Density-dependent effects on parasite growth and parasite-induced host immunodepression in the larval helminth *Pomphorhynchus laevis*

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SUMMARY

Larval helminths exploit the physiology of their intermediate hosts: first, as a resource for energy and space and second by altering the immune system activity to ensure their survival. Whereas the growth pattern under parasite competition has been investigated, the effect of multiple infections on the level of parasite-induced immunodepression in a trophically transmitted helminth has been neglected. In this study, amphipods *Gammarus pulex* were infected in the laboratory by the acanthocephalan *Pomphorhynchus laevis* to investigate how parasite density in the intermediate host affected (i) cystacanth growth and (ii) the level of parasite-induced alterations of the host immune defences, two traits strongly linked to host exploitation. The study highlights that sharing a host is costly. As parasite intensity increases, competition for resources translates into a reduction in cystacanth volume. Immune manipulation is also modulated by density. Interestingly, immunodepression is higher in double-infected hosts compared to hosts with a single infection, suggesting an opportunity for cooperative immune manipulation. However, in higher multiple infections, parasites do not further down-regulate the host immune response, possibly to avoid additional costs that may outweigh the benefits of immunodepression.

Key words: acanthocephalan, competition, exploitation, immunodepression, larval helminth growth, parasite intensity, phenoloxidase.

INTRODUCTION

For parasites with complex life cycles, intermediate hosts represent an energy resource allowing their growth/replication, as well as a vector needed for the transmission to the next host (Poulin, 2007). Co-infections, either by the same or different parasite species, are very widespread. Although co-infecting parasites may cooperate to optimally exploit their host and increase their probability of transmission, host sharing is most of the time associated with costs and/or conflicts (Brown, 1999). Indeed, increasing the number of conspecifics within a host imposes severe competition among parasites for the acquisition of resources. In larval helminths, it is already well established that parasite size often decreases in multiple infections (Dezfuli *et al*. 2001; Brown *et al*. 2003; Steinauer and Nickol, 2003; Fredensborg and Poulin, 2005; Michaud *et al*. 2006; Lagrue and Poulin, 2008). The pressures and the costs of increased parasite intensity are strengthened when the shared host is of relatively small size compared to the parasites, such as for larval acanthocephalans infecting their amphipod intermediate hosts.

Parasite size is a good predictor of adult establishment success (Steinauer and Nickol, 2003) and fecundity (Fredensborg and Poulin, 2005). Nevertheless, because host survival is essential for parasite transmission (and particularly for trophically transmitted helminths with long development), the benefits of increased size will be evolutionarily constrained by the higher probability of host mortality. Parasites should therefore trade their growth against the cost of reduced transmission to the next host (Parker *et al*. 2003; Ball *et al*. 2008). Host damage due to an over-exploitation by parasites may cause an early death of the host and of the parasites before their transmission occurs. Hence, host exploitation will result in a balance between the costs and the benefits of increasing the number of parasites within a host.

In addition to exploiting the resources of the host, helminths interfere with the host immune defences. Immune evasion is a widespread parasite strategy (Schmid-Hempel, 2008) that enables parasites to hide from the immune system or to limit the negative impacts of the host immune response and, thus, to increase the persistence of the parasite within the host, sometimes for years. Helminth parasites are

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masters in their ability to depress the host immune functioning (Maizels *et al*. 2004). Although mechanisms of immune evasion and suppression of immunity have been most investigated and characterized in vertebrate hosts (e.g. Hewitson *et al*. 2009), helminths seem to adopt the same strategies in their mollusc and arthropod intermediate hosts (Loker, 1994; Humbert and Coustau, 2001; Cornet *et al*. 2009*a*).

Haemocytes and the phenoloxidase (PO) cascade are two main effectors of the invertebrate innate immunity involved in the encapsulation and melanization processes (Cerenius and Söderhäll, 2004). The PO enzyme is mainly stored in haemocytes as an inactive pro-enzyme (prophenoloxidase, ProPO), which is rapidly activated upon infection (Labbé and Little, 2009). Both PO and haemocyte levels are associated with disease resistance in crustaceans (Cerenius *et al*. 2003, 2008) and their impairment should enable acanthocephalan macroparasites to develop successfully in the host (Volkmann, 1991; Taraschewski, 2000). In the natural association involving the fish acanthocephalan *Pomphorhynchus laevis* and its amphipod crustacean host *Gammarus pulex*, the parasite depresses the immune defences of intermediate hosts. Haemocytes and the phenoloxidase activity are impaired following infection (Cornet *et al*. 2009*a*). However, if immunodepression seems to be beneficial for the parasites by ensuring their survival within their hosts, it could also set a series of costs. Acanthocephalan-infected hosts are more susceptible to bacterial infections (Cornet *et al*. 2009*a*) and a high environmental risk to contract such secondary opportunistic infections translate into a higher parasite virulence (Cornet and Sorci, 2010). Hence, there should be an optimal level of immunodepression, which is a balance between the costs and benefits. Density-dependent acanthocephalan-induced alterations have been recorded on crustacean intermediate host traits such as survival (Duclos *et al*. 2006) and fecundity (Dezfuli *et al*. 1999). Nevertheless, virtually nothing is known about how parasite intensity may modulate intermediate host immunity in a trophically transmitted parasite and how the number of co-infecting parasites could affect the cost/benefit balance of immunodepression is still unknown.

The present paper aims to investigate, in the *Gammarus-Pomphorhynchus* system, the effects of parasite density (also called intensity) on (i) parasite growth and (ii) parasite-induced immunodepression, and (iii) to assess the potential link between parasite size and the level of immune depression. For this purpose, gammarids were infected with *Pomphorhynchus laevis* in the laboratory and 4 different parasite densities were obtained. I therefore made 2 predictions: first, due to competition for nutrients and space for growth (especially as *G. pulex* is of small size compared to the cystcanth size), individual parasite volume is expected to decrease as the density of parasites increases. Such a crowding effect has already been demonstrated for this parasite species (Dezfuli *et al*. 2001) on wild-caught infected gammarids where the history of infection was unknown. By contrast, although correlative, this study allows a better control of the infection process using experimental infection in the laboratory. Second, the parasite intensity-immunodepression relationship offers different plausible alternatives: (i) all the infected gammarids have the same level of immune defences (i.e. no relationship); (ii) the level of immune defences in multiple-infected hosts is lower than in single-infected hosts and is negatively affected by the parasite intensity (linear relationship, the magnitude of changes (here immunodepression) is expected to increase as the parasite infra-population increases).

MATERIALS AND METHODS

Sampling of hosts and parasites

Gammarus pulex males were collected in June 2008 in a small tributary of the Suzon River at Val Suzon (northern Dijon, France). Animals were maintained in the laboratory under standard conditions (15 °C ±1 °C, light:dark cycle 12:12 h) in well-aerated tanks filled with dechlorinated UV-treated tap water and fed with elm leaves. They were acclimatized for 2 weeks prior to infection experiments. Fish acanthocephalans do not occur at that locality, making the amphipods suitable for experimental infection (Cornet *et al*. 2009*b*).

Pomphorhynchus laevis parasites came from naturally parasitized chubs *Leuscicus cephalus* sampled by electrofishing in the Vouge River at Aubigny en Plaine (southern Dijon). Fish were anaesthetized, killed and dissected within 24 h after sampling. Adult parasites were collected from the intestines; eggs were obtained by dissecting female worms and stored in 400 μl of water.

Infection procedure

In this host-parasite system, it is currently difficult to experimentally control the number of parasites within an infection in a precise manner, as is possible in other systems (see Michaud *et al*. 2006). Due to this constraint, gammarids were exposed to a constant parasite density and those that had been infected with 1, 2, 3 or more parasites were selected *a posteriori*. The potential bias that may have been introduced by this experimental protocol is discussed below.

Controlled infections were made following the procedure of Cornet *et al*. (2009*a*). Briefly, parasite eggs from each sampled female were examined under the microscope (x 200 magnification) to evaluate their number and maturity. Then, 10 suitable clutches

(coming from 3 fish) were pooled and the egg suspension was set at a concentration of 25 eggs/μl.

Prior to parasite exposure, gammarids were food deprived for 24 h. Gammarids were placed by pairs in crystallizing dishes and were provided with approximately 100 eggs/individual deposited on 1 cm² of elm leaf, on which they were allowed to feed for 48 h. Uninfected leaves were provided to the non-infected group. Animals were maintained under standard conditions until cystacanths were detected (usually between 9 and 12 weeks after parasite exposure, Cornet *et al*. 2009*b*). Here, during the 11th week after parasite exposure, gammarids were inspected under a binocular microscope to check for the infection and to evaluate parasite intensity. Gammarids were maintained for 15 days in dishes of 0·2 L with food provided before haemolymph collection. Although there was little evidence of variation in the ontogenic stage of parasites among the different infected groups, this additional time allowed the full development of all parasites into mature cystacanths. Around 20 gammarids harbouring 1 and 2 cystacanths (the most prevalent parasite intensities) were randomly selected, whereas all individuals with higher intensities (far less prevalent) were kept for the experiment.

Haemolymph collection, haemocyte concentration and activities of the ProPO system

Three μl of haemolymph were collected into a sterile, pre-chilled glass capillary and flushed into 20 μl of cold phosphate-buffered saline (Cornet *et al*. 2009*a*). Ten μl were immediately used for haemocyte counting using a Neubauer counting chamber, and samples were frozen in liquid nitrogen and stored at − 80 °C for later phenoloxidase assays.

The activity of naturally activated phenoloxidase enzymes only (therein-after called PO activity) and the activity of the pro-enzymes (ProPO) in addition to that of the PO (therein-after called ProPO activity) were measured for each individual haemolymph extract using a spectrophotometric assay (Cornet *et al*. 2009*b*). The assay was performed using 5 μl of haemolymph extract added to a microplate well containing 20 μl of PBS buffer and either 140 μl of dH_2O to measure PO activity only or 140 μl of chymotrypsin solution (Sigma C-7762, 0·07 mg/ml of dH_2O) to measure ProPO activity. Then 20 μl of L-Dopa solution (Sigma D-9628, 4 mg/ml of dH_2O) were added and the reaction was followed in a microplate reader (Versamax, Molecular Devices) for 40 min at 490 nm. Enzyme activity was analysed using the software SOFT-Max®Pro 4.0 (Molecular Devices) and measured as the slope (V_{max} value) of the reaction curve.

Measurements of haemocyte count and phenoloxidase activity were reported for 1 μl of pure haemolymph.

Dissection and measurements

Gammarids were measured by linear dimension (size of the fourth coxal plate) and dissected to assess the intensity of infection. Cystacanths, with a shape ranging from an ellipsoid to a spheroid, were measured (length and width) and their volumes were estimated using the formula for an ellipsoid $V = \text{length} \times \text{width}^2 \times \pi/6$ (Dezfuli *et al*. 2001). All measurements were taken using a stereoscopic microscope Nikon and Lucia G 4.81 software.

Data analyses

Prior to analyses, immune data (values of haemocyte concentration, PO and ProPO activities) were natural-log transformed to meet the normality assumption. Values shown in Fig. 2 also refer to transformed data.

Mean cystacanth volume per host was calculated by dividing the total volume by the total number of parasites. Variation in mean or total parasite volume in relation to parasite intensity was analysed with ANCOVAs. The ratio variance/mean for parasite volume per host was used to estimate the difference in volume between cystacanths sharing the host.

Variation in immune defences was assessed using linear models. Since the 3 immune parameters were taken on the same individual, immune data were analysed using a multivariate analysis of covariance (MANCOVA, Pillai's trace) with respect to parasite intensity and amphipod size. The MANCOVA is the analogue of univariate ANCOVA when there are multiple response variables recorded for each individual (here, 3 immune measures). Then variation for each parameter was tested independently with univariate tests. Dunnett's post-hoc mean comparison test was used with uninfected gammarids as control. Dunnetts's test is a modified *t*-test specifically designed for comparing each group to a control group (here, any of the infected groups to the non-infected group) (Quinn and Keough, 2002). Otherwise, Tukey HSD pair-wise comparison tests were used, for example when comparing data among infected groups only (groups with different letters were statistically different). The relationships between immune activities and parasite volume were also analysed with ANCOVAs. Parasite intensity was considered as a categorical variable with 4 levels (1, 2, 3, $\geqslant$4). Interaction terms (e.g. parasite intensity × amphipod size) were first tested but were never significant ($P > 0{\cdot}5$) and therefore were dropped from the final models.

All tests were performed using JMP v5.0 for Windows (SAS Institute) and referred to two-tailed tests with significant differences considered at the level of $P \leqslant 0{\cdot}05$.

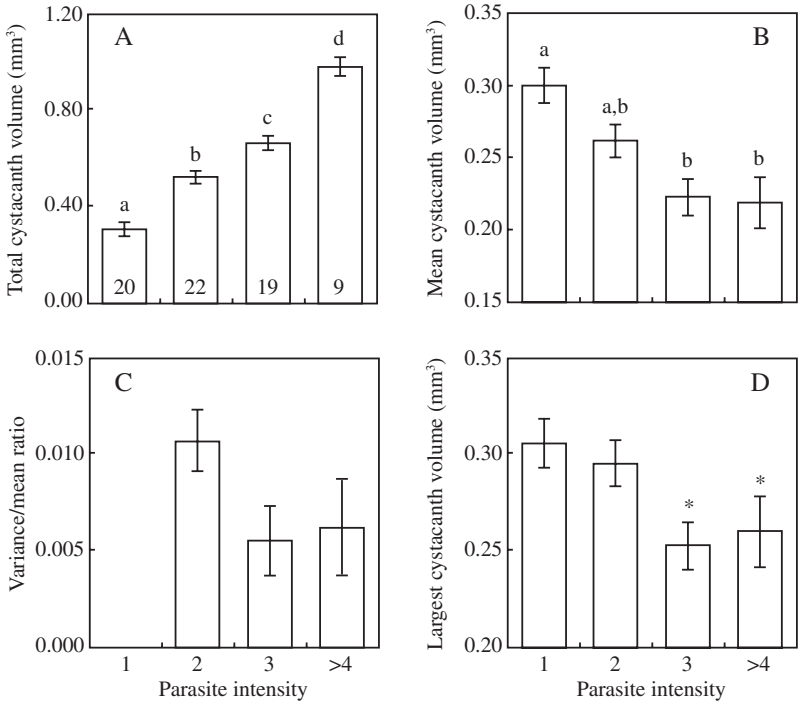

Fig. 1. Volume of cystacanths (in mm^3, mean ± S.E.) in relation to the intensity of infection by the acanthocephalan *Pomphorhynchus laevis* in *Gammarus pulex:* total (A) and mean (B) volume of cystacanths per host (different letters above bars indicate significant differences between means, Tukey HSD, $\alpha = 0{\cdot}05$), (C) variance/mean ratio and (D) volume of the largest cystacanth per host (asterisks indicate groups that significantly differ from the cystacanth volume in single-infection, Dunnett's, $\alpha = 0{\cdot}05$). Sample size of infected hosts is given within bars.

RESULTS

Intensity of infection and parasite volume

The gammarid sample was composed of 26 uninfected and 70 *P. laevis*-infected gammarids, including 20, 22, 19 and 9 animals harbouring respectively 1, 2, 3 and ⩾4 cystacanths. In the last group, the intensity ranged from 4 to 6 parasites per host (mean intensity 4·55). The mean overall size of gammarids was 3·07 ± 0·02 mm (coxal plate) and it did not differ between groups ($F_{4,91} = 0{\cdot}11$, $P = 0{\cdot}9774$).

As predicted, the total volume of cystacanths per host increased with the intensity of infection ($F_{3,66} = 8{\cdot}89$, $P < 0{\cdot}0001$; Fig. 1A) from 0·310 ± 0·026 mm^3 in mono-infected amphipods to 0·985 ± 0·026 mm^3 in heavily infected animals (⩾4 cystacanths). Mean cystacanth volume per host and parasite intensity showed a negative relationship ($F_{3,66} = 8{\cdot}89$, $P < 0{\cdot}0001$; Fig. 1B) ranging from 0·310 ± 0·026 mm^3 in mono-infection to 0·218 ± 0·017 mm^3 in the last category of intensity (⩾4 cystacanths). With increasing parasite intensity, the difference in volume for cystacanths within a host shrank, as shown by the distribution of variance/mean ratios (Fig. 1C; $F_{2,47} = 2{\cdot}70$, $P = 0{\cdot}0781$). It is worth noting that with increasing parasite intensity, the volume of the largest cystacanth within multi-infected hosts also decreased (Fig. 1D; $F_{3,67} = 4{\cdot}33$, $P = 0{\cdot}0076$). The volume of the largest cystacanth from gammarids infected by 3 or ⩾4 parasites was lower compared to the volume of cystacanths found in mono- or bi-infected animals (Dunnett's pair-wise comparison tests with control referring to the cystacanth volume of the mono-infection group; Fig. 1C; * shows groups that differ from the control mono-infection group). The relationships found between parasite intensities and, respectively, the total volume and the mean volume within a host was not affected by the size of gammarids (linear model for total volume per host: parasite intensity $F_{3,65} = 78{\cdot}88$, $P < 0{\cdot}0001$, amphipod size $F_{1,65} = 0{\cdot}19$, $P = 0{\cdot}6598$; average volume per host: parasite intensity $F_{3,65} = 11{\cdot}58$, $P < 0{\cdot}0001$, amphipod size $F_{1,65} = 0{\cdot}02$, $P = 0{\cdot}8899$).

Intensity of infection and levels of immune defence

In agreement with previous studies, *P. laevis* infection (independent of parasite intensity) was related to an alteration of the host immune system compared to uninfected gammarids (Fig. 2). Infected gammarids had a lower haemocyte concentration ($F_{4,91} = 9{\cdot}86$, $P < 0{\cdot}0001$) and a decreased activity of phenoloxidase (PO activity: $F_{4,91} = 14{\cdot}92$, $P < 0{\cdot}0001$; ProPO activity: $F_{4,91} = 27{\cdot}07$, $P < 0{\cdot}0001$) with groups of infected hosts always differing from the basal level of immune defence of uninfected hosts (Dunnett's pair-wise comparison tests with control referring to the 'uninfected' group).

Overall, immune effectors were affected differentially by parasite intensity (Mancova Pillai's Trace $F_{12,195} = 2{\cdot}86$, $P = 0{\cdot}0012$ with parasite intensity

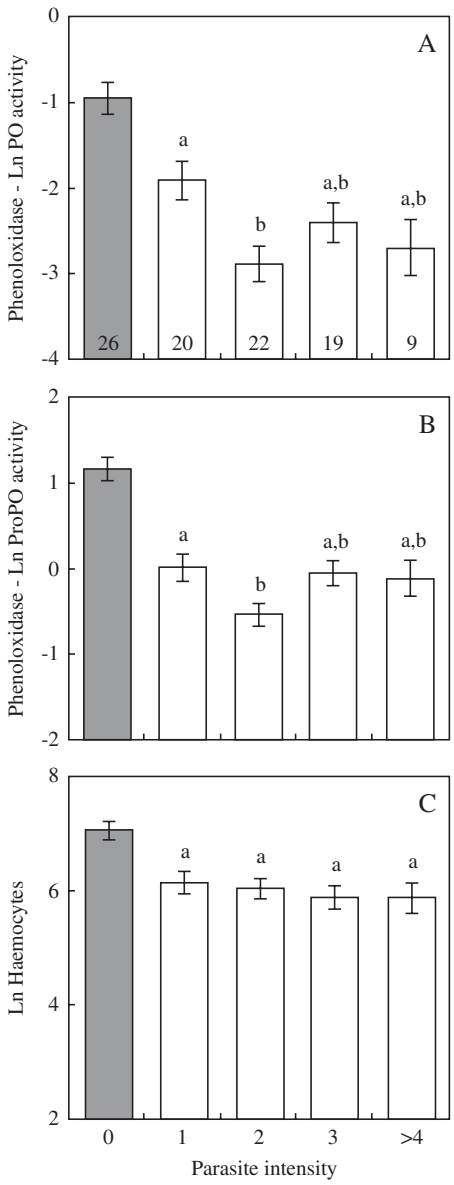

Fig. 2. Levels of immune defence (natural log-transformed, mean ± S.E.) of *Gammarus pulex* in relation to the intensity of infection by *Pomphorhynchus laevis*: (A) PO activity, (B) ProPO activity and (C) number of haemocytes. Grey bars (parasite intensity = 0) refer to uninfected amphipods. Different letters above bars indicate significant differences between groups of infected animals (Tukey HSD, $\alpha = 0{\cdot}05$). Sample size of hosts is given within bars.

$F_{9,195} = 2{\cdot}25$, $P = 0{\cdot}0211$ and amphipod size $F_{3,63} = 4{\cdot}55$, $P = 0{\cdot}0060$). However, whereas parasite intensity explained part of the variation in both phenoloxidase activities (PO and ProPO activity, Table 1, Fig. 2A, B), the haemocyte concentration remained similar across the 4 groups of infected gammarids (Table 1, Fig. 2C). Phenoloxidase activities were significantly lower in double infections (transformed data, mean ± S.E., PO activity $-2{\cdot}903 \pm 0{\cdot}19$, ProPO activity $-0{\cdot}563 \pm 0{\cdot}13$) than in single infections (PO activity $-1{\cdot}937 \pm 0{\cdot}19$, ProPO activity $0{\cdot}004 \pm 0{\cdot}14$). However, intermediate levels of enzyme activity were found for higher parasite intensities (infection with 3 cystacanths: PO activity $-2{\cdot}400 \pm 0{\cdot}19$, ProPO activity $-0{\cdot}059 \pm 0{\cdot}14$ and with $\geqslant 4$ cystacanths: PO activity $-2{\cdot}705 \pm 0{\cdot}28$, ProPO activity $-0{\cdot}122 \pm 0{\cdot}21$) as confirmed by Tukey HSD mean comparison test, $\alpha = 0{\cdot}05$ (see Fig. 2A, B).

Relationships between immune levels and parasite traits

Models including parasite intensity, mean parasite volume and amphipod size (to control for host influence) were run to estimate the influence of cystacanth volume per amphipod on the level of activity of the immune effectors (for PO and ProPO activities only). Overall, parasite volume did not influence either the variation in PO activity (global model $F_{5,64} = 3{\cdot}21$, $P = 0{\cdot}0121$; mean parasite volume $F_{1,64} = 0{\cdot}79$, $P = 0{\cdot}3771$; amphipod size $F_{1,64} = 1{\cdot}15$, $P = 0{\cdot}2873$) or the variation in ProPO activity (global model $F_{5,64} = 3{\cdot}62$, $P = 0{\cdot}0061$; mean parasite volume $F_{1,64} = 1{\cdot}45$, $P = 0{\cdot}2322$; amphipod size $F_{1,64} = 5{\cdot}45$, $P = 0{\cdot}0226$). The parasite volume was unrelated to the variability in the level of immune depression. Again, only the number of cystacanths (parasite intensity) affected the level of PO ($F_{3,64} = 3{\cdot}87$, $P = 0{\cdot}0131$) and ProPO activity ($F_{3,64} = 3{\cdot}71$, $P = 0{\cdot}0159$). Similar results were obtained when the total cystacanth volume was used in the analyses (not shown). Immune defences were likely to be unaffected by the parasite growth (parasite volume) but only affected by the intensity of infection. Nevertheless, the mean volume was highly related to the number of parasites infecting a host; hence it was not surprising it had no effect on its own in explaining the variation on immune defences. However, the relationships between parasite growth and immune effectors can be analysed in single-infected amphipods. Here again, the parasite volume was unrelated to PO activity ($r = 0{\cdot}17$, $n = 20$, $P = 0{\cdot}4779$), ProPO activity ($r = 0{\cdot}21$, $P = 0{\cdot}3694$) and haemocyte concentration ($r = -0{\cdot}34$, $P = 0{\cdot}1765$), or to amphipod size ($r = 0{\cdot}16$, $P = 0{\cdot}4978$).

DISCUSSION

This study reports density-dependent effects within infra-populations of *P. laevis* in their amphipod intermediate hosts *G. pulex*. Variation in parasite growth and parasite-induced immune alterations were recorded and co-varied with an increase in parasite intensity.

Working with complex natural host–parasite systems allows relevant questions on the evolution and ecology of interactions to be addressed. Nevertheless, it also sets a series of limitations. Special attention was taken to minimize the variation in size among hosts to minimize the effect of external factors

Table 1. Effects of parasite intensity of *Pomphorhynchus laevis* and amphipod size on the variation in *Gammarus pulex* immune effectors

Immune effectors	Effects	Sum of squares	D.F.	F	*P*
PO activity	Parasite intensity	10·22	3	4·65	0·0052
	Amphipod size	0·85	1	1·19	0·2790
	Error	48·04	65		
ProPO activity	Parasite intensity	4·04	3	3·64	0·0171
	Amphipod size	2·00	1	5·32	0·0243
	Error	24·24	65		
Haemocytes	Parasite intensity	0·76	3	0·50	0·6835
	Amphipod size	0·001	1	0·002	0·9637
	Error	44·98	65		

on the results. Here, the different densities were not experimentally generated although animals were experimentally infected. Because gammarids were exposed to a constant dose of parasite eggs, there could be some differences between hosts that became infected with several worms and those infected with fewer worms. Infection by 1, 2 or more parasites might not occur randomly but could be affected by an initial difference in host quality and condition or by a difference in the number of ingested eggs, which might be revealed later in the results as effects of parasite density (confounding effects). First, regarding the immune data, gammarids with initially low levels of constitutive immune response might be more prone to the infection. In this case, the observed difference in the immune response between infected and non-exposed gammarids would merely reflect a differential susceptibility, instead of a parasite-induced effect. Cornet *et al*. (2009*a*) compared the level of PO activity in non-exposed, exposed but non-infected and infected hosts. Contrary to the prediction of differential susceptibility, they found that only the infected group had a lower immune activity and that non-exposed and exposed but non-infected hosts had a similar level of PO activity. Overall, these results strongly suggest that the observed variation of PO activity between non-infected and infected gammarids was not due to initial differences in susceptibility. Second, a differential mortality of multi-infected hosts might be hidden by a parasite intensity effect. More gammarids infected with 1 and 2 parasites were obtained during this experiment and were selected at random. By contrast, as there were fewer hosts of higher intensities, only the more resistant ones could have survived until the end of the experiment. Indeed, the small number of heavily infected hosts (parasite intensity $\geqslant 3$) may not be a random sample of multiple infection but are likely to represent a small fraction of high quality hosts that survived, as reported in a trematode-amphipod association (Fredensborg *et al*. 2004). An infection procedure with different parasite dose would have helped to test for such susceptibility effects.

Acanthocephalans are macro-parasites with a complex life cycle and the intermediate hosts represent a vector, essential for transmission, as well as a resource for the larval growth. A reduction in parasite volume is expected to occur as a direct cost arising from a competition for resources between co-infecting parasites. In agreement with previous studies on several species (Dezfuli *et al*. 2001; Heins *et al*. 2002; Brown *et al*. 2003; Steinauer and Nickol, 2003; Fredensborg and Poulin, 2005; Michaud *et al*. 2006), the results showed that the total volume of *P. laevis* cystacanths within a host increased constantly with intensity. However, the total volume might not increase linearly for much higher intensities as a constraint of space for growth (not assessed in the study, but see Michaud *et al*. 2006). Since the vector survival is essential for parasite transmission, the optimal growth is a balance between the positive effects of conspecifics on host exploitation and the negative effects on host mortality (see also Ball *et al*. 2008). As a consequence, and to limit host mortality due to an excessive parasite load, the mean cystacanth volume decreased with intensity in their amphipod host, suggesting density-dependent effects on growth and intra-specific competition for the host resources. These results (increase in parasite total volume and decrease in individual parasite volume) are more likely fitting to the prediction of an adaptive life-history strategy than of a simple response to resource constraints (Parker *et al*. 2003). However, a measure of fitness trait (e.g. host survival) would be useful to ascertain that the observed intensity-dependent changes in parasite growth are adaptive.

In addition, competition in multi-infected hosts was strengthened. The volume of cystacanths tended to be more homogeneous (i.e. lower variance/mean ratio values) and the largest cystacanth within a host was smaller when hosts harboured at least 3 parasites whereas there was no difference for smaller intensities (see also Heins *et al*. 2002). This pattern is likely to be the result of a direct competition due to crowding effects rather than a delayed development since infection occurred only once, and that gammarids could not have contracted further infection later in

the experiment. Finally, no evidence of a host size effect on the growth of parasites was found. This may be related to the similar size of gammarids used for the experimental infection.

Helminths are immuno-modulatory parasites. Down-regulation of invertebrate immunity by complex life cycle parasites is always seen as a strategy to avoid clearance and to persist within the host until transmission. In that way, the acanthocephalan *P. laevis* relies on an immunodepressive strategy and induces a reduction of both the phenoloxidase activity and haemocytes (this study, Cornet *et al*. 2009*a*,*b*). The change in immune status in infected hosts could also be seen as a host response resulting from a reallocation of resources from the immune system to the host maintenance (to compensate for the loss of resources diverted by the parasite) or reproduction. However, a shift of resources towards reproduction is less likely since acanthocephalans induce a partial or total castration of their hosts (Bollache *et al*. 2002; Dezfuli *et al*. 2008). The proximal mechanisms of such immune activity down-regulation in infected hosts are not yet known in this particular host-parasite association (mainly due to the difficulties of working with a non-model species), but given the literature, it is reasonable to say that immunodepression is achieved by excretory-secretory (E-S) molecules released by the cystacanth in the body cavity that would interfere with the immune effectors (Guillou *et al*. 2007; Hanington *et al*. 2010).

Could the intensity of immunodepression be affected by parasite density? In other words, is the level of immunodepression the same among infected hosts whatever the number of parasites they harbour or is this level decreasing as the parasite density increases? It is worth mentioning that in the *G. pulex*-*P. laevis* association, one cystacanth is able to induce the significant immunological alterations. In addition, according to Poulin (1994) and given the fact that the parasite infrapopulation size is small in this host-parasite system, the magnitude of parasite-induced changes is likely to be less or not affected by the parasite intensity. Here, contrary to this expectation, I found that host physiological exploitation based on the ability to depress the level of immune defences of *G. pulex* was modulated by the presence of conspecifics. The activity of the ProPO system was found to be lower in hosts harbouring 2 cystacanths compared to mono-infected hosts. For higher intensities, phenoloxidase activities were intermediate and did not statistically differ from the activity level measured in hosts with 1 or 2 cystacanths respectively. However, PO activity tended to be lower than the ProPO activity in high infection intensity. The immunodepression level may be difficult to interpret for higher intensity because of potential differences in host initial condition and/or mortality that could have generated a non-random sample of heavily infected hosts. This should not be the case when considering the double-infected hosts. It is worth noting that not all the branches of the immune system were affected by infection intensity. Haemocyte concentration was lower in infected hosts than in uninfected hosts but did not differ between the 4 classes of parasite densities. This might be related to the mode of immunodepression that could act differently on haemocytes and on the ProPO cascade.

The higher immunodepression in double-infected amphipods would be consistent with the fact that immunodepression effects could be cumulative and dose dependent, especially if they rely on E-S molecules. However, no relationship was found between cystacanth growth and the magnitude of immunodepression, suggesting that only the number of parasites per host accounted for the variation of the phenoloxidase activities. In the field, about 20% of gammarid infections include multiple worms (L. Bollache, personal communication, Outreman *et al*. 2002). A non-negligible part of the parasite population shares an intermediate host with conspecifics (usually just one). Hence, the opportunity for cooperative immunodepression may exist. The reduction in immune function to a potential optimum in double infection, but not beyond it in higher intensity infection, would therefore make sense.

Why did immunodepression not decrease linearly with parasite intensity? First, immune manipulation may be costly (Poulin, 1994) and immune interference is likely to trade against other life-history traits such as growth, although such an assumption has never been tested. Second, gammarids were infected with a pool of eggs coming from 10 parasites with no possibility to control for the relatedness of co-infecting parasites (molecular tools are not currently available). The probability of having non-related parasites is likely to have increased in multiple infections. Decreasing the relatedness of parasites is expected to increase competition and decrease investment in common beneficial traits (Read and Taylor, 2001). Third, one might also keep in mind that transmission to the definitive host is the ultimate goal of larval acanthocephalans. Thus, severe host exploitation will result in an increased mortality of the host. In this system, parasite virulence (infection-induced host mortality) is environmentally modulated and sensitive to the pressure of opportunistic pathogens (Cornet and Sorci, 2010). Hence, parasites have to trade their immunodepressive effects against the risk of host death. Thus, it would be maladaptive for the parasites to induce a severe immunodepression in multiple-infected hosts ($n \geqslant 3$) since it could facilitate secondary infections and exacerbate the host background mortality (Cornet and Sorci, 2010).

Intra-specific competition among cystacanths in multi-infected hosts is likely to have negative consequences on parasite fitness in the final host. Especially, larval parasite volume is a reliable

estimator of adult establishment success and survival in the definitive host (Steinauer and Nickol, 2003) as well as fecundity (Fredensborg and Poulin, 2005). In addition to the negative effects of multi-infection on host resource depletion and reduced survivorship (Duclos *et al*. 2006), selection should prevent infection with high parasite intensity. Both data on growth and immunodepression suggest that costs emerged when more than 2 parasites shared a host: parasite volume was markedly reduced and the level of immune depression did not decrease further. Here, whereas the immune manipulation was higher in hosts infected by 2 parasites (a pattern also found for the behavioural manipulation in this *Gammarus-Pomphorhynchus* association, Franceschi *et al*. 2008), it does not necessarily mean that it is optimal for parasite transmission. Indeed, it is quite difficult to answer whether co-infecting parasites rather profit or suffer (in term of adult fitness) from this increased immunodepression. This should be tested. Unfortunately, failure to complete the parasite cycle under laboratory conditions as has been done for other systems (Steinauer and Nickol, 2003; Fredensborg and Poulin, 2005) prevents a firm conclusion from being drawn at this stage. However, this represents an interesting issue for later investigations.

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