their life-cycles is to survive within the host, which mainly depends on the parasite capacity to circumvent the host immune response (Loker, 1994). Immune evasion might be achieved either through molecular mimicry, when the parasite prevents its immune detection by mimicking host epitopes (Damian, 1964; Salzet et al., 2000; Zambrano-Villa et al., 2002), or through direct alteration of the host immune system that leads to immunosuppression (Duvaux-Miret et al., 1992; de Jong-Brink, 1995). These mechanisms may involve parasite excretory–secretory products that impair or inhibit both humoral and cellular effectors of the host immune system (Duvaux-Miret et al., 1992; Shelby et al., 2000; Humbert and Coustau, 2001; Labrosse et al., 2003; Guillou et al., 2007). Molecules such as lectins and mucins are of importance in host–parasite interactions and avoidance of host immune processes (Loukas and Maizels, 2000; Theodoropoulos et al., 2001). Nevertheless, immunosuppression may not only be beneficial for the parasite as it could potentially favour subsequent infections by other pathogens (Graham, 2008), which could then challenge the already established immunosuppressing parasite (Hurst et al., 2003; Wedekind and Little, 2004). This is particularly the case for endoparasites with complex life cycles. These parasites often use one or more invertebrate intermediate hosts in which they undergo successive growth events until they become infective to their definitive host, usually a vertebrate (Lafferty, 1999). These parasites need to ensure their own survival through immune evasion in the intermediate host until transmission to a suitable definitive host. However, a reduction of immunocompetence might increase the probability of host death by favouring super-infections by pathogens. Hence, parasites that rely on immune depression mechanisms have to balance the benefits and the costs of their

* Corresponding author. Tel.: +33 380396240; fax: +33 380396231.
E-mail address: stephane.cornet@u-bourgogne.fr (S. Cornet).

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immunosuppressive effect to ensure both their own survival and that of their intermediate host until transmission has occurred.

Rigaud and Moret (2003) found that natural infection by parasites with complex life cycles (the acanthocephalans *Pomphorhynchus laevis* and *Polymorphus minutus*) was associated with a reduced activity of the enzyme phenoloxidase (PO) in their intermediate host, the amphipod crustacean *Gammarus pulex*. This suggests that these parasites are potentially able to depress the activation of the prophenoloxidase (ProPO) system, an important component of the immune system in invertebrates (see below). In rivers of Burgundy (Eastern France), three species of acanthocephalan parasites, *P. laevis*, *Pomphorhynchus tereticollis* (see Perrot-Minnot, 2004), and *P. minutus*, currently infect *G. pulex* as an intermediate host before being transmitted via predation to their definitive host (fish and water birds, respectively Kennedy, 2006). Gammarids ingest parasite eggs released in the faeces of their definitive hosts. After hatching, the parasite larva (acanthor) passes through the gut wall and develops in the haemocoel until reaching a cystacanth stage. During their development, acanthocephalans are therefore exposed to the gammarid immune system. The immune system of crustaceans provides innate immunity, which relies on both cellular (Johansson et al., 2000) and humoral (Bachère et al., 1995) components. Pathogens entering the host haemocoel are usually phagocytosed or encapsulated by haemocytes. These reactions are accompanied by the proteolytic activation of the ProPO system (Cerenius and Söderhäll, 2004) that leads to the activation of the key enzyme, PO, which synthesises melanin and is also used to signal non-self, as well as kill and isolate internal parasites.

The extent to which acanthocephalans affect the gammarid immune system has not been well characterised. Rigaud and Moret (2003) found a correlation between infection and low PO activity, but there is a lack of knowledge of whether the differential immune activity between uninfected and parasitised animals is the consequence of parasite infection or the cause of differential infection (only animals with low PO activity could be infected). Moreover, whether or not the parasites alter several immune pathways and the general consequences of such alterations on host immunity to other pathogens are not known. In addition, there is now clear evidence showing that acanthocephalans are able to affect several phenotypic traits of *G. pulex* such as behaviour (Bethel and Holmes, 1973; Cézilly et al., 2000; Bauer et al., 2005; Lagrue et al., 2007), reproduction (Bollache et al., 2002) and physiology (Bentley and Hurd, 1996; Plaistow et al., 2001), to favour host exploitation and their own transmission. It is still unknown whether these multiple alterations in phenotype are related or independent. Pleiotropy, or a single change inducing a cascade of effects, could explain multiple changes and the exploration of this aspect of parasite manipulation has yet to be explored (Cézilly and Perrot-Minnot, 2005). In particular, the developing field of neuropsychoimmunology proposes that the immune and the nervous systems might be connected (Maier and Watkins, 1999; Adamo, 2002, 2006). There is now clear evidence that both systems share common molecular effectors such as neuromodulators (serotonin, octopamine and dopamine) (Adamo, 2002; Demas, 2004), which could be targeted by manipulative parasites. Indeed, recent work has demonstrated that the changes in behavioural responses of *G. pulex* to light when infected by acanthocephalans (infected animals are attracted by light instead of being repulsed) specifically involve the serotonergic system (Tain et al., 2006), which also affects immunity in other model systems (Baines et al., 1992; Mössner and Lesch, 1998). The confirmation or affirmation of potential links between immune and behavioural alterations by parasites would provide important insights about the mechanisms and therefore the evolution of parasitic manipulation.

This study examined potential immune manipulation by acanthocephalans of the gammarid immune system, by addressing four questions. We first used experimental infections under controlled laboratory conditions (Franceschi et al., 2008) to confirm the immunodepressive effect of this parasite on *G. pulex*. Second, we examined whether acanthocephalans affect several compartments of the immune system, by studying changes in humoral (two activities of the ProPO system) and cellular immunity (haemocyte concentration) in *G. pulex* naturally parasitised with three acanthocephalan species: *P. laevis*, *P. tereticollis* and *P. minutus*. Third, we estimated the potential cost of the observed immune depression, by investigating the probability of infection by bacteria in gammarids according to their status of infection by acanthocephalans. Finally, we investigated relationships between these parasitic immune modifications and behavioural changes associated with the infection in both experimentally and naturally-parasitised gammarids.

2. Materials and methods

2.1. Sampling

Gammarus pulex were collected in the River Ouche at Dijon in October 2006 and 2007 to study, respectively, *P. laevis* and *P. tereticollis* infection and in the River Bèze at Noiron sur Bèze in June 2007 for *P. minutus* infection. Since three different samplings occurred, in all the following analyses we compared acanthocephalan infections with a set of uninfected gammarids sampled at the same time and at the same site. Prevalence of different acanthocephalan species was relatively low (usually between 1% and 2%, e.g. Lagrue et al., 2007), so infected animals were actively sought and consequently the samples did not reflect the natural prevalence of infection. Infected gammarids could easily be identified as the parasite appeared as orange–red dots through the cuticle of the host. Animals were maintained in the laboratory under standard conditions (15 ± 1 °C, light:dark cycle 12:12 h) in aerated tanks filled with de-chlorinated u.v.-treated tap water and fed ad libitum with elm leaves. At the end of the experiments, all individuals were measured (size of the fourth coxal plate) using a stereoscopic microscope (Nikon SMZ-10A) and a video analysis system (VTO 232, Linkam Scientific Instruments). They were then dissected and the parasite species identified following Perrot-Minnot (2004). Behavioural activity, level of immune defences and immunocompetence (resistance to a bacterial challenge) were estimated the day after sampling (see below).

2.2. Laboratory infection experiments

Controlled infections were made following Franceschi et al. (2008). Both gammarid host *G. pulex* and parasite *P. laevis* were collected in the River Ouche. In the laboratory, uninfected gammarids were acclimated to laboratory conditions for 15 days prior to the experiment. Only males were used. Parasites were taken from naturally-parasitised definitive hosts, the chub, *Leuciscus cephalus*. Fish were anaesthetized, killed and dissected. Adult parasites were immediately collected from the intestines. Eggs were obtained from female worms, placed in 400 μL of water and parasite tissues were preserved in ethanol for molecular species identification. Chub may be infected by several acanthocephalan species that could not be reliably identified morphologically, so we used a PCR-based method (see details in Franceschi et al., 2008) to identify *P. laevis*. Egg maturity was evaluated under a microscope (200× magnification) and nine clutches with more than 75% of mature eggs were kept for experimental infection with 972 parasites. Prior to parasite exposure, gammarids were deprived of food for

24 h. Gammarids were placed in pairs in crystallizing dishes and an egg suspension (100 eggs per individual) was deposited on 1 cm^2 of dry elm leaf, on which gammarids were allowed to feed for 48 h. Uninfected leaves were provided to the control group. After the exposure period, gammarids were rinsed and placed in groups of 18 in 0.5 L aquaria. From the fifth week (the time from which infection begins to become apparent, Franceschi et al., 2008), gammarids were inspected under a binocular microscope to detect parasite infection. Parasite larvae can be detected through the host cuticle from the late acanthella stage of their development. Infected gammarids were isolated in 0.2 L dishes and at the same time, some individuals from the control group (uninfected and not exposed to parasites) were also isolated. Parasite development was then followed until it reached the cystacanth stage and measures of phototaxis and haemolymph extraction were made 2 weeks after the parasites reached this stage. At the end of the experiment (130 days), 30 exposed animals that were not infected were also tested. Three categories of gammarids were obtained: 'control' (animals not exposed to parasites eggs), 'uninfected' (animals exposed to parasite eggs but not infected) and 'infected' (exposed to parasite eggs and infected).

2.3. Behavioural measurements

Pomphorhynchus laevis and P. tereticollis alter the phototaxic behaviour of gammarids (Cézilly et al., 2000; Perrot-Minnot, 2004). Reaction to light was quantified as described by Perrot-Minnot (2004). Gammarids were individually placed into a horizontal plastic tube filled with oxygenated water, composed of a dark zone and a light zone of equal sizes, under light intensity of 1300 lux. After an acclimatisation of 5 min, the position of the gammarid was recorded every 30 s for 5 min. A score of 0 was given when the tested animal was found in the opaque half of the tube and a score of 1 was given when the gammarid was found in the transparent side. Thus, at the end of the trial, the final score could range from 0 (strongly photophobic) to 10 (strongly photophilic).

Infection by *P. minutus* in *G. pulex* is associated with a change in the vertical distribution and clinging behaviour of gammarids (Cézilly et al., 2000; Bauer et al., 2005). We used the method of Bauer et al. (2005) to assess these behavioural changes. A single gammarid was introduced to a vertical tube filled with 500 ml of aerated water, where five levels each of 5 cm were defined. After 5 min of acclimatisation, individual positions were recorded every 30 s in a time-interval of 5 min, and a score ranging from 1 (bottom level) to 5 (top level) was given. At the end of each experiment, the sum of the scores ranged from 10 (always in the bottom section) to 50 (always in the top section). After being tested for behaviour, gammarids were kept in vials on ice for haemolymph extraction. Behavioural measurements were assessed in both naturally- and experimentally-infected gammarids (i.e. for which haemolymph was collected for immune assays).

2.4. Haemolymph collection, haemocyte concentration and activities of the ProPO system

Haemolymph extracts were taken as described by Cornet et al. (2007) by wounding gammarids between the seventh and eighth dorsal segment with very fine scissors. Two microlitres of haemolymph (i.e. approximately 75% of the total volume) were collected into a sterile, pre-chilled glass capillary and flushed with 20 μL of cold PBS (8.74 g NaCl, 1.78 g Na_2HPO_4, $2H_2O$, 1 L distilled water, pH 6.5). Ten microlitres were immediately used for the determination of haemocyte concentrations and samples were frozen in liquid nitrogen and stored at −80 °C for immunological assays.

Haemocyte concentration was determined using a counting chamber (Neubauer Improved) as the number of cells counted in 0.1 μL and reported for 1 μL of pure haemolymph. This was done in naturally-infected gammarids only.

For each individual haemolymph extract, the activity of naturally-activated PO enzymes (hereinafter called PO activity) and the activity of the proenzymes (ProPO) in addition to that of the PO (hereinafter called total activity) were measured using a spectrophotometric assay (Cornet et al., 2007). PO activity was quantified without further activation, whilst total activity required the activation of the ProPO into PO with chymotrypsin. After thawing on ice, 5 μL of haemolymph extract were added to a microplate well containing 20 μL of PBS buffer and either 140 μL of distilled water to measure PO activity only or 140 μL of chymotrypsin solution (Sigma C-7762, 0.07 mg mL^{-1} of distilled water) to measure total activity. Then, 20 μL of L-Dopa solution (Sigma D-9628, 4 mg mL^{-1} of distilled water) was added to each well. The reaction was allowed to proceed at 30 °C in a microplate reader (Versamax, Molecular Devices) for 40 min. Readings were taken every 15 s at 490 nm and analysed using the software SOFT-Max®-Pro. 4.0 (Molecular Devices). Enzyme activity was measured as the slope (V_{max} value) of the reaction curve during the linear phase of the reaction and reported to the activity of 1 μL of pure haemolymph. Activities of the ProPO system were assessed in both naturally- and experimentally-infected gammarids.

2.5. Immune challenge and bacterial clearance

Gammarids were exposed to a bacterial pathogen to assess the efficiency of the immune system to eliminate an infection. We used a bacterial strain of *Escherichia coli* (strain CIP 103410, Institute Pasteur, Paris, France) resistant to tetracycline. The day before the experiment, one colony was allowed to grow overnight in 20 mL of broth (10 g bactotryptone, 5 g yeast extract, 10 g NaCl, 1 L of distilled water, pH 7.0) at 37 °C in a shaking incubator. The solution was then centrifuged (4 °C, 400*g*, 30 min) and the bacteria washed twice with PBS buffer. Bacterial concentration was evaluated under a compound microscope using a counting chamber (Neubauer Improved) and set at 8×10^4 bacteria per microlitre.

Crustacean exposure to bacterial infection was made by injection (Gorman and Paskewitz, 2000). Gammarids were briefly immobilized on sticky gum. A small hole was made laterally on the animal's third dorsal segment using a fine sterile needle. Then 0.5 μL of the bacterial solution was injected into the animal's haemocoel using a Hamilton syringe equipped with a fine needle (gauge 33). After injection, gammarids were kept in water at a controlled temperature (15 ± 1 °C) for 8 h prior to haemolymph extraction. For bacterial counting, haemolymph extracts were preferred to whole body extracts because haemolymph contains the first line of immune effectors, and also because whole body homogenisation would release digestive enzymes stored in gastric caeca and potentially attack bacteria in a manner unrelated to an immune response. Each individual provided 2 μL of haemolymph that were collected into a 0.5 mL microcentrifuge tube containing 198 μL of PBS buffer. After homogenisation, 100 μL of the mixture were spread on agar Petri dishes containing 20 μg mL^{-1} tetracycline. Petri dishes were incubated overnight at 37 °C and colonies (colony forming unit, CFU) were counted. The number of colonies is expected to be inversely proportional to the immune defence level. The bacterial clearance efficiency was investigated in naturally-infected gammarids only.

2.6. Statistical analyses

Behavioural data were not normally distributed and were therefore analysed using non-parametric Wilcoxon statistical tests.

Haemocyte concentration and PO activities (PO and total activities in both studies) were analysed using a multivariate analysis of

variance (MANOVA, Pillai's trace) with respect to 'infection status' and 'sex' as fixed factors and 'size' as the covariate. The assumption for parametric tests was ensured by the square-root transformation of haemocyte concentration and the natural-log transformations of PO activities and size. The best statistical model was obtained by starting with models including all higher order interactions between explanatory variables and proceeding with a stepwise simplification of models, removing non-significant highest order interaction terms. The factor 'sex' was not included in the analyses from experimental infection since only males were used.

In experimental infections, we tested for differences in variances of PO and total enzyme activities (untransformed data), to account for potential differences in variation between groups. The Brown-Forsythe and O'Brien tests were used because of their Type I error and power properties (Ramsey and Ramsey, 2007). Because they provided similar results, we only show results from the O'Brien's test, which is more powerful for small and unequal sample sizes (Ramsey and Ramsey, 2007).

Data from the bacterial clearance assays were natural-log transformed and first analysed using univariate analyses of variance (stepwise backward procedure) with 'infection status' and 'sex' as fixed factors and 'size' as the covariate. In all cases, the status of infection explained patterns of resistance to the bacterial challenge; we therefore showed the model including the status of infection only for each parasite species.

Correlations between behavioural and immune traits were investigated using non-parametric Spearman's correlation tests. Otherwise, Pearson's correlation tests were used. Multiple comparisons were corrected using the Bonferroni method.

Data were analysed using JMP v5.0 for Windows (SAS Institute, Cary, USA). All tests were two-tailed with significant differences considered at the level of $P \leqslant 0.05$.

3. Results

3.1. Experimental assessment for immune depression induced by P. laevis

We obtained 3.51% *G. pulex* infected by *P. laevis* (24 infected versus 683 uninfected). Most were infected by one cystacanth of *P. laevis*; only three multiple infections occurred. All *P. laevis*-infected animals were therefore analysed together.

Both PO and total activity were strongly influenced by infection status (Table 1, Fig. 1). 'Control' and 'uninfected' groups exhibited similar levels of PO and total activity, which were significantly higher than those of the 'infected' group (Fig. 1). Host size was also negatively correlated with total activity ($r = -0.34$, $n = 59$, $P = 0.0072$). For PO activity, the variances did not differ between 'control' and 'uninfected' groups (O'Brien test: $F_{1,43} = 1.33$, $P = 0.2546$), nor between control and infected groups ($F_{1,26} = 1.13$, $P = 0.2971$). For the total enzyme activity, the variances did not differ between 'control' and 'uninfected' groups ($F_{1,43} = 0.07$, $P = 0.7906$), but the variance was significantly lower in the 'infected' group than in the 'control' group ($F_{1,26} = 8.60$, $P = 0.0069$).

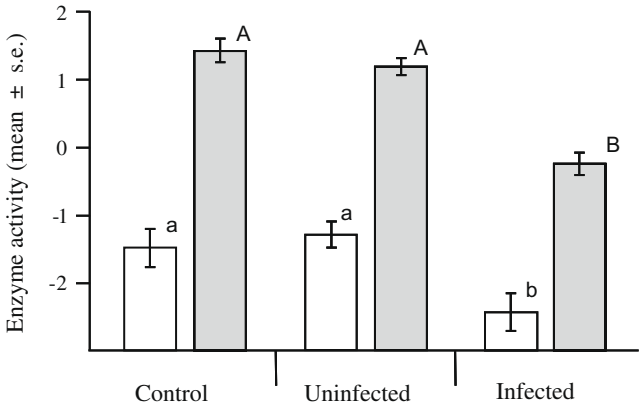

Fig. 1. Activity of the ProPO system (natural-log transformation) of *Gammarus pulex* from the experimental study according to their status of experimental infection by *Pomphorhynchus laevis*: 'control' ($n = 14$), 'uninfected' ($n = 31$), 'infected' ($n = 14$). Open bars: PO activity, dark bars: total activity. Within each group of analyses, different letters above the bars denote significantly different values (Dunnett's pairwise comparison test, control treatment refers to 'control', $\alpha = 0.05$).

3.2. Assessment of immune depression in different immune compartments in three acanthocephalan species

Mixed infections (25%) were recorded for the two *Pomphorhynchus* spp., but none of the immune traits was affected by the intensity of infection; the same situation applied in *P. laevis* and in *P. tereticollis* infections (*t*-tests, all $P > 0.05$, data not shown). We therefore compared effects of the infections between unparasitised and parasitised animals in further analyses.

Lower PO activity (Fig. 2A), total activity (Fig. 2B) and haemocyte concentration (Fig. 2C) were strongly affected by the infection status by *P. laevis*, *P. tereticollis* and *P. minutus* (Table 2). Size was in general not related to immunological parameters, except in gammarids infected with *P. laevis* where PO activity and haemocyte load were negatively correlated to animal size (respectively, $r = -0.36$, $n = 82$, $P = 0.0008$ and $r = -0.27$, $n = 82$, $P = 0.0144$). We also found gender differences in PO activity for the two *Pomphorhynchus* spp. (Table 2), with contrasting patterns according to the experiment: females had more PO activity than males in the

Table 1
Multivariate (Pillai's trace) and univariate analyses of variance investigating variance for phenoloxidase (PO) and total activity measured in experimentally infected *Gammarus pulex* as a function of infection status and size

Models	Source of variation	*num d.f.*[a], *den d.f.*[b]	*F*	*P*
MANOVA	Global model	6, 110	7.52	**<0.0001**[c]
	Infection status	4, 110	9.15	**<0.0001**
	Size	2, 54	2.89	0.0642
ANOVA PO activity	Global model	3, 55	3.79	**0.0152**
	Infection status	2, 55	5.50	**0.0066**
	Size	1, 55	<0.01	0.9780
ANOVA total activity	Global model	3, 55	21.78	**<0.0001**
	Infection status	2, 55	25.46	**<0.0001**
	Size	1, 55	4.85	**0.0317**

[a] Numerator degrees of freedom.
[b] Denominator degrees of freedom.
[c] Significant values shown in bold.

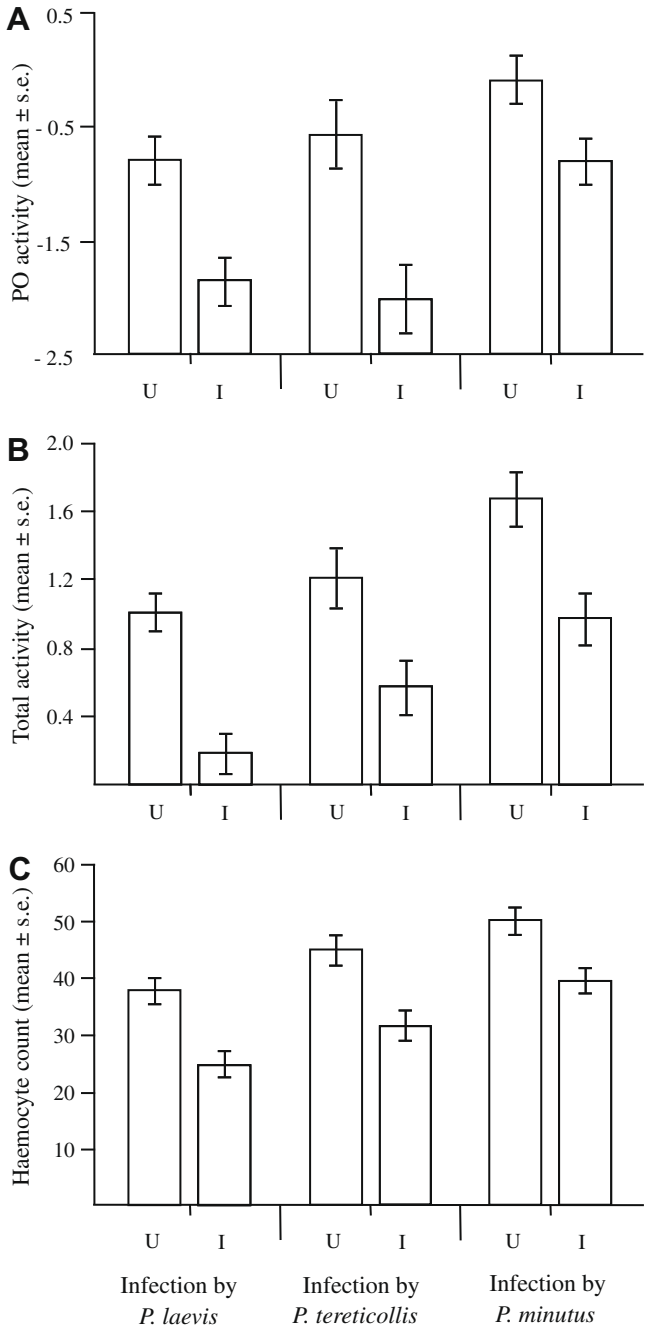

Fig. 2. Comparison of immune effectors according to the status of natural infection (U, uninfected; I, infected) by *Pomphorynchus laevis* (U, $n = 43$; I, $n = 39$), *Pomphorynchus tereticollis* (U, $n = 26$; I, $n = 27$) and *Polymorphus minutus* (U, $n = 35$; I, $n = 41$) for (A) PO, (B) total phenoloxidase activity (natural-log transformation) and (C) haemocyte concentration (square-root transformation).

experiment involving *P. laevis* (females −0.19 ± 0.31, males −1.55 ± 0.15) but less in the experiment involving *P. tereticollis* (females −2.47 ± 0.51, males −1.07 ± 0.23).

3.3. Acanthocephalan infection and bacterial super-infection

Acanthocephalan infection was associated with a reduction of resistance to the experimental bacterial challenge, as shown by the larger number of bacterial colonies in infected animals compared to uninfected ones (Fig. 3). This was observed for infection by *P. laevis* ($F_{1,40} = 9.13$, $P = 0.0044$), *P. tereticollis* ($F_{1,49} = 29.21$, $P < 0.0001$) and *P. minutus* ($F_{1,55} = 30.08$, $P < 0.0001$).

3.4. Assessment of correlations between behavioural changes and immune depression

Across the three parasite species, size was unrelated to scores of behaviour both for uninfected and for infected gammarids (Spearman's rank correlation coefficient, all $P > 0.05$) and sex had no influence (all Wilcoxon tests, all $P > 0.05$). In the two *Pomphorhynchus* spp. parasite load did not influence the level of behavioural modifications (Wilcoxon test: *P. laevis* $Z = 0.98$, $P = 0.322$; *P. tereticollis* $Z = -1.14$, $P = 0.241$). These factors were not taken into account in further analyses.

Pomphorhynchus laevis-infected gammarids were more highly attracted by light than uninfected ones (Fig. 4) ($Z = -3.68$, $P = 0.0002$ in experimental infections; $Z = 6.02$, $P < 0.0001$ in natural infections). This was also true for infections with *P. tereticollis* ($Z = -3.69$; $P = 0.0002$) (Fig. 4). *Polymorphus minutus* infection was associated with a modification of the vertical distribution (Fig. 4). Uninfected *G. pulex* were most frequently located at the bottom level, whereas infected animals were more frequently found in upper levels of the water column ($Z = -3.34$, $P = 0.0008$).

In natural infections, neither variation in scores of reaction to light nor scores of vertical distribution for infected individuals were significantly correlated with variation in immunological parameters, and this absence of relationship was found across the three acanthocephalan species (Table 3). This pattern was also found in laboratory-infected gammarids where phototaxis scores of *P. laevis*-infected animals were unrelated to PO ($r_s = 0.24$, $n = 14$; $P = 0.4024$) or total activity ($r_s = 0.25$, $n = 14$; $P = 0.3721$).

4. Discussion

The experimental infection study revealed that a lower level of immune defence is associated with acanthocephalan infection. If the low level of immunity was the cause of a difference in infection, instead of its consequence, then exposed animals should have separated into two distinct groups compared to the unexposed control group: the uninfected animals with higher levels of defence and the infected ones with lower levels of defence. The control group should also have exhibited greater variation in defence levels than the two exposed groups (initial variation should be reduced in the two exposed group because they should contain either resistant or sensitive individuals). Our data showed that the basal level of immunity showed no difference in the activity of the ProPO system between 'controls' and 'uninfected' gammarids (exposed to eggs and uninfected), whilst that of 'infected' gammarids was reduced. In addition, the pattern of variation was no greater in the 'control' group compared to exposed groups. This suggests that the success of infection did not result from differential levels of immunocompetence, but rather that the cause of differences in immune levels was due to parasite infection. The only difference in variances was found between the 'control' and 'infected' groups for the total enzyme activity, suggesting that parasites homogeneously reduce this activity.

Studies of natural infections revealed that the level of immune defence of *G. pulex* is strongly affected by the three acanthocephalans *P. laevis*, *P. tereticollis* and *P. minutus*. In all cases, we found that the activity of the ProPO system and the concentration of haemocytes were lower in parasitised gammarids. Even if the experimental study had only been conducted using *P. laevis*, we could confidently conclude that patterns of immune alteration were relatively similar for the three parasite species. These results therefore confirm and extend those of Rigaud and Moret (2003), obtained on other gammarid populations. *Pomphorhynchus* and *Polymorphus* belong to two different groups in the Palaeacanthocephala taxon (García-Varela et al., 2000) Therefore, the fact that

Table 2
Multivariate (Pillai's trace) and univariate analyses of variance investigating variance for phenoloxidase (PO) activity, total activity and haemocyte concentration of *Gammarus pulex* infected with *Pomphorhynchus laevis*, *Pomphorhynchus tereticollis* and *Polymorphus minutus* as a function of infection status, size and sex

Parasite species	Immune parameter	Source of variation	*num d.f.*[a], *den d.f.*[b]	*F*	*P*
MANOVAs					
P. laevis		Global model	9,234	6.39	**<0.0001**[c]
		Infection status	3,76	12.40	**<0.0001**
		Size	3,76	5.39	**0.0020**
		Sex	3,76	3.01	**0.0352**
P. tereticollis		Global model	9,147	2.43	**0.0140**
		Infection status	3,47	6.32	**0.0011**
		Size	3,47	0.36	0.7759
		Sex	3,47	1.34	0.2703
P. minutus		Global model	9,216	2.15	**0.0263**
		Infection status	3,70	4.90	**0.0038**
		Size	3,70	1.10	0.3521
		Sex	3,70	1.56	0.2057
ANOVAs					
P. laevis	PO activity	Global model	3,78	12.91	**<0.0001**
		Infection status	1,78	14.50	**0.0003**
		Size	1,78	8.87	**0.0039**
		Sex	1,78	6.70	**0.0115**
	Total activity	Global model	3,78	10.22	**0.0001**
		Infection status	1,78	27.09	**<0.0001**
		Size	1,78	0.36	0.5458
		Sex	1,78	0.71	0.4014
	Haemocytes	Global model	3,78	9.10	**<0.0001**
		Infection status	1,78	16.45	**0.0001**
		Size	1,78	4.96	**0.0288**
		Sex	1,78	1.87	0.1746
P. tereticollis	PO activity	Global model	3,49	5.43	**0.0026**
		Infection status	1,49	9.81	**0.0029**
		Size	1,49	0.16	0.6895
		Sex	1,49	4.21	**0.0455**
	Total activity	Global model	3,49	2.89	**0.0443**
		Infection status	1,49	4.99	**0.0300**
		Size	1,49	0.11	0.7361
		Sex	1,49	1.55	0.2190
	Haemocytes	Global model	3,49	4.33	**0.0087**
		Infection status	1,49	12.29	**0.0010**
		Size	1,49	0.26	0.6121
		Sex	1,49	0.05	0.8182
P. minutus	PO activity	Global model	3,72	3.32	**0.0244**
		Infection status	1,72	6.00	**0.0167**
		Size	1,72	1.58	0.2126
		Sex	1,72	2.60	0.1108
	Total activity	Global model	3,72	5.57	**0.0017**
		Infection status	1,72	9.89	**0.0024**
		Size	1,72	3.15	0.0798
		Sex	1,72	3.63	0.0607
	Haemocytes	Global model	3,72	4.80	**0.0041**
		Infection status	1,72	12.14	**0.0008**
		Size	1,72	0.22	0.6385
		Sex	1,72	3.17	0.0788

[a] Numerator degrees of freedom.
[b] Denominator degrees of freedom.
[c] Significant values shown in bold.

both the activity of the ProPO system and the haemocyte concentration were negatively affected in the same way by the three different species suggests a common ability of Palaeacanthocephala to alter the immune physiology of their intermediate hosts.

This ability is likely to be the result of an adaptation of parasites to *G. pulex*, rather than simply a side effect of a parasitic infection in gammarids, for the following reasons. First, not all parasites infecting *G. pulex* alter immune physiology of their host. In a recent work studying the infection of *G. pulex* with the cestode, *Cyathocephalus truncatus*, Franceschi et al. (2007) did not find any effect of the parasite on activity of the ProPO system, which suggests that the effects on the immune system of a same host is likely to be parasite-specific. Second, no modification in the level of defence was observed in *Gammarus roeseli* which lives in sympatry with *G. pulex*, and is commonly infected by the same acanthocephalan species (Rigaud and Moret, 2003; Moret et al., 2007). This suggests that alteration of immune physiology is also host-specific. Finally, it is worth noting that infections by different acanthocephalan species do not induce the same changes in all the physiological attributes of *G. pulex*. For example, carotenoid uptake from the host differs markedly between *Polymorphus* and *Pomphorhynchus* (Gaillard et al., 2004).

The mechanisms by which these acanthocephalans depress both the cellular and humoral immune defences of *G. pulex* are not known. Since enzymes are mainly synthesised and stored in immune cells, the reduction and impairment of haemocytes may

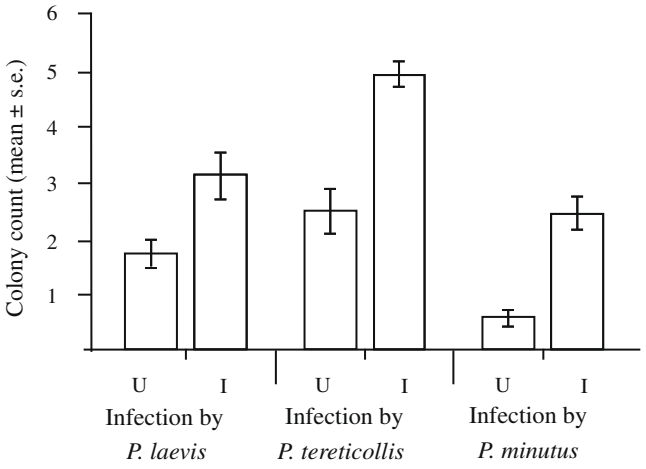

Fig. 3. Comparison of live bacteria (number of colony forming units, natural-log transformation) after infection by *Escherichia coli* according to the status of natural infection by *Pomphorynchus laevis* (U, $n = 22$; I, $n = 20$), *Pomphorynchus tereticollis* (U, $n = 25$; I, $n = 36$) and *Polymorphus minutus* (U, $n = 28$; I, $n = 29$).

have led to the reduction of protein titres and enzymes involved in the melanization response (Shelby et al., 2000). Parasites and parasitoids could limit the action of their host cellular immune responses through excretory–secretory products affecting the number of haemocytes and their function (Webb and Luckhart, 1996; Richards and Edwards, 2000; Humbert and Coustau, 2001; Labrosse et al., 2003). These excretory–secretory products could also interfere and block host plasma melanization by inhibiting the ProPO activating cascade (Shelby et al., 2000; Asgari et al., 2003; Gomes et al., 2003). In our acanthocephalan-gammarid systems, although traces of melanization are sometimes found on the cystacanths (personal observations), they are never encapsulated. Acanthocephalan parasites are often surrounded by a capsule which acts as a protective barrier. In associations between *Echinogammarus stammeri* with *P. laevis* and *P. minutus*, haemocytes in the vicinity of the cystacanths are rare and in many instances partially or completely disintegrated (Dezfuli et al., 1992; Dezfuli and Giari, 1999). Moreover, and consistent with the observation that the melanization pathway is impaired during the process of infection, it has been shown in the cockroach *Periplaneta americana* that the cystacanth of the acanthocephalan *Moniliformis moniliformis* is surrounded by a capsule made of agranular haemocytes (Ravindranath and Anantaraman, 1977). In that case, the degranulation of haemocytes after encapsulation occurred but any trace of melanization observed was probably due to inhibition of enzymes of the ProPO pathway by the acidic mucopolysaccharides of the parasite integument (Ravindranath and Anantaraman, 1977). Cytological observations of the host–parasite interface and inspection of excretory–secretory products that may be released by acanthocephalans would help to understand the physiological changes induced by parasites in *G. pulex*.

The benefit of immune depression is obvious as it may improve parasite survival within the intermediate host body cavity. However, the reduced immunity of *G. pulex* to a bacterial infection when infected with any of the three acanthocephalan parasites suggests that immune depression might also be costly. Immune depression may favour successful subsequent infection by other pathogens (Cox, 2001; Graham, 2008), which could then compete with the already established immunosuppressing parasite. Furthermore, by altering immune activities, acanthocephalans may enhance the probability of their intermediate host dying from a secondary infection before transmission to their definitive host. It is therefore likely that acanthocephalan parasites may have to optimise both their own survival and that of their intermediate host until transmission occurs to the definitive host.

Finally, our study confirmed previous observations on the ability of each acanthocephalan species to alter the behaviour of

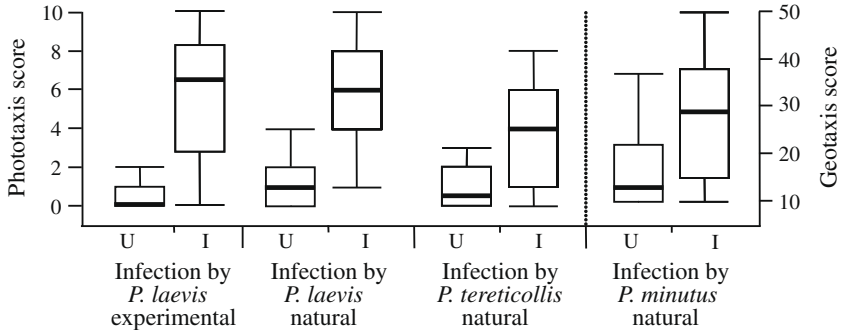

Fig. 4. Behavioural scores of *Gammarus pulex* according to their infection status by *Pomphorynchus laevis* (experimental infections: U, $n = 14$; I, $n = 14$; natural infections: U, $n = 43$; I, $n = 39$), *Pomphorynchus tereticollis* (U, $n = 26$; I, $n = 27$) and *Polymorphus minutus* (U, $n = 35$; I, $n = 41$). Lines are medians, boxes are interquartile range and bars are interdecile range.

Table 3
Non-parametric correlations (Spearman's r_s) between immune parameters (phenoloxidase (PO) and total activity, haemocyte concentration) and phototaxis for *Gammarus pulex* infected by *Pomphorhynchus laevis* ($n = 44$) and by *Pomphorhynchus tereticollis* ($n = 27$) and geotaxis for gammarids infected by *Polymorphus minutus* ($n = 41$)

	Phototaxis				Geotaxis	
	P. laevis		*P. tereticollis*		*P. minutus*	
	r_s	*P*	r_s	*P*	r_s	*P*
PO activity	−0.039	0.8132	−0.296	0.1331	0.053	0.7377
Total activity	−0.258	0.1128	−0.198	0.3201	0.058	0.7144
Haemocyte concentration	−0.320	0.0471	0.136	0.4975	0.062	0.6973

None of these correlations was significant after a correction of Bonferroni.

G. pulex (Bethel and Holmes, 1973; Cézilly et al., 2000; Bauer et al., 2005). Gammarids naturally infected with each *Pomphorhynchus* species were found to be more photophilic and those naturally infected with *P. minutus* were found more often in the upper level of the water column. Behavioural changes observed in naturally-infected gammarids with *P. laevis* were also observed in experimentally infected gammarids with the same parasite, confirming the results of Franceschi et al. (2008), but this time using a sympatric combination of hosts and parasites.

The three species of acanthocephalan parasites studied here are therefore able to manipulate both the immune system and the behaviour of their intermediate host, *G. pulex*, and these two traits appear to be ancestral to the group (see Section 4 above and Moore, 2002). This multidimensional manipulation is therefore interesting to investigate as it may provide insights into the mechanisms and therefore the evolution of parasitic manipulation (Cézilly and Perrot-Minnot, 2005). We did not find any relationship in parasitised gammarids between behavioural scores and immune parameters either in naturally or in experimentally infected animals. These results therefore suggest that the processes involved in each case are likely to be independent. Alternatively, the basal cues could be the same (e.g. due to pleiotropy), but the ultimate phenotypic expression could depend on different physiological processes. The absence of correlation between these traits illustrates the complexity of the parasitic manipulations. Here the link between behavioural and immunological alterations was investigated at the phenotypic level. However, the importance of neuromodulators in both systems should not be neglected (Maier and Watkins, 1999; Adamo, 2002, 2006) and offers perspectives for future research.

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