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The effects of parasite age and intensity on variability in acanthocephalan-induced behavioural manipulation

Nathalie Franceschi *, Alexandre Bauer, Loïc Bollache, Thierry Rigaud

Université de Bourgogne, Laboratoire Biogéosciences, UMR CNRS 5561, Equipe Ecologie Evolutive, 6 Boulevard Gabriel, 21000 Dijon, France

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Abstract

Numerous parasites with complex life cycles are able to manipulate the behaviour of their intermediate host in a way that increases their trophic transmission to the definitive host. *Pomphorhynchus laevis*, an acanthocephalan parasite, is known to reverse the phototactic behaviour of its amphipod intermediate host, *Gammarus pulex*, leading to an increased predation by fish hosts. However, levels of behavioural manipulation exhibited by naturally-infected gammarids are extremely variable, with some individuals being strongly manipulated whilst others are almost not affected by infection. To investigate parasite age and parasite intensity as potential sources of this variation, we carried out controlled experimental infections on gammarids using parasites from two different populations. We first determined that parasite intensity increased with exposure dose, but found no relationship between infection and host mortality. Repeated measures confirmed that the parasite alters host behaviour only when it reaches the cystacanth stage which is infective for the definitive host. They also revealed, we believe for the first time, that the older the cystacanth, the more it manipulates its host. The age of the parasite is therefore a major source of variation in parasite manipulation. The number of parasites within a host was also a source of variation. Manipulation was higher in hosts infected by two parasites than in singly infected ones, but above this intensity, manipulation did not increase. Since the development time of the parasite was also different according to parasite intensity (it was longer in doubly infected hosts than in singly infected ones, but did not increase more in multi-infected hosts), individual parasite fitness could depend on the compromise between development time and manipulation efficiency. Finally, the two parasite populations tested induced slightly different degrees of behavioural manipulation.

Keywords: Acanthocephalans; Gammarids; Behavioural manipulation; Experimental infections

1. Introduction

Parasite-induced phenotypic changes are widely documented phenomena, especially in trophically transmitted parasites characterised by a complex life-cycle (e.g. recent reviews in Moore, 2002; Thomas et al., 2005). Numerous parasites have developed the ability to modify the behaviour of their intermediate hosts, an alteration that enhances predation by definitive hosts, therefore favouring parasite transmission (Lafferty, 1999; McCurdy et al., 1999; Seppälä et al., 2004; Perrot-Minnot et al., 2007). This classic example of extended phenotype (Dawkins, 1982) has been widely studied over the last 10 years, with the principal focus being on the adaptive nature of this so-called "behavioural manipulation" (e.g. Moore, 1983; Poulin, 1995; Lagrue et al., 2007a). However, our understanding of how the manipulative processes evolve is still unclear, because the causes and outcomes of the intraspecific variation observed in the intensity of changes remain poorly understood (Thomas et al., 2005).

Amongst parasites altering host behaviour, the Acanthocephala are considered one of the most relevant biological models. In their seminal experiments, Bethel and Holmes (1973, 1977) used acanthocephalans to formally test whether behavioural changes induced in the intermediate host were associated with an increased predation risk by definitive hosts. Afterwards, other papers, combining

* Corresponding author. Tel.: +33 380 39 62 28; fax: +33 380 39 62 31.
E-mail address: nathalie.franceschi@u-bourgogne.fr (N. Franceschi).

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experimental and field studies, showed that infection with other acanthocephalan species was also linked to adaptive behavioural manipulation (e.g. Moore, 1983; Lagrue et al., 2007a). In fact, intermediate host manipulation as a transmission strategy appears to be an ancestral trait in acanthocephalans (Moore, 2002; Kennedy, 2006). *Pomphorhynchus laevis* is a fish acanthocephalan, widely distributed in Europe, which uses several crustacean amphipod species as intermediate hosts (Crompton and Nickol, 1985; Perrot-Minnot, 2004; Kennedy, 2006). Several studies have shown that this parasite induces numerous behavioural alterations in the amphipod *Gammarus pulex*, modifying the gammarid's phototactic behaviour (Brown and Thompson, 1986; Bauer et al., 2000; Cézilly et al., 2000; Perrot-Minnot, 2004; Tain et al., 2006) or anti-predator behaviour (Kaldonski et al., 2007). Although the adaptive value for the parasite of such phenotypic changes has recently been confirmed (Lagrue et al., 2007a), these behavioural modifications also showed considerable variation in their intensity. Focusing on the phototaxis inversion exhibited by *P. laevis*-infected *G. pulex* – on average, uninfected gammarids are photophobic, with little variation within this trait (Perrot-Minnot, 2004), whereas infected ones are attracted to light (Cézilly et al., 2000) – it is clear that some wild-caught infected individuals are profoundly manipulated, whilst others are not affected by infection. These differences are linked to differences in brain serotonergic activity (Tain et al., 2006).

In acanthocephalans, two proximate factors have the potential to explain inter-individual differences in behavioural modifications. On one hand, the developmental stage of the parasite may modulate the intensity of behavioural changes in their intermediate host. Indeed, in two different acanthocephalan species, Bethel and Holmes (1974) and Sparkes et al. (2006) observed that modified behaviours were observed only when the parasite larvae become infective for the definitive host (cystacanth stage). However, it is not known if ageing affects the intensity of manipulation during the cystacanth stage. On the other hand, the parasite intensity (i.e. the number of parasites within infected hosts, Margolis et al., 1982) may also induce variation in the host phenotypic changes. McCahon et al. (1991) found increased drift behaviour in *G. pulex* with an increasing number of *P. laevis*, but Bauer et al. (2000) and Cézilly et al. (2000) found no effect of parasite intensity on phototaxis behaviour. However, these two factors are potentially not independent: parasite larval development has been demonstrated to be intensity-dependent in a number of parasite species (e.g. Michaud et al., 2006; Lagrue et al., 2007b), including acanthocephalans (Benesh and Valtonen, 2007). Therefore, parasite development, parasite intensity and their consequences on host phenotype alteration should be studied simultaneously, as has been done in a few cases (e.g. Lagrue et al., 2007b). Behavioural manipulation in acanthocephalans has been studied in naturally-infected systems, where infection dynamics and intensity were not controlled. Experimental infections have been used to study the developmental course of some acanthocephalan species (Hynes and Nicholas, 1957; Oetinger and Nickol, 1982; Brattey, 1986; Barger and Nickol, 1999; Duclos et al., 2006; Benesh and Valtonen, 2007), but they were usually massive infection experiments, with a limited control of the level of multiple infections, and were not used to test the host's behavioural manipulation.

Therefore, the three main objectives of this study were, using experimental infections of *G. pulex* by the acanthocephalan *P. laevis*: (i) to monitor the occurrence and consequences of multiple infections, according to the parasite exposure doses; (ii) to test, using repeated measures, the effects of the developmental stages of the parasite on the behaviour of the host; and (iii) to compare the behavioural alterations induced by *P. laevis* at different infection intensities.

2. Materials and methods

2.1. Origin of hosts and parasites

Gammarus pulex, used for experimental infections, were collected in July 2006 in a small tributary of the Suzon River, located in Burgundy, eastern France (N 47° 24.21′; E 4° 52.97′). In surveys over 10 years, *P. laevis* parasites were never found in this population (L. Bollache, unpublished data), and this population can therefore be considered naïve for the parasite. At this site, gammarids may harbour a larval cestode, *Cyathocephalus truncatus* (Franceschi et al., 2007), and a microsporidian, *Pleistophora mulleri* (K. Monceau and T. Rigaud, unpublished data). Individuals infected by these parasites were discarded during sampling. Some amphipod species can be infected by asymptomatic, vertically-transmitted microsporidia which potentially confound behavioural changes induced by acanthocephalans (Haine et al., 2005). Even though these parasites have never been found in *G. pulex* (T. Rigaud, unpublished data) we used diagnostic PCR, as described in Haine et al. (2004), to control for these infections. None of the 31 females and 44 males randomly picked in our samples was found to be infected (K. Monceau and T. Rigaud, unpublished data), whilst the prevalence of infected females reaches 58% in populations of other amphipod species (Haine et al., 2004). In the laboratory, the gammarids were acclimated for 15 days prior to infection experiments, in groups of 500 individuals, in well-aerated aquaria of 37 × 55 × 10 cm containing water at 15 ± 1 °C and elm leaves for food, under a 12:12 h light:dark cycle.

Parasite eggs were taken from naturally-parasitised chubs (*Leuciscus cephalus*). Fish were caught in two rivers, the Ouche and the Vingeanne (Burgundy), both tributaries of the Saône River and located 40 km from each other. Fish were anaesthetised, killed and dissected within 24 h after collection. Adult parasites were immediately collected from the fish intestines and eggs were obtained by dissecting female worms. Eggs from each female were placed in

400 μL of water and parasite tissues were preserved in 300 μL of alcohol for species identification.

2.2. Parasite molecular identification

In Burgundy, gammarids and fish may be infected by two closely-related species of acanthocephalan parasites, *P. laevis* and *Pomphorhynchus tereticollis*. These two species cannot be reliably distinguished based on morphology, and thus a molecular method was used for parasite identification (Perrot-Minnot, 2004). A total of 40 parasites from eight different fish (four fish per river) were examined. Parasite DNA was extracted according to a modification of the method of Gloor et al. (1993). A small piece of tissue was ground up in 300 μL Tris–EDTA Buffer with 10 μL Proteinase K (Promega), and incubated at 57 °C for 30 min. After Proteinase K inactivation at 90 °C for 3 min, the solution was centrifuged for 4 min at 13,200 *g* at 4 °C. One hundred microlitres of the supernatant was collected and diluted in 500 μL of ultrapure sterilised water. A diagnostic PCR was used to amplify a portion of the internal transcribed spacer (ITS) rDNA gene, according to Perrot-Minnot (2004), with some modifications. The primers used for PCR (BD1f: 5′GTCGTAACAAGGTTTCC GTA3′, Perrot-Minnot (2004) and AC/ITS1r: 5′TTGC GAGCCAAGTGATTCAC3′, M.J. Perrot-Minnot and R.A. Wattier, unpublished data) generated amplification products of 320 bp for *P. laevis* and 350 bp for *P. tereticollis* (M.J. Perrot-Minnot and R.A. Wattier, unpublished data). PCR reactions were performed in a final volume of 10 μL, containing 3 μL of template DNA, 200 μM of each nucleotide, 200 nM of each primer, and 2.5 U of Taq DNA polymerase (Promega) with the manufacturer's buffer containing 2.5 mM $MgCl_2$. Thermal cycling was performed using an initial denaturation at 95 °C for 3 min, followed by 39 cycles at 95 °C (20 s), 50 °C (45 s), and 65 °C (45 s). A final incubation of 5 min at 65 °C was performed to completely extend the amplified product. The sizes of PCR products were verified by the electrophoresis of 10 μL of PCR product in an agarose gel (2%) using DNA size standards (100 bp ladder, Fermentas). A negative control (reaction solution without template DNA) and two positive controls (template DNA from already-identified *P. laevis* and *P. tereticollis*, Perrot-Minnot, 2004) were carried out for each PCR reaction. From these analyses, we obtained a result of 72.5% of *P. laevis*.

2.3. Infection procedure and analysis

Parasite eggs from each female were examined under a Nikon microscope (20×) to evaluate their maturity (mature eggs contain a developed larval stage called acanthor, see Crompton and Nickol, 1985). Only clutches with more than 75% mature eggs were kept for the experiments. For each source of parasites (Ouche and Vingeanne Rivers), the eggs from six different *P. laevis* females, extracted from four different fish, were mixed. The number of eggs was then estimated by averaging the counts made under a microscope in 10 samples of 1 μL. After dilution with water, suitable egg exposure doses were obtained.

Prior to infection, gammarids were deprived of food for 24 h. Two gammarids were placed in a dish of 6 cm diameter, filled with water at 15 ± 1 °C. The egg suspension at suitable concentration was deposited on a 1 cm^2 dry elm leaf. The deposit of acanthocephalan eggs on amphipod food has been shown to increase the probability of infection, compared with eggs placed directly in the water or incorporated in balls of flour and oil (preliminary tests, not shown). Indeed, parasite eggs often anchor to the vegetation which forms the diet of amphipods (references in Kennedy, 2006). The infected leaf was then placed in the dish, and the gammarids were allowed to feed on it for 48 h. Uninfected leaves were provided to control groups. Preliminary tests revealed that a dose of 100 eggs per gammarid provided an acceptable ratio of infection success versus multiple infection rate (results not shown). To obtain different parasite intensities, four treatments were created with parasites from the Ouche River, corresponding to different exposure doses: 25, 50, 100 and 200 parasite eggs per gammarid. With parasites from the Vingeanne River, only the 100 dose was used. This last experimental series allowed us to confirm that the phenomena observed with the Ouche River population can be replicated with parasites from another source. The control group consisted of *G. pulex* maintained under the same conditions as the experimental series, but unexposed to parasite eggs. For each treatment, 150 gammarids (50% females, 50% males) were used.

At the end of the exposure period, the gammarids were rinsed and placed in aquaria of 0.5 L, under a 12:12 h light:dark cycle. Fifteen individuals undergoing the same treatment were randomly assigned to each aquarium. These aquaria were filled with dechlorinated, UV-treated and aerated tap water at a temperature of 15 ± 1 °C. The water was changed automatically six times each day, all aquaria receiving water from the same storage tank.

The number of surviving gammarids was checked every week. From the fifth week, all gammarids were also inspected once a week under a binocular microscope to detect the presence of parasites. Larval parasites can be detected through the host cuticle from the late acanthella stage of their development. Acanthella and cystacanth stages can be easily distinguished, as the acanthella appears as a translucent light-orange and shapeless parasite, whereas the cystacanth forms an opaque and bright-orange sphere. As soon as an acanthella was detected, the gammarid was isolated in a plastic dish of 0.20 L filled with water at 15 ± 1 °C. At the same time, uninfected individuals from the control treatment were also isolated. The parasite development was then followed once a week, and the date on which the parasites reached the cystacanth stage was noted (thereafter called "early cystacanth" stage). The prevalence (number of infected hosts/total number of surviving gammarids) was calculated 75 days post-exposure. The intensity of infection (number of parasites per infected

host) was estimated on the same date and also checked at the end of the experiment by dissecting all animals. Since intensity data did not satisfy homoscedasticity conditions, even after data transformations, we created three categories for analyses, each describing parasite intensity, hosts infected by a single parasite, hosts infected by two parasites and hosts infected by more than two parasites. The development time of the parasites was estimated by the time lapse between the day of exposure and the day when the parasites reached the early cystacanth stage.

Survival data were analysed using the Cox regression method. Two separate analyses were conducted to analyse host survival. The first was performed prior to the detection of the acanthella stage, i.e. during the major growth period of the parasite (Duclos et al., 2006). The second analysis was carried out between the detection of acanthella and the end of the experiment, when the parasite growth is slower. During the growth phase of the parasite, we first tested the effect of the exposure dose with infections involving parasites from the Ouche River and included the following factors: dose (control/dose 25/dose 50/dose 100/dose 200 eggs), a random 'aquarium' factor nested within the 'dose' factor (to take into account the variability between aquaria within each dose category), sex of the host and the interaction between dose and sex. During this growth phase, we also tested the effect of parasite origin using exposures with the dose of 100 eggs, for the following factors: parasite origin (control/parasites from the Ouche River/parasites from the Vingeanne River), a random 'aquarium' factor nested within the 'origin', sex of the host and the interaction between origin and sex. The Cox regression model analysing survival between the acanthella and cystacanth stages included the following fixed factors: treatment (control/infection by parasites from the Ouche River/infection by parasites from the Vingeanne River), sex of the host, parasite intensity and the interaction between treatment and sex.

Prevalence was analysed using two separate logistic regressions. The first tested the effect of exposure dose for parasites from the Ouche River, including exposure dose and sex of host as fixed factors, and a random 'aquarium' factor nested within the 'dose' factor. The second tested the effect of parasite origin for the dose 100, including parasite origin and sex of host as fixed factors, and an 'aquarium' factor nested within the 'dose' factor. Parasite intensity was analysed only for infected individuals using logistic regressions, but the nested 'aquarium' factor was not entered into the model, because in series where infection was weak, infected individuals came from too few aquaria.

Development time to the "early cystacanth" stage was analysed using an ANOVA, after data transformation using a Box–Cox procedure to meet normality and homoscedasticity (Quinn and Keough, 2002). The model included the following fixed factors: origin of parasites (Ouche/Vingeanne), intensity of infection (one parasite/two parasites/more than two parasites), sex of the host (males/females) and the interactions among these factors.

Failure in parasite development was estimated by counting the number of parasites that remained at the acanthella stage until the end of the experiment (145 days), and were thus considered dead. The proportion of dead parasites was then analysed using an ANOVA (on arcsin-square root transformed data) with the same factors as those selected for the development time analysis: origin of parasites, intensity of infection, sex of the host and interactions.

2.4. Behavioural measurements

The reaction to light of isolated individuals was measured as described in Perrot-Minnot (2004) and Franceschi et al. (2007). A single gammarid was introduced into a horizontal tube filled with well-aerated water, with a dark zone and a light zone of equal size. After a 5 min period of acclimatisation, the position of the gammarid was recorded each 30 s for 5 min. At each observation, a score of 0 was given if the individual was located in the dark area and a score of 1 was given if it resided in the lighted area. At the end of each trial, summed scores ranged from 0 (always in the dark, strongly photophobic) to 10 (always in the light, strongly photophilic). Phototaxis was measured three times for each individual during parasite development: the day following the appearance of acanthella (acanthella stage), the day after the parasite reached the cystacanth stage (early cystacanth stage), and 4 weeks after this second measurement (late cystacanth stage). At the end of the experiments, all host individuals were dissected and measured (body height at the level of the fourth coxal plate basis; Bollache et al., 2002) using a Nikon SMZ 1500 stereoscopic microscope and Lucia G 4.81 software.

Phototaxis scores met neither normality nor homoscedasticity conditions, even after data transformation. We therefore used non-parametric tests for analyses. The Wilcoxon rank-sum test was used for bivariate analyses and the Kruskal–Wallis test for multivariate analyses. To test if there were individual changes in phototaxis during parasite development, analyses were carried out using the non-parametric paired Wilcoxon signed-rank test. Differences in phototaxis scores between series were analysed using ANOVAs, after data transformation using a Box–Cox procedure to meet normality and homoscedasticity.

The ANOVA models presented are the one minimising the Akaike Information Criterion (AIC, Quinn and Keough, 2002). Post-hoc tests were Tukey Honest Significant Difference (HSD) ($\alpha = 0.05$). Analyses were performed using JMP 6.0 Software (SAS Institute Inc.) and all tests were two-tailed. P values < 0.05 were considered significant.

3. Results

3.1. Characteristics of infection and effects on host survival

With parasites from the Ouche River, the higher the exposure dose, the higher the prevalence (Fig. 1a, Whole model: $\chi^2_{38} = 109.53$, $P < 0.0001$; dose: $\chi^2_3 = 41.64$,

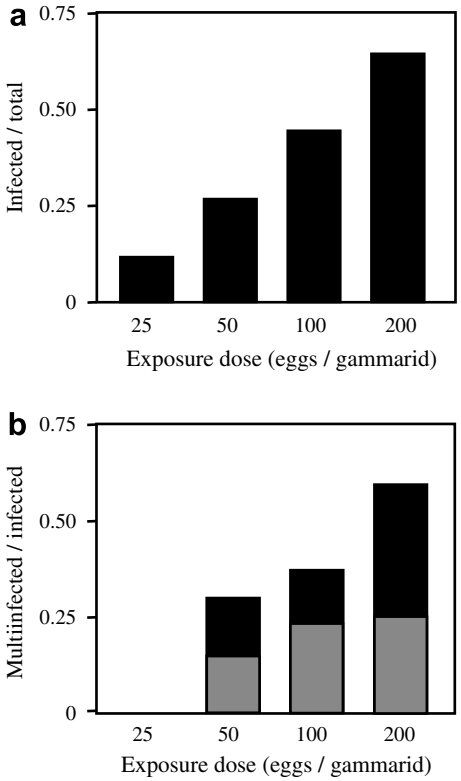

Fig. 1. Effect of the dose of *Pomphorhynchus laevis* eggs supplied to *Gammarus pulex* on parasite prevalence (a) and proportion of multiply infected hosts (b) 75 days post-exposure. The intensity was expressed as the proportion of hosts infected by more than one parasite (grey part of the bar: two parasites; black part of the bar: more than two parasites, amongst which 69.3% were infected by three parasites).

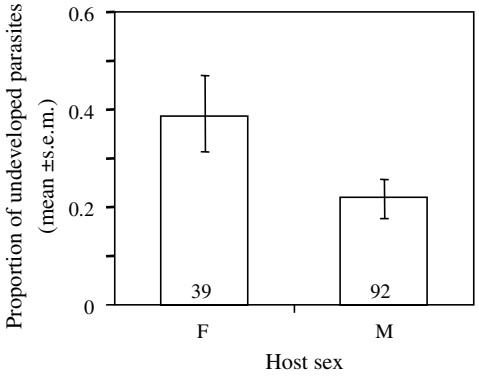

Fig. 2. Proportion of *Pomphorhynchus laevis* that did not reach the cystacanth stage, according to host sex. F: females, M: males. The numbers on the histograms are sample sizes.

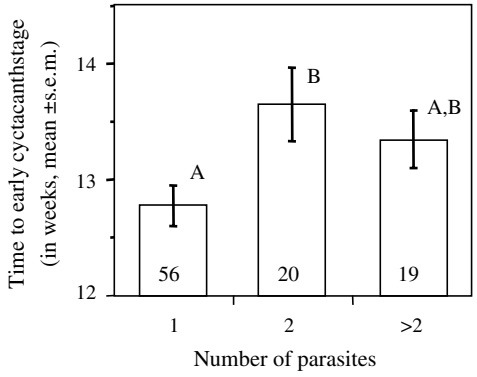

Fig. 3. Development time of *Pomphorhynchus laevis* (time to reach the early cystacanth stage), compared with the number of parasites hosted by the gammarid. The numbers in the histograms are sample sizes. Levels not connected by the same letter are significantly different after a Tukey HSD post-hoc test ($\alpha = 0.05$).

$P < 0.0001$; aquarium [dose]: $\chi^2_{34} = 60.71$, $P = 0.003$; sex: $\chi^2_1 = 0.36$, $P = 0.55$) and the intensity (Fig. 1b, Whole model: $\chi^2_4 = 17.67$, $P = 0.001$; dose: $\chi^2_4 = 16.06$, $P = 0.001$; sex: $\chi^2_1 = 0.68$, $P = 0.41$). The maximal intensity found was eight parasites in an individual exposed to the maximal dose. For the 100 dose, there was no significant effect of the parasite origin or host sex, either on prevalence (Whole model: LR $\chi^2_{20} = 29.82$, $P = 0.07$) or intensity (Whole model: LR $\chi^2_2 = 0.51$, $P = 0.77$).

The proportion of parasites that did not reach the cystacanth stage was influenced by host sex (ANOVA, model reduced from a model including host sex, parasite origin, parasite intensity and their interactions: Global model: $F_{4,130} = 3.13$, $P = 0.02$; effect of parasite origin: $F_{1,130} = 3.08$, $P = 0.08$; effect of parasite intensity: $F_{2,130} = 2.26$, $P = 0.11$; effect of host sex: $F_{1,130} = 4.08$, $P = 0.04$). The proportion of failure was higher in females than in males (Fig. 2). Hosts in which no parasite developed were removed from the analyses described below. In infected individuals, the time necessary to reach the early cystacanth stage was influenced by multiple infection status ($F_{2,92} = 3.97$, $P = 0.02$), whilst host sex, parasite origin and all interactions were not significant and thus removed from the statistical model. Parasites developed faster in single infections, whereas parasites in doubly infected hosts had the longest development time (Fig. 3).

We found no significant effect of *P. laevis* infection on host survival rate within the limit of the doses investigated, either during the parasite growth phase or during the development from acanthella to cystacanth. During parasite growth, the two separate Cox regression models revealed no effect of parasite dose or parasite origin, but revealed an effect of host sex in the two cases (Whole model testing the dose effect: $\chi^2_{54} = 211.34$, $P < 0.0001$; dose: $\chi^2_4 = 3.08$, $P = 0.54$; aquarium [dose]: $\chi^2_{45} = 80.53$, $P = 0.0009$; sex: $\chi^2_1 = 133.05$, $P < 0.0001$; interaction between dose and sex: $\chi^2_4 = 2.68$, $P = 0.61$; Supplementary Fig. S1. Whole model testing the parasite origin: $\chi^2_{32} = 125.71$, $P < 0.0001$; parasite origin: $\chi^2_2 = 0.08$, $P = 0.96$; aquarium [origin]: $\chi^2_{27} = 40.80$, $P = 0.04$; sex: $\chi^2_1 = 84.19$, $P < 0.0001$; interaction between origin and sex: $\chi^2_2 = 1.63$, $P = 0.44$; Supplementary Fig. S2). About 20% of females versus 80% of males survived after 75 days. During post-acanthella development, we found no significant effect on host survival (Cox regression whole model: $\chi^2_8 = 14.12$, $P = 0.08$).

3.2. Phototaxis behaviour

In experiments with parasites from the Ouche River, we observed no effect of exposure dose on the phototaxis behaviour of hosts, either at the acanthella stage (Kruskal–Wallis: $\chi^2_3 = 2.00$, $P = 0.57$, $n = 69$), at the early cystacanth stage ($\chi^2_3 = 0.86$, $P = 0.84$, $n = 74$) or the late cystacanth stage ($\chi^2_3 = 3.64$, $P = 0.30$, $n = 46$). For further analyses, we therefore pooled all animals infected with *P. laevis* from the Ouche River.

At the acanthella stage, there was no difference between gammarids infected by parasites from the Ouche River and uninfected controls (Wilcoxon test: $Z = -0.68$, $P = 0.49$), nor between those infected by parasites from the Vingeanne River and uninfected controls ($Z = 0.14$, $P = 0.88$) (Fig. 4). When parasites reached the early cystacanth stage, infected *G. pulex* – both those infected by parasites from the Ouche River ($Z = -6.56$, $P < 0.0001$) and from the Vingeanne River ($Z = 4.00$, $P < 0.0001$) – were more strongly attracted to light than uninfected ones (Fig. 4). The same phenomenon was found, in an accentuated form, at the late cystacanth stage (Fig. 4) ($Z = 8.30$, $P < 0.00001$ and $Z = 5.71$, $P < 0.0001$, for parasites from the Ouche and from the Vingeanne Rivers, respectively). At the end of the experiment, gammarids that remained uninfected in the experimental series obtained a similar phototaxis score to the uninfected control individuals (Fig. 4, Kruskal–Wallis: $\chi^2_2 = 3.37$, $P = 0.19$). For all these analyses, host size was not correlated with phototaxis score (Spearman correlations, all $P > 0.10$, results not shown), and phototaxis was not significantly different between host sexes (Wilcoxon tests, all $P > 0.09$, results not shown).

Analysis by repeated measures showed that there was an unambiguous increase in the phototaxis score between the acanthella and the young cystacanth stages in infected individuals (Wilcoxon signed-rank test (WSRT): $P < 0.0001$, $n = 65$ for individuals infected with parasites from the Ouche River; $P = 0.01$, $n = 19$ in individuals infected with parasites from the Vingeanne River) (Fig. 4). An increase was also observed between early and late cystacanth stages (WSRT: $P < 0.0001$, $n = 45$ for individuals infected with parasites from the Ouche River; $P = 0.002$, $n = 16$ for individuals infected with parasites from the Vingeanne River). This increase was still present when multiply infected individuals were removed from the analysis (WSRT: $P < 0.0001$, $n = 29$ for individuals infected with parasites from the Ouche River; $P = 0.01$, $n = 12$ for individuals infected with parasites from the Vingeanne River), indicating that this pattern is not due to a potential sampling effect on multiply infected gammarids, where the ratios of mature to immature cystacanths could have increased with age. Such an increase was not found in uninfected control individuals during the same periods (WSRT: $P = 0.48$, $n = 40$ and $P = 0.81$, $n = 32$, respectively) (Fig. 4). Concomitantly with this increase in median phototaxis scores between early and late cystacanths, variances around the average values were also different (Levene's test: $F_{1,155} = 21.16$, $P < 0.0001$), the variation in behavioural manipulation being higher in young cystacanths than in mature ones (Fig. 4).

The increase in phototaxis scores between the two first stages (acanthella and early cystacanth) was influenced by the infection intensity and also the parasite origin (ANOVA, model reduced from a model including host sex, parasite origin, parasite intensity and their interactions: Global model: $F_{3,80} = 5.54$, $P = 0.002$; effect of parasite intensity: $F_{2,80} = 6.41$, $P = 0.003$; effect of parasite origin: $F_{1,80} = 4.37$, $P = 0.04$). Parasites from the Ouche River induced a more marked behavioural change than those from the Vingeanne River (Fig. 5a). *Gammarus pulex* infected by two cystacanths of *P. laevis* exhibited a greater change in phototaxis score than the singly infected ones, whilst individuals infected by more than two parasites showed an intermediate score between these two extremes

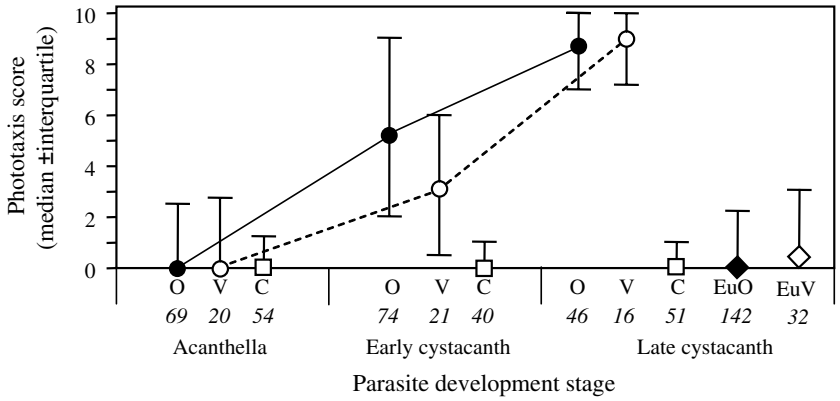

Fig. 4. Phototaxis scores of *Gammarus pulex* individuals according to their infection status and to the developmental stages of the parasites. C: control series (individuals not exposed to the infection); O: individuals infected by parasites from the Ouche River; V: individuals infected by parasites from the Vingeanne River; EuO: individuals exposed to parasites from the Ouche River that remained uninfected; EuV: individuals exposed to parasites from the Vingeanne River that remained uninfected. Sample sizes are in italics. Variations in sample size between two developmental stages result from individuals that were not detected as infected at the previous stage (e.g. unseen acanthella) or death of individuals. See text (Section 3.2) for sample sizes used in repeated measure analyses.

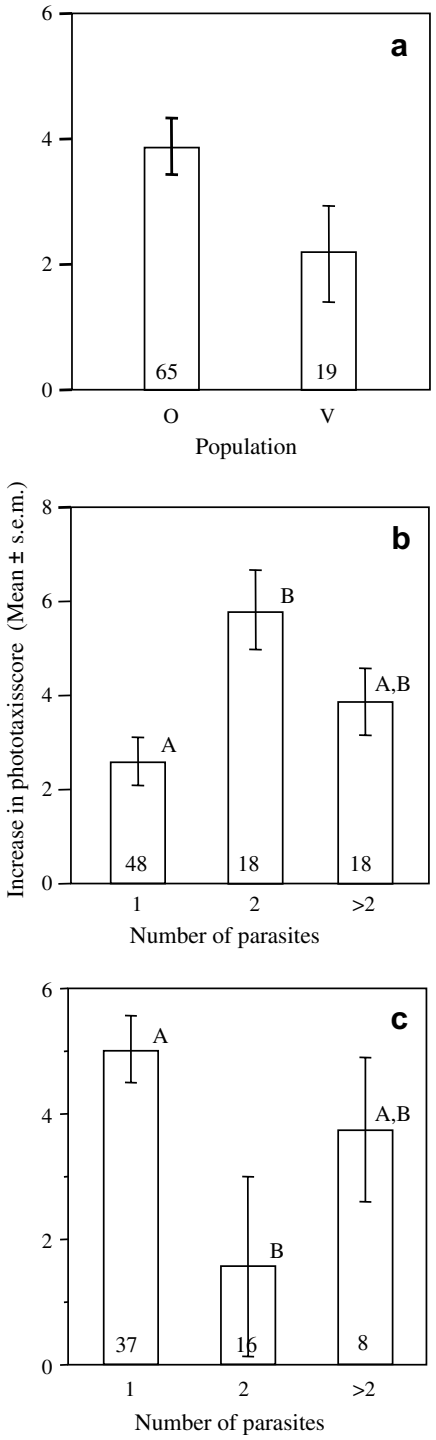

Fig. 5. Increase in phototaxis score in infected *Gammarus pulex* compared with the developmental stages of *Pomphorhynchus laevis*. (a) Increase in phototaxis score between the "acanthella" and "early cystacanth" stage, compared with the origin of the parasites; (b) increase in phototaxis score between the "acanthella" and "early cystacanth" stage, compared with the number of parasites in each host; (c) increase in phototaxis score between "early cystacanth" and "late cystacanth" stages, compared with the number of parasites in each host. O: Ouche River, V: Vingeanne River. The numbers in the histograms are sample sizes. Levels not connected by same letter are significantly different after a Tukey HSD post-hoc test ($\alpha = 0.05$).

(Fig. 5b). The increase in phototaxis score between the two cystacanth stages (early and late) was also influenced by the number of parasites, but not by parasite origin (ANOVA, model reduced from a model including host sex, parasite origin, parasite intensity and their interactions: effect of parasite intensity: $F_{2,58} = 3.19$, $P = 0.048$). As a mirror of the preceding stage, singly infected hosts showed a greater increase than those with two parasites (Fig. 5c).

4. Discussion

This study revealed that the larval maturation of the acanthocephalan *P. laevis* and its intensity are sources of variation in the behavioural manipulation induced in its host *G. pulex*. Moreover, our experimental procedure allowed the analysis of several characteristics of the infection.

We found an increase in parasite prevalence and intensity related to an increase in exposure dose. However, the doubling of the exposure dose induced a doubling of prevalence up to the dose of 100 eggs per gammarid. The mean intensities calculated from our experiments for the first three doses (between 1 and 1.5 parasites per host) are similar to those found in the wild by Outreman et al. (2002), but the average value of two parasites per host reached in our highest exposure dose has never been found in the wild. We found no significant effect of parasite dose or parasite intensity on host survival. Experimental infection studies in the amphipod host *Hyalella azteca* had revealed pathogenic effects of acanthocephalans, with no effect on survival when parasites were present at "low intensity" (in fact intensities less than three parasites per host, comparable with those obtained in the present study) and an increase in mortality when the levels of infection were very high (Duclos et al., 2006). However, since this last finding was obtained with intensities far higher than those encountered in natural populations (>16 parasites per host, Duclos et al., 2006), its biological significance is questionable. The continuum of parasite intensities obtained in the present study allowed us to study the outcome of multiple infections on parasite development. We found that multiple infections increased the average parasite development time. This result suggests there is competition amongst parasites for resources within the host, as observed in other host–parasite systems (Michaud et al., 2006; Benesh and Valtonen, 2007), which can have potential consequences on parasites fitness (discussed below with the effect of multiple infections on *G. pulex* behavioural manipulation). However, parasite development failures were not affected by parasite intensity, but rather by host sex, failures being more frequent in female hosts. This is consistent with the hypothesis of a higher investment of females in the immune function (Rolff, 2002), a situation already observed in wild populations of *G. pulex* (Rigaud and Moret, 2003; Cornet et al., 2007). Overall, our results suggest that the development of *P. laevis* in *G. pulex* is modulated in multiple infections and by host characteristics (gender).

The intensity of the behavioural change induced by *P. laevis* in its host was strongly influenced by parasite developmental stage. We believe our repeated measurement experiment provides the first formal confirmation of the observations made on natural infections in a similar host–parasite system (Bethel and Holmes, 1973): behavioural change in the intermediate host is only observed when the parasite becomes able to infect the definitive host. In addition, we also found an effect of cystacanth age, a trait impossible to detect using natural infections. Older cystacanths induced a higher degree of manipulation compared with young ones, with a reduced variation around the high phototaxis scores. This has two main implications. At a mechanistic level, it suggests that the proximal factor responsible for behavioural change (Tain et al., 2006) increases or accumulates with cystacanth age. At an ecological level, this increase in behavioural change may explain a part of the variation observed in natural populations; indeed, each sample taken at a given moment could be a mixture of infections of different ages. Given that the phototaxis of wild-caught infected *G. pulex* rarely reach the high median values and the low variance obtained at the end of our experiments (Bauer et al., 2000; Cézilly et al., 2000; Perrot-Minnot, 2004), it seems that old infections are not commonly found in the wild. This is in line with the findings of Lagrue et al. (2007a) who showed that behavioural changes favour predation by fish, and therefore do not allow individual infections to be maintained for a long time in *G. pulex* populations.

Our study also revealed an effect of infection intensity on host behaviour manipulation. *Gammarus pulex* infected by two parasites produced a higher phototaxis score than the singly infected ones, but a higher parasitic intensity induced an intermediate score between these two extremes. No such difference was detected in the study of Cézilly et al. (2000), probably due to the absence of control of the infection dynamics. Such a phenomenon probably points to a density-dependent effect, as previously evidenced for some parasites (Ebert et al., 2000; Brown et al., 2003). With up to two parasites, the effect on host behaviour seems to be cumulative, but above this intensity, parasites may compete for resources, limiting their individual capacity to change host behaviour. Alternatively, there may be a greater level of damage inflicted on the host at high parasite intensities. Such damage nevertheless seems to be limited, as we found no effect of parasite infective dose or parasite intensity on host survival. Parasite intensity has contrasting effects on parasite development time (negative effect) and on the ability of young parasites to manipulate host behaviour (positive effect) with, in both cases, a maximum reached at intermediate intensities. These effects have potential consequences for parasite fitness. Indeed, parasites in double infections reach their infective stage later than parasites in single infections. Everything else being equal, such relatively late manipulation increases the probability of the gammarid dying before its predation by the final host, which represents a cost for the parasite. On the other hand, the stronger manipulation may decrease the time required to reach a final host and somehow compensate for the cost, a phenomenon yet to be formally confirmed (however, see discussion in Poulin, 2007 about optimal "manipulative effort").

Finally, it is worth noting that the occurrence of behavioural manipulation at the infective stage and the subsequent increase with parasite age were found for the two parasite populations, suggesting wide-ranging phenomena across *P. laevis* populations. *Pomphorhynchus laevis* from the Ouche River nevertheless induced much stronger behavioural modification than those from the Vingeanne River at the early cystacanth development stage. As the parasite eggs used in our experiments came from two different populations, this difference could be due to differences in parasite genotypes, or to differences in environmental conditions during egg maturation. We attempted to limit the environmental variation by sampling parasites in the same definitive host species and by taking eggs from parasite females of approximately the same condition of maturity, and we found no inter-population difference in parasite ability to infect their hosts, but further experiments are needed to address the causes of this aspect of variation due to parasites.

In summary, our experimental procedure revealed variation in the intensity of behavioural manipulation due to acanthocephalan infection. The intensity increases with larval parasite age, with parasite intensity under a threshold, and is variable according to parasite origin. This is the first step towards our understanding of the variation of behavioural manipulation. However, we investigated the effects on one host population only, a population allopatric with the parasites used. This had the advantage of demonstrating the effect of infection on naïve hosts, but there is now increasing evidence that local adaptation plays a crucial role in host–parasite evolution (Kawecki and Ebert, 2004). The intensity of behavioural manipulation could also be modulated by local adaptation of parasites to their hosts (or the reverse, hosts being locally adapted to their parasites, see Moret et al., 2007). This hypothesis therefore needs to be tested by experiments involving different hosts and parasite populations (Kawecki and Ebert, 2004).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpara.2008.01.003.

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