Differential phenoloxidase activity between native and invasive gammarids infected by local acanthocephalans: differential immunosuppression?

T. RIGAUD[1]* *and* Y. MORET[2]

[1] *Equipe Ecologie-Evolutive, UMR CNRS 5561 Biogéosciences, Université de Bourgogne, 6 Boulevard Gabriel, 21000 Dijon, France*
[2] *Department of Animal and Plant Sciences, University of Sheffield, Sheffield S10 2TN, UK*

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SUMMARY

Manipulative endoparasites can alter the behaviour and the physiology of their intermediate hosts in ways that increase the probability of successful transmission to the final host. This requires that the parasite is able to circumvent its host's immune defence. Successful immune evasion may depend on host–parasite coevolutionary history and the appearance of new hosts invading the local host population may promote local parasite maladaptation. To test this hypothesis, we examined the effect of 2 acanthocephalan parasites, *Pomphorhynchus laevis* and *Polymorphus minutus*, on the immunity of their local and new invasive gammarid intermediate hosts, respectively *Gammarus pulex* and *Gammarus roeseli*. We found that infection by each parasite was correlated with a decrease, at different degrees, of the standing level of immune defence in local hosts – measured as the phenoloxidase (PO) enzyme activity – whereas invasive hosts infected by *P. laevis* had their PO-enzyme activity enhanced. These results suggest that these acanthocephalans evade their local host immune response through immunosuppression but cannot evade the immune response of their new invasive host. The potential role of this maladaptation on the success of invasive species is discussed.

Key words: acanthocephalans, amphipods, local adaptation, coevolution, immunocompetence, phenoloxidase.

INTRODUCTION

Endoparasite fitness crucially depends on survival within the host body cavity, which is mainly based on the parasite capacity to evade the host immune defence (Loker, 1994; Damian, 1997). Such an interaction with the host immune system is assumed to result from long co-evolutionary processes between the host and the parasite (Zambra-Villa *et al*. 2002). Consequently, infection of novel hosts might be compromised, as the parasite may not manage to evade successfully the new host immune system. This may have important implications for parasite maintenance when successful competitors invade native host populations.

Biological invasions provide an interesting context to study such host–parasite relationships. Differential migration rates between parasites and their hosts can cause local parasite maladaptation. For instance, a high host gene flow compared to that of the parasite may prevent local adaptation of the parasite (Gandon *et al*. 1996). Recent empirical data are in favour of differential migration rate between introduced species and their parasites (Torchin *et al*. 2003). In addition, Torchin, Lafferty & Kuris (2002) showed that invasive species (in a marine environment) are less affected by local parasites compared to populations in their native range. More specifically, Dunn & Dick (1998), studying parasitism in native and invasive species of gammarids (Crustacea, Amphipoda), found that native species are more often infected by local parasites than invaders, suggesting that parasites may not be adapted to the new hosts.

Gammarus pulex and *Gammarus roeseli* are two gammarid species that occur in sympatry in rivers of Burgundy (eastern France). *Pomphorhynchus laevis* and *Polymorphus minutus* are two acanthocephalan parasites that commonly use *G. pulex* as an intermediate host before being transmitted via predation to their definitive host, respectively fish and water birds (Crompton & Nickol, 1985). These parasites also infect *G. roeseli* in Burgundy (Bauer *et al*. 2000). The gammarids are orally infected when ingesting parasite eggs released in the faeces of the definitive host. At its infective stage (acanthor), the parasite passes through the gut wall of the crustacean host to occupy its haemocoel. There, the parasite changes into acanthella stage, and finally cystacanth stage that alters the behaviour of the intermediate host to favour predation by the definitive host (Bakker, Mazzi & Zala, 1997; Bethel & Holmes, 1973; Cézilly, Grégoire & Bertin, 2000). Prior to this,

* Corresponding author: Equipe Ecologie-Evolutive, UMR CNRS 5561 Biogéosciences, Université de Bourgogne, 6 Boulevard Gabriel, 21000 Dijon, France. Tel: +33 380 39 39 45. Fax: +33 380 39 62 31. E-mail: thierry.rigaud@u-bourgogne.fr

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however, the cystacanth will face the host immune response within the host haemocoel. While *G. pulex* is a native species in France, *G. roeseli* is a recent colonizer (around 80 years) of Central European origin (Karaman & Pinkster, 1977; Jazdzewski, 1980; Roux, Roux & Opdam, 1980), and shares the same local parasite community as *G. pulex*. This increases the probability for local acanthocephalans to end up in this new host. However, unlike the observation in *G. pulex*, *P. laevis* does not alter the behaviour of *G. roeseli*, possibly because they are maladapted to this new host (Bauer *et al.* 2000). Survival of local acanthocephalans within *G. roeseli* will also depend on their capacity to evade the immune response of this new host with which they share a short co-evolutionary period. It is therefore predicted that parasite maladaptation to invasive hosts, in terms of immune evasion, should provide a strong advantage to invaders over native competitors.

Here, we compared, using animals collected in the wild, one of the most important components of the immune system of *G. pulex* and *G. roeseli* facing acanthocephalan parasites. Immunity of crustaceans, like other invertebrates, is innate and parasite infections activate multiple systemic responses, including phagocytosis and encapsulation by haemocytes (Ratcliffe *et al.* 1985; Hoffmann, Reichhart & Hetru, 1996; Gillespie, Kanost & Trenczeck, 1997), and accompanying melanization reactions (Söderhäll, Cerenius & Johansson, 1996; Söderhäll & Cerenius, 1998). These latter are based on the prophenoloxidase (proPO) cascade, which is a common and generalized response to invasion by a parasite (Söderhäll & Cerenius, 1998). The operation of the cascade is indicated by the phenoloxidase (PO)-enzyme activity in the haemolymph and can be monitored by measuring the rate of conversion of a phenol substrate into quinone, which then polymerizes to form melanin. Endoparasites evading the PO response through molecular mimicry are not expected to affect the standing level of PO-enzyme activity of the host haemolymph. In contrast, the PO-enzyme activity should be down-regulated when endoparasites immunosuppress the host. We therefore measured the PO-enzyme activity in *G. pulex* and *G. roeseli*, using a correlative approach, in relationship with the infection by *P. laevis* and *P. minutus* parasites.

MATERIALS AND METHODS

Gammarid sample and measures

Gammarids used in this study were collected in July 2002 in the river Tille at Les Maillys (Burgundy, eastern France). At this site, *G. pulex* and *G. roeseli* live in sympatry and both are presumably exposed to the local acanthocephalan parasites *P. laevis* and *P. minutus*. Gammarids were sampled using the kick-sampling method described by Hynes (1954). Two samples were collected for the PO-enzyme activity test: in the first one, around 50 animals from each species were collected randomly. Then, owing to the low parasite prevalence found in these samples, individuals infected by acanthocephalan parasites were actively sought, until around 20 infected hosts were found for each species. This sample will therefore not reflect the actual parasite prevalence. Parasitized individuals can be distinguished from non-parasitized ones because acanthocephalans can be seen through the transparent crustacean cuticle as bright orange or yellow dots. The animals were then transferred according to gammarid species into pots filled with water from the river and provided with oxygen. The pots were kept at low temperature on ice packs during the travel to the laboratory. In the laboratory all animals were kept in aquaria under standard conditions (15 °C) fed with dead leaves before being used for the trial. Measurements described below were made within the 24 h following sampling.

For the study, each individual was isolated in a 1·5 ml Eppendorf tube 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 and a video-analysis system VTO 232 from Linkam scientific instruments (Bollache, Gambade & Cézilly, 2000). A haemolymph sample was collected for each individual to measure PO-enzyme activity (see below). Animals were then dissected in Ringer's solution under a stereoscopic microscope to count and identify the acanthocephalan species in their body cavity. To control for a potential interaction between host reproduction and parasite effect, we recorded whether females were gravid (with eggs in their ventral incubating pouch or marsupium). This control was needed, because Plaistow, Troussard & Cézilly (2001) showed that *P. laevis* was able to depress lipid reserves only in reproductive females (i.e. when energetic demand is stronger). Therefore, a decrease in PO activity in reproductive infected females could indicate a general decrease in body condition, and not a direct parasite effect.

Haemolymph collection and PO activity

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). For this, the telson of the gammarids was removed with dissecting scissors to create a hole out of which haemolymph was collected. Gammarids were injected with sodium cacodylate buffer through the second tergite behind the head, using a 1 ml disposable syringe (Clinipak U-40 Insulin, Pharma-Plast) and the perfused liquid (buffer plus haemolymph) was collected through the posterior hole into 1·5 ml Eppendorf tubes. Samples

Table 1. Analysis of covariance following a stepwise regression (backward elimination procedure) for *Gammarus pulex* PO values (square-root transformed) as a function of infection by *Pomphorhynchus laevis* and *P. minutus*, host sex and host size (covariate)

Source of variation	Sum of squares	D.F.	*F*	*P*
Size	86·192	1	17·947	<0·001
Sex	20·225	1	4·211	0·044
Infection 1 {Uninfected *vs* Infected}	151·035	1	31·448	<0·001
Infection 2 {*P. laevis vs P. minutus*}	20·582	1	4·285	0·042
Size*Sex	28·324	1	5·897	0·018
Error	321·802	67		

Global Model: $F_{5,72}=19{\cdot}141$; $P<0{\cdot}001$.

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 microtitre plate wells containing 140 μl of cold distilled water, 20 μl of cold saline phosphate buffer (PBS: 8·74 g NaCl; 1·78 g $Na_2HPO_4.2H_2O$; 1000 ml distilled water; pH 6·5). Then 20 μl of cold L-dopa solution (4 mg per ml of distilled water) were added into each well and the reaction allowed to proceed for 40 min at 30 °C in a microtitre plate reader (Versamax, Molecular Devices). Readings were taken at 490 nm and analysed using SOFTmax®PRO 4.0 software (Molecular Devices). Enzyme activity was measured as the slope (Vmax value) of the reaction curve during the linear phase of the reaction (Barnes & Siva-Jothy, 2000) within a time-scale between 5 and 30 min after the reaction mix was made.

Statistics

As, in our sample, *G. pulex* was the only host harbouring the two acanthocephalan parasites, *P. laevis* and *P. minutus*, we tested the effect of these two parasites on the PO activity using an analysis of covariance (ANCOVA) model including parasite infection, sex of the host as categorical factors, host size as covariate, and the second order interactions between these factors. We used a backward stepwise procedure to remove non-significant factors or interactions ($P>0{\cdot}05$).

As *P. laevis* was the only acanthocephalan found in both gammarid species, *G. pulex* and *G. roeseli*, we first tested the effect of the infection by the parasite on the level of PO activity (Vmax values) of the two host species together. Then the effect of *P. laevis* was analysed in each host species separately. In all analyses, we used an ANCOVA including a factor 'species' in addition to the other factors and covariates used in the above tests. Similarly to the above tests, we used a backward-stepwise procedure to remove non-significant interactions. To analyse the effect of reproductive status in females (gravid *vs* non-gravid), the same type of analysis was made, excluding males and adding a reproductive status as categorical factor.

Statistical analyses were done on transformed dependent variables to meet the assumptions of normality. Vmax values were natural-log transformed for analyses involving both gammarid species together or *G. roeseli* alone and square root transformed for analyses involving *G. pulex* alone. All the statistical analyses were done using JMP 3.2 software (SAS Institute, 1997).

RESULTS

We used data from 73 *G. pulex* (32 males and 41 females) and 59 *G. roeseli* (31 males and 28 females). While the resident intermediate host *G. pulex* was infected by both species of acanthocephalans (19 infected by *P. laevis* and 15 infected by *P. minutus*), *G. roeseli* was infected by *P. laevis* only (20 individuals).

Effect of P. laevis *and* P. minutus *on the PO-enzyme activity of* G. pulex

The size of gammarids within each host species had a large effect on the level of PO activity measured (Table 1). This could be due to the method used to collect haemolymph: large gammarids provided more haemolymph and consequently had higher level of PO activity compared to small individuals. Low PO-enzyme activities were associated with acanthocephalan infection (Fig. 1, Table 1), but the enzyme activity was lower in *G. pulex* infected by *P. laevis* than in *G. pulex* infected by *P. minutus* (Fig. 1, Table 1). However, the variance in PO activity did not differ between uninfected and acanthocephalan-infected individuals (variances on square-root transformed data were 7·32 ($n=39$) and 4·19 ($n=34$), respectively; O'Brien's ANOVA testing the equality of variances: $F_{1,71}=2{\cdot}32$, $P=0{\cdot}13$). The gammarid PO-enzyme activity was higher in females than in males. However, the slopes of the regression between enzyme activity and gammarid size differed significantly between sexes (Table 1),

Table 2. Analysis of covariance following a stepwise regression (backward elimination procedure) for PO values as a function of infection by *Pomphorhynchus laevis*, host species, host sex and host size (covariate)

((A) Model including *Gammarus pulex* and *G. roeseli* (PO values natural-log transformed). (B) Model with *G. pulex* only (PO values square-root transformed). (C) Model with *G. roeseli* only (PO values natural-log transformed).)

Source of variation	Sum of squares	D.F.	*F*	*P*
A				
Size	11·107	1	24·580	<0·001
Host species	16·885	1	37·367	<0·001
Infection	7·607	1	16·835	<0·001
Host species∗Infection	5·464	1	12·092	<0·001
Infection∗Size	5·676	1	12·562	<0·001
Error	50·157	111		
Global Model: $F_{7,116}=32{\cdot}451$; $P<0{\cdot}001$.				
B				
Size	82·356	1	18·572	<0·001
Infection	143·778	1	32·423	<0·001
Sex	17·570	1	3·962	0·052
Sex∗Size	26·171	1	5·902	0·019
Error	235·025	53		
Global Model: $F_{4,57}=25{\cdot}497$; $P<0{\cdot}001$.				
C				
Size	3·649	1	8·005	0·007
Infection	3·069	1	6·732	0·012
Sex	0·157	1	0·344	0·560
Infection∗Sex	2·426	1	5·322	0·025
Infection∗Size	3·342	1	7·331	0·009
Error	24·163	53		
Global Model: $F_{5,58}=3{\cdot}182$; $P=0{\cdot}014$.				

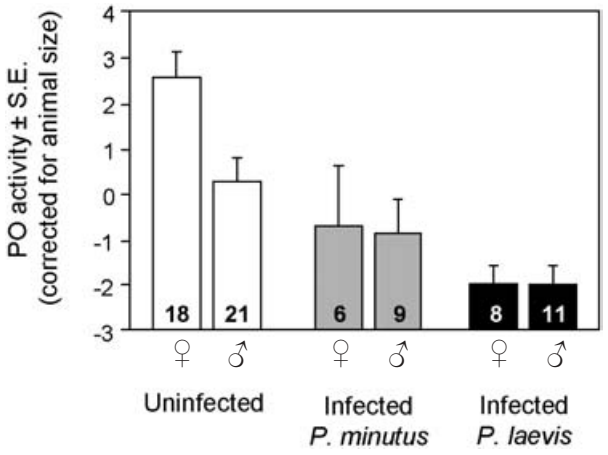

Fig. 1. Values of PO activity (Vmax values in units per minute) corrected for size in *Gammarus pulex*, according to sex and infection status by *Pomphorhynchus laevis* and *P. minutus*. Correction for size was made by using the residuals of the regression square-root of size against PO activity. The numbers within the columns are sample size. Note that this representation using residuals is just for an easy reading, avoiding the confounding size data. For the statistical analysis (Table 1), size was used as covariate in an ANCOVA analysis.

with large females having higher PO-enzyme activity than large males (results not shown).

Effect of the infection by P. laevis *on the PO-enzyme activity of* G. pulex *and* G. roeseli

Size again had a large effect on the level of PO activity (Table 2), for the same reason mentioned above. The 2 gammarid species had different levels of PO activity, with *G. roeseli* having around 2/3 the enzyme activity of *G. pulex* in uninfected individuals (Table 2A; Fig. 2). This, despite that *G. roeseli* was larger on average than *G. pulex* (Wilcoxon, $Z=-2{\cdot}07$; $P=0{\cdot}039$). Infection by *P. laevis* influenced the level of PO activity in both host species, but in different ways (Fig. 2) (The factor 'infection' and the interaction 'species∗infection' were both significant, see Table 2A). While the infection was associated with a low PO activity in the local host *G. pulex* (Fig. 2, Table 2B), it was associated with a high enzyme activity in the invading host *G. roeseli* (Table 2C, PO activity (untransformed data)= $18{\cdot}99\pm2{\cdot}26$ and $32{\cdot}58\pm9{\cdot}26$ for uninfected and infected hosts, respectively). In females, reproductive status had no significant effect on the level of PO activity: the stepwise procedure never included this factor in the analysis, neither in interaction with the infection status or alone (the significant factors retained were the same as those described in Table 2A, the factor 'sex' being removed). Reproductive females therefore had similar levels of PO activity compared with non-reproductive ones.

While males and females of *G. roeseli* had a similar general level of PO activity, they responded differently to the infection by *P. laevis* (Table 2C, Fig. 2). Infection induced no significant effect in males (one-way ANOVA: $F_{1,26}=0{\cdot}21$, $P>0{\cdot}60$) while it is associated with an increase in females ($F_{1,29}=3{\cdot}98$, $P=0{\cdot}05$). There was also a significant interaction between infection by *P. laevis* and the size of *G. roeseli* for the level of the PO activity (Table 2C). In fact, the positive relationship between size and level of PO activity was stronger in infected than in uninfected individuals (results not shown). In *G. pulex*, as seen earlier, the relationship between PO activity and size co-varied differently according to gender of the crustacean (Table 2B).

DISCUSSION

The main result from our study is that the level of PO-enzyme activity was lower in individuals of the local host *G. pulex* infected by the acanthocephalan parasites *P. laevis* and *P. minutus*, while the infection of the invading host *G. roeseli* by *P. laevis* was associated with a higher PO-enzyme activity. Independently of parasite infection, we also observed that the local gammarid species had a higher general level of PO-enzyme activity than the invasive one. However,

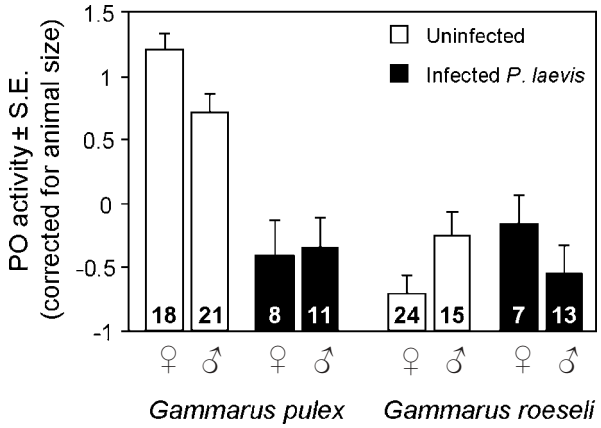

Fig. 2. Values of PO activity (Vmax values in units per minute) corrected for size in *Gammarus pulex* and *G. roeseli*, according to sex and infection status by *Pomphorhynchus laevis*. Correction for size was made by using the residuals of the regression natural-log of size against PO activity. The numbers within the columns are sample size. Note that this representation using residuals is just for an easy reading, avoiding the confounding size data. For the statistical analysis (Table 2), size was used as covariate in an ANCOVA analysis.

this latter result can hardly be interpreted as it may result from fundamental differences in the biology of the two species.

Considering *G. pulex* alone, the PO-enzyme activity of infected and uninfected gammarids by the 2 acanthocephalan parasites could suggest that the parasites successfully infected hosts with poor immune defence while immunocompetent hosts may have successfully cleared the infection. But several lines of results are not in accordance with this hypothesis. First, individuals infected by *P. laevis* and *P. minutus* showed different levels of PO-enzyme activity. Second, the variance was not different between infected and uninfected *G. pulex*, while a strongly-reduced variance would be expected in infected individuals in the case of infection of immunodeprived hosts by parasites. Finally, the PO-enzyme activity has been found to be enhanced in parasitized *G. roeseli*. We therefore believe that the 2 species of parasites immunosuppress to different degrees their local host *G. pulex*, to evade its immune response. Such a mechanism was recently shown to explain the low levels of immune response in mosquitoes infected by malaria parasites (Boëte, Paul & Koella, 2002), and is also known in several other parasite–invertebrate systems (Christensen & LaFond, 1986; Strand & Pech, 1995). One possible explanation for the decrease of PO activity could be that parasites induce a general decrease in individual quality, including immune function. Our results in females do not support this hypothesis: previous results showed that lipid reserves are decreased in reproductive females infected by *P. laevis* relative to non-reproductive ones (Plaistow *et al.* 2001), but we failed to find evidence of any effect of reproductive status on the PO activity. It is therefore unlikely that the decrease in immunocompetence is directly due to a general decrease in body condition.

It is likely that the acanthocephalan *P. laevis* did not manage to efficiently interact with the immune system of *G. roeseli*, which mounted an immune response through the increase of PO-enzyme activity. *P. laevis* parasites could be maladapted to immunosuppress the new invader host, *G. roeseli*. This suggestion would be in the line with the inability of *P. laevis* to manipulate *G. roeseli*'s behaviour (Bauer *et al.* 2000), and also in line with the observations of Hynes & Nicholas (1958), who described that acanthocephalans infecting a 'wrong' amphipod host species are more frequently melanized and have an impeded development. Such a maladaptation of the local parasites to new hosts may give an advantage to invasive species when colonizing new areas and could have helped *G. roeseli* to successfully colonize Burgundy's rivers.

Another important result revealed by this study is that sex of the host had an important effect on the standing level of PO-enzyme activity. Males of *G. pulex* tend to have lower PO-enzyme activity than females and this difference is increased when the gammarids become bigger. Interestingly, *P. laevis* prevalence and abundance was found to be higher in males than in females in *G. pulex* (Outreman *et al.* 2002), and our observation may explain this difference in parasite load. Such a difference between males and females may result from different strategies of investment to immunity by males and females according to the pay off in terms of fitness. Females would possibly gain more fitness through increased longevity by investing more into immunity, while males would gain fitness by increasing mating rates (Rolff, 2002). Furthermore, large male gammarids are the most successful in pairing with females (Bollache *et al.* 2000; Bollache, Gambade & Cézilly, 2001). Consequently, these large males may achieve higher mating rates than smaller ones. Mating has been found recently to decrease general immune defence in insects (Rolff & Siva-Jothy, 2002). Higher mating rates could have lead to the relatively low PO-enzyme activity in large *G. pulex* males. We did not find any sex difference in the level of PO-enzyme activity in *G. roeseli* in the absence of parasites, but immune responses to parasite infection were dependent of the host gender. Immune responses of females to *P. laevis* infection were stronger than these of males. For the same reason as mentioned above for *G. pulex*, it might be more beneficial for females than for males to invest in immunity (Rolff, 2002).

In conclusion, this correlative study suggests that the acanthocephalan parasites *P. laevis* and *P. minutus* immunosuppress their intermediate gammarid hosts. However, local parasites would be maladapted to immunosuppress new invasive intermediate

hosts. This maladaptation of the local parasites to new hosts may have provided a considerable advantage to invaders over native competitors. *G. roeseli* has been reported to be infected by *P. laevis* in Czechoslovakia and Hungary, in its native area (Moravec & Scholz, 1991; T. Rigaud, personal observation). Future studies will allow comparisons of the immune evasion capacity of parasites from native *versus* non-native origins, and therefore formally test the maladaptation hypothesis.

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