Intraspecific conflict over host manipulation between different larval stages of an acanthocephalan parasite

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Abstract
Competitive interactions between cointecting parasites are expected to be strong when they affect transmission success. When transmission is enhanced by altering host behaviour, intraspecific conflict can lead to ‘cointection exclusion’ by the first-in parasite or to a ‘sabotage’ of behavioural manipulation by the youngest noninfective parasite. We tested these hypotheses in the acanthocephalan parasite Pomphorhynchus laevis, reversing phototaxis in its intermediate host Gammarus pulex. No evidence was found for cointection exclusion in gammarids sequentially exposed to infection. Behavioural manipulation was slightly weakened but not cancelled in gammarids infected with mixed larval stages. Therefore, cointecting infective and noninfective larvae both suffered competition, potentially resulting in delayed transmission and increased risk of mortality, respectively. Consequently, noninfective larva is not just a ‘passive passenger’ in the manipulated host, which raises interesting questions about the selective pressures at play and the mechanisms underlying manipulation.

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Introduction
Intraspecific or interspecific antagonism in resource exploitation arises from either joint use (competition) or distinct and incompatible uses of a common resource. Under particular conditions, exploitation of a common resource can also promote cooperation and increase the per capita profitability of the resource. In host–parasite systems, the ‘host as a resource’ generally provides multiple services for parasites, notably nutrients and habitat. It is also used as a ‘vehicle’ for transmission (see the ‘host-as-vehicle’ analogy of Lafferty, 1999 and Lafferty et al., 2000), particularly by parasites with complex life cycles. The conditions for antagonism and cooperation to evolve between such parasites when they exploit a common host may therefore be broader and more diverse than in typical cases of competition simply for food or mates.

Mixed infections involving at least two species of parasites (heterospecific infections) or two strains (mono-specific infections) are common in nature (Read & Taylor, 2001). Apart from competition for establishment, growth or reproduction (Parker et al., 2002; Lively, 2009; Mideo, 2009), one obvious opportunity for antagonism over host exploitation lies in the different optimal strategies for transmission of parasite individuals or species. Cointecting parasites may have divergent interests over the optimal timing or place to be to reach their next hosts or in the optimal level of virulence. Such evolutionary conflicts have been studied predominantly in ‘manipulative’ parasites with complex life cycles, which induce phenotypic changes in their intermediate host in ways that increase the probability of completing their life cycle, a phenomenon called ‘behavioural manipulation’ (see Moore, 2002 for a review). For instance, many trophically transmitted parasites enhance their intermediate host vulnerability to predation by final hosts, especially by altering their host’s anti-predatory defences. Increasing the probability of trophic transmission to a final host by infective parasites is a highly virulent exploitation strategy, which may be in complete contradiction with another cointecting parasite species targeting a different final host.

Lafferty et al. (2000) identified at least three outcomes of interspecific conflicts between parasites sharing a
common intermediate host but targeting different definitive hosts: (i) avoiding intermediate hosts already manipulated by another parasite; (ii) destroying the manipulator; or (iii) overpowering it to either manipulate the host in a different way (‘hijacking’, Lafferty et al., 2000) or to neutralize manipulation (‘sabotage’, Thomas et al., 2002). This latter outcome has been observed for instance between the vertically transmitted microsporidia Dictyocaulus sp. and the acanthocephalan parasite Polymorphus minutus, coinfesting the amphipod Gammarus roeseli (Haine et al., 2005). Such scenarios are also relevant to intraspecific conflicts in manipulative parasites when larvae at different levels of maturity infect the same intermediate host, specifically when one parasite is infective for the next host in the life cycle whereas its coinfective conspecific is not. For a parasite, reaching its infective stage of parasite development (Franceschi et al., 2008) may facilitate sequential coinfection. Opportunistic scenarios are present (Parc de la Colombière, Burgundy, Eastern France, 47°17′50.62″N; 5°2′35.85″E). Both fish parasite species alter the reaction to light of G. pulex (Tain et al., 2006) but P. tereticollis may exhibit more seasonality in the intensity of behavioural manipulation and is also much less prevalent in this locality (M.-J. Perrot-Minnot, unpublished data; Perrot-Minnot, 2004). Gammarids were visually inspected to select individuals infected with P. laevis cystacanths (with or without an acanthella) and uninfected individuals. We analysed only animals infected with one or two cystacanths (gammarids with heavier parasite loads were much rarer). Phototaxis was measured within a week of collection, following the protocol of Perrot-Minnot (2004). Individual reaction to light was scored in a 5-min choice test by recording the amphipod’s position in the dark or the light side of a tube every 30 s. For each individual, phototaxis summed scores ranged from 0 (highly photophobic) to 10 (highly photophilic) (Perrot-Minnot, 2004). Dissection under a binocular microscope was used to confirm the infection status of each individual.

Experimental study
Two 4-week staggered exposures of G. pulex to P. laevis eggs were performed to obtain a sequential infection with...
larval stages of different maturity levels (cystacanth and acanthella) in the same host. Then, host behavioural manipulation was measured by recording G. pulex reaction to light. The first experimental infection is thereafter called ‘i1’ and the second experimental infection ‘i2’ (Fig. 1).

**Experimental infections**  
*Gammarus pulex* were exposed to parasites following the procedure of Franceschi et al. (2008). Briefly, parasite eggs were retrieved from females collected in the intestine of chub (*Leuciscus cephalus*) caught in the Vouge River (47°08'03.65"N, 5°10'45.61"E). Eggs used in the first and the second infection were collected in December 2008 and January 2009, respectively. Molecular identification of parasites was performed based on size polymorphism of the internal transcribed spacer 1 (ITS1) rDNA gene, following Franceschi et al. (2008). We used 7 *P. laevis* females from 5 different fish for the first experimental infection and 10 *P. laevis* females from 5 different fish for the second experimental infection.

*Gammarus pulex* used for experimental infections were collected in December 2008 in a small tributary of the Suzon River, Burgundy, eastern France (47°24'12.6"N; 4°92'58.2"E). They were acclimated in the laboratory for 15 days pre-exposure in dechlorinated, UV-treated and aerated tap water at a temperature of 15 ± 1 °C, under a 12 : 12 h light : dark cycle. Throughout the experiment, gammarids were fed with conditioned elm leaves. Male gammarids were given a 1 cm² piece of elm leaf on which a parasite eggs suspension had been deposited. After 48 h, the gammarids were rinsed and placed in groups of 10 individuals in aquaria of 0.5 L. For the i1 exposure, a dose of 100 eggs per gammarid was used. Based on previous observations, we expected this dose to give the number of mono- and bi-infected gammarids necessary to compare simultaneously bi-infected gammarids (two cystacanths from i1) to sequentially bi-infected gammarids (one cystacanth from i1 and one acanthella from i2). For i2, an exposure dose of 50 eggs per gammarid was used, because this dose increases the proportion of mono-infected gammarids relative to pluri-infected gammarids (Franceschi et al., 2008), thus the number of sequentially bi-infected gammarids with only one acanthella from i2. Half of the gammarids from i1 was re-exposed to parasite eggs from i2, whereas the other half was maintained with a single exposure (Fig. 1). *Gammarus pulex* unexposed to parasite eggs but maintained under the same conditions as the exposed ones were used as controls. From the sixth week post-exposure, all gammarids were inspected once a week under a dissecting microscope to detect the presence of parasites. Larval parasites can be detected through the host cuticle from the late acanthella stage of their development. We carefully monitored parasite development as soon as the acanthellae from i1 were detected (translucent light-orange and shapeless larval stage). Infected and control gammarids were then isolated in plastic dishes of 0.2 L filled with dechlorinated, oxygenated and UV-treated tap water at 15 ± 1 °C. Parasite development was followed twice a week to record the day the parasite switched from acanthella to cystacanth. Developmental time was recorded as the time lapse in days between the day of exposure and the day the parasitic...
parasite reached the cystacanth stage. Development of i2 parasites was monitored in the same way.

**Behavioural measurements**

Reaction to light of isolated individuals was measured as previously described. To estimate when the possible behavioural switch in infected gammarids occurs, three repeated measures of phototaxis were carried out over the course of parasite development (Fig. 1). Phototaxis was first measured 9 days after the appearance of cystacanth in i1 (P1, Fig. 1). The second behavioural measure P2 was performed as soon as the i2 acanthellae were detected through the host cuticle. The third phototaxis measure on the same individuals was performed about 9 days after i2 parasites in sequentially infected gammarids developed into cystacanths (P3, Fig. 1). At the end of these behavioural assays, gammarids were dissected and parasites were counted.

**Statistical analyses**

Phototaxis score data were analysed using nonparametric tests, because data did not meet homoscedasticity conditions even after transformation. Prevalences and parasite loads associated to i1 and i2 experimental infections were compared using Fisher exact test (two-tailed) and Mann–Whitney test (two-tailed), respectively. Mann–Whitney two-tailed test with normal approximation for large samples was used for pairwise comparisons. When necessary, we corrected for multiple testing by applying the Šidák correction to derive threshold probability for individual tests (Sokal & Rohlf, 1995). The timing of behavioural manipulation in experimentally infected gammarids was analysed using Friedman tests. Statistical analyses were performed using JMP software version 7.0.1 (SAS Institute, Cary, NC, USA), except Friedman tests which were carried out using R software version 2.11.1 (R Development Core Team, www.r-project.org).

**Results**

**Behaviour of field-collected gammarids**

Overall, 4082 gammarids were dissected, among which 393 were parasitized by one or two *P. laevis* cystacanths. Because we intentionally collected gammarids with visual signs of infection, this rate is much higher than the natural prevalence of *P. laevis* (0.4–0.7%) recorded in this population in 2004–2005 (Lagrué et al., 2007; M.-J. Perrot-Minnot, unpublished data). Among hosts harbouring one or two cystacanth(s), 24 were coinfected with one acanthella. The phototaxis score of infected animals was compared to that of 423 uninfected gammarids.

Gammarids coinfected with one acanthella and one cystacanth did not differed significantly in their reaction to light from gammarids infected with one cystacanth (Mann–Whitney two-tailed test: $Z = -2.24$, $P = 0.025$, which was nonsignificant after Šidák correction), although they were 33% more photophobic (Fig. 2). However, they were significantly more photophobic than gammarids infected by two cystacanths, but not as photophobic as uninfected ones ($Z = -2.49$, $P = 0.013$, and $Z = 3.52$, $P = 0.0004$, respectively; these differences remained significant after Šidák correction) (Fig. 2).

**Experimental infections**

The prevalence of primary infection of healthy gammarids exposed to parasite eggs (proportion of gammarids infected with one or more cystacanth) did not differ significantly between the two experimental exposures i1 and i2 (Table 1a, b). Nor for experimental exposure i2 did prevalence differ between new primary infections of gammarids and sequential infections of superparasitized gammarids (Table 1a, b). In addition, i2 prevalence was comparable in gammarids for which i1 failed (exposed to i1 but not infected) and in singly exposed gammarids (Table 1a, b).

Parasite load after i2 single exposure was significantly lower than that after i1 first exposure (Table 1b), confirming the effect of egg exposure dose (50 eggs per gammarid in i2 vs. 100 eggs in i1). Mean load of i2 parasites developing to the cystacanth stage did not differ between previously unexposed and previously infected or exposed hosts (i.e. those for which the initial i1 infection failed) (Table 1a, b). Finally, developmental time of i2 parasites when developing alone or as a superinfection was comparable (Table 1b).

For gammarids infected at i1 that harboured one or two cystacanths, a significant increase in phototaxis was observed as the cystacanth matured (respectively Friedman test: $\chi^2 = 22.26$, $P < 0.0001$; $\chi^2 = 21.26$, $P < 0.0001$; Fig. 3). By contrast, phototaxis score was...
Table 1: Infection success of *Pomphorhynchus laevis* in *Gammarus pulex* following two independent experimental infections in the laboratory.

Infection success was measured as the prevalence (proportion of gammarids harbouring one or more cystacanth(s)), the parasite load (median and quartiles of the number of parasites in infected hosts) and the parasite developmental time (median and quartiles of the interval from exposure to parasite eggs to the formation of a cystacanth, in days). (a) Infection success is given for four distinct infection states, according to the number of time gammarids were exposed to eggs: once for [1] and for [2], twice for sequentially bi-infected hosts in which i1 succeeded [3] or failed [4]. (b) Statistical analysis of infection success in paired comparisons, according to host status (see text for details).

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<thead>
<tr>
<th>Host status</th>
<th>N</th>
<th>Prevalence (%)</th>
<th>Parasite load*</th>
<th>Developmental time (days)*</th>
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<tr>
<td>(a)</td>
<td></td>
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<td>[3] sequential superinfection (i1 + i2)</td>
<td>91</td>
<td>27.2</td>
<td>1 [1–2]</td>
<td>68</td>
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<tr>
<td>[4] i2 exposure of i1 exposed gammarids in which i1 failed</td>
<td>72</td>
<td>26.9</td>
<td>1 [1–3]</td>
<td>70</td>
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(b) Paired comparisons according to host status

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<td>[1] vs. [2]</td>
<td>0.99</td>
<td>−3.45</td>
<td>0.0006</td>
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<tr>
<td>[2] vs. [3]</td>
<td>0.14</td>
<td>−0.88</td>
<td>0.38</td>
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<td>[2] vs. [4]</td>
<td>0.13</td>
<td>−1.51</td>
<td>0.13</td>
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*Interquartiles are written in square brackets.

Fig. 3: Phototaxis scores of experimentally infected *Gammarus pulex* according to host infection status and parasite developmental stage. Phototaxis of sequentially bi-infected gammarids (1 cyst + 1 acanth) is compared to that of mono-infected gammarids (1 cyst), of simultaneous bi-infected gammarids (2 cyst) and of uninfected gammarids. P1, P2 and P3 refer to the timing of the phototaxis test and therefore to the age of the parasites (see Fig. 1). Full lines connecting symbols indicate a significant increase in phototaxis scores over time, unlike symbols connected by dotted lines, which show a stable pattern of phototaxis scores after a Friedman test. Sample sizes are indicated below X-axis.

Discussion

A conflict between acanthella and cystacanth over behavioural manipulation is expected based on opposite implications of predation of the intermediate host (death for the former vs. possible transmission for the latter).
Intraspecific conflict over parasite-induced host manipulation

Such a conflict can lead to either a ‘sabotage’ of behavioural manipulation by the acanthella or to ‘coinfection exclusion’ by the cystacanth.

Experimentally, *P. laevis*-infected *G. pulex* were no less or more susceptible to a second infection than were uninfected gammarids. Therefore, we have no evidence for exclusion of a second intruder by the established cystacanth. Nor could we detect a role of immunosuppression of *G. pulex* by acanthella of *P. laevis* in determining infection success of *P. laevis*. Previous studies showed that immunosuppression of *G. pulex* by *P. laevis* occurs (Rigaud & Moret, 2003; Cornet et al., 2009), but our current results suggest it is either not induced before the cystacanth stage or that the *P. laevis*-induced proPO immunosuppression is not involved in *G. pulex* defence against *P. laevis* larvae. The onset of pro-PO immunosuppression in the course of *P. laevis* development should be established. Furthermore, developmental time of the second parasite appeared not affected by previous infection with an older parasite, in contrast with previous studies on cestodes (Jäger & Schjørring, 2006).

Concerning the sabotage of behavioural manipulation, reactions to light of naturally infected and laboratory-infected gammarids were quite similar. In both types of reactions to light of naturally infected and laboratory-cystacanth. Nor could we detect a role of immunosuppression in the course of *P. laevis* development should be established. Furthermore, developmental time of the second parasite appeared not affected by previous infection with an older parasite, in contrast with previous studies on cestodes (Jäger & Schjørring, 2006).

Concerning the sabotage of behavioural manipulation, reactions to light of naturally infected and laboratory-infected gammarids were quite similar. In both types of hosts, sequentially bi-infected *G. pulex* were less photophobic than uninfected ones and therefore remained partially manipulated. The 33% increase in gammarid photophobia when harbouring an acanthella in addition to the original cystacanth was not statistically significant. Therefore, we have no clear and strong evidence for the sabotage hypothesis in *P. laevis*. Nevertheless, the presence of an immature stage of larval parasite was not neutral for its more mature conspecific. First, *G. pulex* infected with an acanthella and a cystacantra were significantly more photophobic than gammarids infected with two cystacanths, a difference that could nevertheless be because of the cystacanth load. In addition, phototaxis scores in gammarids harbouring both an acanthella and a cystacanth did not increase as the cystacanth matured, whereas they did in mono-infected and simultaneously bi-infected gammarids (as previously observed by Franceschi et al., 2008). This stable pattern over time at an intermediate level of manipulation indicates that the presence of an acanthella slightly decreased or delayed the behavioural manipulation of cystacanth-infected hosts. Note that this temporal pattern could not be investigated with naturally infected hosts. The temporal dynamic of manipulation thus seems to be a key parameter in the competitive interaction between the acanthella and the cystacanth; it may ‘take time’ for a parasite to reverse phototaxis, and manipulation could be either accelerated by a coinfecting cystacanth or delayed by a coinfecting acanthella. To our knowledge, the only other study to address sabotage in mixed larval stages infection failed to find it. Indeed, Sparkes et al. (2004) showed that the acanthella of the acanthocephalan parasite *Acanthocephalus dirus* was unable to lessen the alteration of host phenotype induced by the cystacanth consisting in a nearly complete depigmentation of the isopod body. However, these authors considered a phenotypic alteration that is already partially occurring at the acanthella stage, although less pronounced than at the cystacanth stage (the colour change induced by the acanthella and the cystacanth ranged from over 40% to over 80% of the body, respectively). A suppression of manipulation would therefore seem unlikely, whereas in our model system, *P. laevis* acanthella alone did not induce any change in phototaxis (Franceschi et al., 2008).

Despite the absence of complete sabotage, our study nevertheless shows that the acanthella is not a passive ‘passenger’ in its intermediate host, with respect to host behaviour. It disrupts or delays, albeit weakly, the manipulative effect of a mature cystacanth. This disturbance may result from a type of apparent competition between the acanthella and the cystacanth (Mideo, 2009), in which each conspecific protagonist indirectly decreases the fitness payoff of host exploitation by the other. The host offers a variety of resources prone to a ‘tragedy of the common’ for coinfesting parasites, most commonly addressed in the case of within-host competition for nutrients (Parker et al., 2002; Rankin et al., 2007; Lively, 2009; Mideo, 2009). Was the pattern of competition between acanthella and cystacanth reported here ‘tragic’? A general criterion for a ‘tragedy of the common’ to occur is the decreased overall fitness of the group following to selfish competition. Franceschi et al. (2010) showed that gammarids have a decreased survival probability once infected by *P. laevis*; staying for a longer time in the intermediate host is therefore costly for the parasite. In addition, the acanthella lacks several anatomical (proboscis muscles) and physiological (glycogen reserves) attributes necessary for establishing successfully in its definitive host (see Nickol, 1985; Taraschewski, 2000), thus being ingested by a fish is costly for *P. laevis* at that stage. If the intermediate level of behavioural manipulation in sequentially coinfected hosts translates into an intermediate level of exposure to predators, it may diminish the individual payoffs of both acanthella and cystacanth in terms of survival. While this direct link between intensity of manipulation and intensity of predation should be quantified specifically (Cézilly et al., 2010), this hypothesis is realistic because exposure to light should make the infected gammarids more visible to fish predators foraging in the river current.

The absence of a complete counteracting effect of an acanthella on cystacanth-induced manipulation raises the question of whether selection pressure is strong enough for sabotage to evolve. Selection pressure could be weak because of a low risk of experiencing the cystacanth-acanthella conflict. This risk diminishes at low prevalence, because the chances of coinfection with mixed larval stages decrease as parasite prevalence decreases. Prevalence is indeed a key parameter for sabotage to evolve (Thomas et al., 2002) and more
generally for the evolution of strategies that optimize parasite transmission (Thomas et al., 1998). Pomphorhynchus laevis prevalence in the populations sampled could have been too variable and/or too weak to induce a selection pressure high enough for adaptive strategies to fully evolve in response to such a conflict. The lack of complete sabotage by the acanthella and the lack of coinfection exclusion from the cystacanth reported here are consistent with this interpretation. However, the fact that the acanthella is not a ‘passive passenger’ raises the question of whether the observed competition between the two larval stages is a by-product of two opposing manipulations. Parker et al. (2009) and Hammerschmidt et al. (2009) showed that there is a strong selective pressure on manipulative parasites to ‘actively’ protect their host from predation before maturation and then to switch to an increased predation after maturation. These opposite behavioural manipulations should be achieved even in absence of interaction between parasites. In mixed larval stage infections, these two antagonistic manipulations could be in conflict, leading to an intermediate phenotype. According to our results, sequentially bi-infected gammarids harbouring two cystacanths were still slightly more photophobic than gammarids infected by two cystacanths of the same age, a difference that was close to significance. From a functional viewpoint, this long-lasting disturbance of cystacanth-induced behavioural manipulation by the younger parasite suggests profound modifications of the host’s brain, and potentially an important time lapse to recover from them. Reversed phototaxis of G. pulex infected by cystacanths of P. laevis is related to an important increase in immunoreactivity to serotonin (5-HT) in the host brain (Tain et al., 2006). The possibility that the targets of the acanthella and the cystacanth in the crustacean central nervous system experience competitive interactions at the molecular level between antagonistic products of acanthella and cystacanth, such as 5-HT receptors or 5-HT transporters, therefore appears to be an interesting perspective.

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