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Differential influence of *Pomphorhynchus laevis* (Acanthocephala) on brain serotonergic activity in two congeneric host species

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The physiological mechanisms by which parasites with complex life cycles manipulate the behaviour of their intermediate hosts are still poorly understood. In Burgundy, eastern France, the acanthocephalan parasite *Pomphorhynchus laevis* inverses reaction to light in its amphipod host *Gammarus pulex*, but not in *Gammarus roeseli*, a recent invasive species. Here, we show that this difference in manipulation actually reflects a difference in the ability of the parasite to alter brain serotonergic (5-HT) activity of the two host species. Injection of 5-HT in uninfected individuals of both host species was sufficient to inverse reaction to light. However, a difference in brain 5-HT immunocytochemical staining levels between infected and uninfected individuals was observed only in *G. pulex*. Local adaptation of the parasite to the local host species might explain its inability to manipulate the behaviour and nervous system of the invasive species.

Keywords: host manipulation; 5-HT; *Gammarus* spp.; biological invasion

1. INTRODUCTION

Several parasite species with complex life cycles, such as trematodes, cestodes and acanthocephalans, bring about changes in the physiology and/or behaviour of their intermediate hosts that appear to enhance trophic transmission to final hosts (Moore 2002; Thomas *et al.* 2005). This phenomenon known as ‘host manipulation’ is currently regarded as a classic example of an extended phenotype (Dawkins 1982). However, two important aspects of host manipulation deserve further consideration. First, although the ecological and the evolutionary aspects of parasitic manipulation have drawn considerable attention (see reviews in Moore 2002; Thomas *et al.* 2005), its mechanistic basis remains poorly understood (Adamo 2002; De Jong-Brink & Koene 2005; Klein 2005). Second, although behavioural manipulation can show extensive variation between infected individuals belonging to the same host population (Cézilly *et al.* 2000; Tain *et al.* 2006), between host populations (Kennedy 2003) and between host species (Bauer *et al.* 2000), the functional significance of variability

in host manipulation remains largely unexplored (Cézilly & Perrot-Minnot 2005).

In Burgundy (eastern France), the acanthocephalan *Pomphorhynchus laevis* exploits two congeneric and sympatric amphipod species: the native *Gammarus pulex* and the recent invader, *Gammarus roeseli*. The parasite appears to survive and grow equally well in both the hosts, as indicated by the frequency and size of mature cystacanths in both the hosts (M. J. Perrot-Minnot & F. Cézilly 2006, unpublished data). Uninfected *G. pulex* typically show strong photophobic behaviour, while infected individuals are strongly photophilic (Cézilly *et al.* 2000). Recently, it has been shown that altered reaction to light in *G. pulex* infected by *P. laevis* is related to brain serotonin (5-HT) levels (Tain *et al.* 2006). Injection of 5-HT (but not that of octopamine) in uninfected *G. pulex* induced attraction to light, thus mimicking the effect of infection. Furthermore, infected individuals showed a 40% increase in brain 5-HT immunoreactivity. By contrast, infected and uninfected *G. roeseli* were equally photophobic, suggesting that *G. roeseli* is able to resist manipulation attempts by *P. laevis* (Bauer *et al.* 2000). However, the effect of *P. laevis* on brain 5-HT activity in *G. roeseli* has not been documented. If the alteration of brain 5-HT activity underlies host manipulation by *P. laevis*, one would expect to observe no change in brain 5-HT immunoreactivity in *G. roeseli* infected by *P. laevis*. Alternatively, reaction to light might not be functionally linked to 5-HT in *G. roeseli*, thus explaining the apparent resistance of the host species to manipulation by *P. laevis*. The present study was specifically designed to address this point. We used both injections of 5-HT followed by behavioural assays and immunocytochemistry to assess the relationship between 5-HT levels and reaction to light in both infected and uninfected *G. pulex* and *G. roeseli*.

2. MATERIAL AND METHODS

(a) Collection and maintenance of *Gammarus pulex* and *Gammarus roeseli*

Samples were collected in Burgundy (eastern France) in July 2003 and May 2004 (for behavioural and pharmacological assays), and in August 2005 (for studies of 5-HT brain immunoreactivity). Gammarids were maintained in laboratory aquariums at 14°C, under a constant (12 : 12 h) photoperiod.

(b) Behavioural assays

The apparatus used to measure phototaxis consisted of a horizontal, half light–half dark plastic tube (23 × 3 cm) containing aerated dechlorinated and UV-treated water at 14°C (see Tain *et al.* 2006 for details). Infected and uninfected individuals were placed in the apparatus and their position recorded every 30 s over a 5 min period. Behaviour was scored as the number of times each individual was recorded in the light half of the tube. Phototaxis scores therefore ranged from 0 (strongly photophobic) to 10 (strongly photophilic). Sample sizes are shown within individual figures.

(c) Injections

Excess, but non-lethal, doses of 5-HT (at 2.35×10^{-3} M; $5 \mu\text{g } \mu\text{l}^{-1}$ in 1 μl of vehicle solution) were injected into infected and uninfected individuals as previously described (Helluy & Holmes 1990; Tain *et al.* 2006). Controls consisted of octopamine (at 2.64×10^{-3} M; $5 \mu\text{g } \mu\text{l}^{-1}$) and vehicle solution (Crustacean Ringer solution) injections.

(d) 5-HT activity and neuronal architecture

Previous studies have shown that immunocytochemistry provides a reliable estimate of 5-HT activity in invertebrates (Fickbohm *et al.* 2001; Molaei & Lange 2003; Benzid *et al.* 2006).

(i) Brain preparation and immunocytochemistry

Individuals (sampled in August 2005) exhibiting high (8–10) or low (0–2) phototaxis scores were selected for immunocytochemistry.

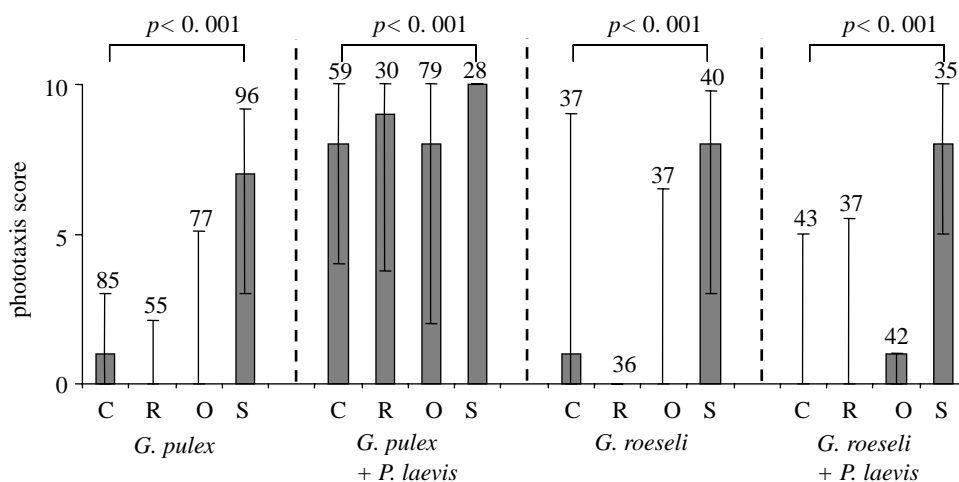


Figure 1. Phototactic behaviour of *P. laevis* infected/uninfected *G. pulex* and *G. roeseli* (C) and those injected with Ringer solution (R), octopamine ($5 \mu\text{g } \mu\text{l}^{-1}$) (O) or 5-HT ($5 \mu\text{g } \mu\text{l}^{-1}$) (S). Figure shows median phototaxis scores, interquartiles, sample size and significance as determined by Kruskal–Wallis test. All comparisons to control individuals (with the exception of serotonin injections) are non-significant (*post hoc* tests, $p > 0.05$).

Brains of infected/uninfected male *G. pulex* and *G. roeseli* were dissected in cold crustacean Ringer solution and fixed overnight in 4% paraformaldehyde in phosphate-buffered saline (PBS). Brains were then rinsed in PBS with 0.2% Triton X-100 (PBSTX) and incubated for 4 h in 4% goat serum. Brains were washed then incubated overnight at 4°C with rabbit anti-5-HT (Sigma diluted to 1 : 500 in PBSTX). Once rinsed, brains were incubated for 4 h with Alexa Fluor 488 goat anti-rabbit (Molecular probes) diluted to 1 : 50 in PBSTX. After washing, brains were viewed with a Leica confocal microscope. Sample sizes are shown within figures.

(ii) *Image analysis and optical microdensitometry*

Individual brains were scanned within a single frame using a 20× objective. As previously described, brains were imaged from 50 regularly spaced horizontal scans and the composites were examined for morphological and serotonergic differences between infected and uninfected controls using Leica Confocal Software-LITE (LCS-lite; Tain *et al.* 2006). To give an estimation of 5-HT within individual brains, we measured the level of labelling within the region encompassing the tritocerebrum from the lateral projections to the medial projections and ventrally to include the TGN cell body (Tain *et al.* 2006).

(e) *Statistical analysis*

All statistical analyses were carried out using JMP software (v. 5, SAS Institute, Cary, NC, USA). Kruskal–Wallis tests and *post hoc* comparisons (Siegel & Castellan 1988) were used to assess behavioural differences, as behavioural scores were not normally distributed. ANOVA was used to determine differences between levels of 5-HT labelling. *Post hoc* tests were used to determine 2×2 differences. Data were log-transformed prior to ANOVA analysis to meet normality criteria.

3. RESULTS

Scores for phototaxis differed between groups (control, ringer, octopamine and serotonin) in both uninfected and infected gammarids of each species (Kruskal–Wallis test, $p < 0.001$ in all cases; figure 1). Uninfected *G. pulex* showed strong photophobia (figure 1). By contrast, infected *G. pulex* showed a reversal of this behaviour, being significantly more photophilic than uninfected individuals (Mann–Whitney test, $Z = 6.29$, $p < 0.001$). Uninfected *G. roeseli* also exhibited negative phototaxis, although with a larger variation compared to uninfected *G. pulex*. However, reaction to light did not differ between uninfected and infected *G. roeseli* (Mann–Whitney test, $p = 0.20$).

Only the injection of 5-HT resulted in an alteration of phototaxis in both uninfected *G. pulex* and

uninfected *G. roeseli* (*post hoc* test, $p < 0.001$ in both the cases), with individuals showing a reversal from photophobic to photophilic behaviour. Control individuals (both saline and octopamine injected) failed to show any alteration in reaction to light (figure 1). In addition, 5-HT injection tended to enhance photophilic behaviour in infected *G. pulex* (*post hoc* test, $p = 0.056$), and induced it in infected *G. roeseli* (*post hoc* test, $p < 0.001$), whereas control injections yielded no significant change (figure 1).

Finally, brain 5-HT immunocytochemical staining was significantly higher in infected *G. pulex* compared with uninfected individuals (ANOVA; $F_{1,18} = 12.5$, $p = 0.002$; figure 2) whereas no such difference was observed when comparing brain 5-HT immunocytochemical staining between uninfected and infected *G. roeseli* (ANOVA; $F_{1,13} = 0.005$, $p = 0.95$; figure 2).

4. DISCUSSION

Specificity in infection (Kennedy 2006) and the ability to manipulate the behaviour of intermediate hosts (Moore 2002; Thomas *et al.* 2005) are the two important aspects of the evolution of complex life cycles (Parker *et al.* 2003). However, little is known about the specificity in manipulation of hosts by parasites (see also Moore & Gotelli 1996; Cézilly & Perrot-Minnot 2005). Recently, Tain *et al.* (2006) provided evidence that three different acanthocephalan species sharing the same intermediate host had different effects on host behaviour. Specifically, they found that the two fish acanthocephalans, *P. laevis* and *Pomphorhynchus tereticollis*, did concomitantly alter reaction to light and brain 5-HT immunoreactivity in *G. pulex*, whereas no such effects were observed in gammarids infected with the bird acanthocephalan parasite *Polymorphus minutus*. Here, we address the specificity of behavioural manipulation in two congeneric intermediate hosts infected by a single common parasite (Bauer *et al.* 2000). Our results show that the differential susceptibility of *G. pulex* and *G. roeseli* to manipulation by *P. laevis* (Bauer *et al.* 2000) is reflected in the contrasting effect of the parasite on brain 5-HT immunocytochemical

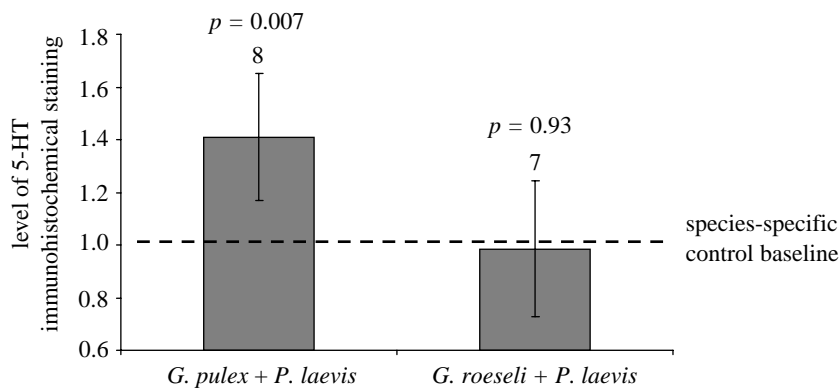


Figure 2. Optical densitometric measures of 5-HT immunoreactivity within the tritocerebral region of *G. pulex*/*G. roeseli* infected with *P. laevis*, relative to uninfected controls (dotted line). Figures show standardized means against species-specific controls, associated 95% CI, sample size and significance as determined by ANOVA.

staining in the two species. Moreover, 5-HT injections reversed the phototactic responses of uninfected individuals of both *G. pulex* and *G. roeseli*.

Our results confirm that 5-HT is involved in the alteration of reaction to light in arthropod hosts infected by manipulative parasites (Helluy & Thomas 2003; Tain *et al.* 2006). 5-HT injections reversed the phototactic responses in both *G. pulex* and *G. roeseli*. Since large doses of 5-HT had to be injected in order to mimic the effect of infection (see Helluy & Holmes 1990), the possibility exists that 5-HT or its metabolites were binding with non-5-HT receptors to produce the observed change in host behaviour. However, 5-HT brain immunocytochemical staining was significantly augmented in manipulated hosts. A classical interpretation of the dramatic increase in brain 5-HT immunoreactivity observed in *P. laevis*-infected *G. pulex* would be that the chemical is not being released and is therefore building up inside neurons. In this case, one would expect that removing 5-HT, rather than adding it, would mimic the effect of infection. However, it is possible that *P. laevis* can induce *G. pulex* to massively increase its production of 5-HT.

As *G. roeseli* individuals injected with 5-HT showed strong reversal of phototaxis, the inability of *P. laevis* to reverse reaction to light in *G. roeseli* was clearly not due to the absence of a link between 5-HT and phototaxis in the intermediate host species. We therefore conclude that *G. roeseli* can, to some extent, resist manipulative attempts by *P. laevis*. However, since little is known about the precise molecular pathways by which manipulative parasites modify the behaviour and nervous system of their hosts (De Jong-Brink & Koene 2005), it is only possible to speculate about the origin of apparent resistance to manipulation by *P. laevis* in *G. roeseli*. Considering the interplay between the immune system and the nervous system, Adamo (2002) argued that host manipulation by parasites may have evolved as a consequence of the interaction between parasites and the immune system of their host. The absence of behavioural alteration in *G. roeseli* infected by *P. laevis* might therefore be a direct consequence of the reduced ability of the parasite to evade its host's immune defences. Interestingly, Rigaud & Moret (2003) observed that the level of phenoloxidase enzyme activity was lower in *G. pulex* infected by *P. laevis* compared with

uninfected individuals, whereas the opposite was true in *G. roeseli*. This suggests that acanthocephalans may be adapted to suppress the immune response of their local host, but not that of their invasive host (see also Hynes & Nicholas 1958). In Burgundy, *G. roeseli* is a recent invading species of Ponto-Caspian origin (Jazdewski 1980). One possibility is that the process of invasion in itself selects for particular immunocompetent genotypes (see Lee & Klasing 2004). Alternatively, local strains of *P. laevis* may have coevolved with *G. pulex*, which constitutes the predominant intermediate host in rivers of Burgundy, and specialization in manipulation might then be regarded as some sort of local adaptation. For instance, Kennedy (2003) reported that reversed phototactic behaviour was commonly observed in *G. pulex* infected by *P. laevis* in two rivers in England where the parasite has been present for a long time, but was absent in infected conspecifics from a river where the parasite has only recently been introduced. The absence of behavioural alteration in *G. roeseli* might also be interpreted in terms of optimal manipulative effort. Since uninfected *G. roeseli* show a larger variation in photophobic behaviour than uninfected *G. pulex* (see interquartile range in figure 1), there might be little benefit in manipulating a host that is already likely to be exposed to predation, particularly if manipulation incurs a cost (see Poulin *et al.* 2005).

To understand the coevolution of manipulation in parasite and host lineages, and to establish its adaptive function, it is crucially important that the mechanistic basis of parasite manipulation is unravelled (Cézilly & Perrot-Minnot 2005). In this respect, interactions between acanthocephalan parasites and their amphipod intermediate hosts might prove to be a particularly rewarding model for future investigations.

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