

PROTECTION FIRST THEN FACILITATION: A MANIPULATIVE PARASITE MODULATES THE VULNERABILITY TO PREDATION OF ITS INTERMEDIATE HOST ACCORDING TO ITS OWN DEVELOPMENTAL STAGE

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Many trophically transmitted parasites with complex life cycles manipulate their intermediate host behavior in ways facilitating their transmission to final host by predation. This facilitation generally results from lowering host's antipredatory defenses when the parasite is infective to the final host. However, a recent theoretical model predicts that an optimal parasitic strategy would be to protect the intermediate host from predation when noninfective, before switching to facilitation when the infective stage is reached. We tested this hypothesis in the fish acanthocephalan parasite *Pomphorhynchus laevis* using the amphipod *Gammarus pulex* as intermediate host. Gammarids parasitized by noninfective stage of *P. laevis* (acanthella) hid significantly more under refuges than uninfected ones. In addition, acanthella-infected gammarids were less predated upon by trout than uninfected ones. As predicted, a switch toward decreased antipredatory behavior of *G. pulex* and enhanced vulnerability to predation was found when *P. laevis* reached the stage infective to its final host. The parasites appear to be able to exploit plasticity in host antipredatory responses, and shift the host optimal response toward their own optimal balance.

KEY WORDS: Behavioral switch, intermediate host protection, parasite manipulation, *Pomphorhynchus laevis*, predation enhancement, predation suppression.

In parasites with multihost life cycles, completion of the life cycle critically relies on several transmission steps. Strategies to enhance transmission probability between successive hosts have arisen several times in different lineages of parasites, particularly in trophically transmitted parasites (i.e., those transmitted through predation of infected intermediate hosts by definitive hosts) (Chubb et al. 2010). Spectacular examples are known where parasite larvae change the behavior of their intermediate hosts, in ways increasing their vulnerability to predation by the definitive host, hence their transmission (see Moore 2002 for a review). Most

of the time, there is a match between the parasite developmental stage and the onset of this so-called behavioral manipulation: changes in behavior appear in the intermediate host when the parasite reaches the stage infective to a definitive host. Before this stage, the parasite larvae invest mainly in growth during what we could call a "noninfective phase." This phase is characterized by the parasite's inability to establish in the definitive host (see Nickol 1985 for a review on acanthocephalan parasites), and many studies have shown that none of the changes in host phenotype facilitating parasite's transmission are observable at this phase

(e.g., Seppälä et al. 2005; Franceschi et al. 2008). Therefore, until reaching the infective stage, parasite growth and survival contribute the most to future transmission chances.

Parasite survival in intermediate host depends on several factors related to host exploitation, but first of all on survival of the host itself. Decreasing predation risks of the intermediate host thus seems a basic way for a noninfective larva to increase its fitness. In a recent theoretical model, Parker et al. (2009) showed that a parasite strategy that induces a switch from “predation suppression” (during non infective phase) to “predation enhancement” (as soon as the infective stage is reached) in the intermediate host is selectively advantageous. Parker et al. (2009) even emphasize the fact that “predation suppression” should evolve more easily than “predation enhancement,” especially because the former does not need to be host-specific contrary to the later.

This model has some empirical support in trophically transmitted parasites. Hammerschmidt et al. (2009) showed that copepods infected by the noninfective stage of the cestode *Schistocephalus solidus* increased their recovery time after a shock and decreased their general activity, two behaviors presumably reducing predation risk. As soon as the parasite reached its infective stage, a behavioral switch was observed, possibly increasing vulnerability to predation. Reductions of risky behavior have also been found in vector-borne parasites (although this transmission mode does not involve predation of one host by another). Parasites of both human and avian malaria change the behavior of their mosquito vector before reaching the infective stage, in a way supposedly reducing their contact with vertebrates and possibly increasing the vector survival (respectively, Anderson et al. 1999; Koella et al. 2002). These studies shed light on protective behavior of hosts parasitized by immature parasite stages, but they did not directly address whether such protective behavior translates into decreased mortality risks. Yet, this demonstration remains essential to conclude to adaptive protection by noninfective parasites (Cézilly et al. 2010).

This study aimed at investigating if the noninfective stage of the acanthocephalan parasite *Pomphorhynchus laevis* induces protective behavior in its intermediate host. *Pomphorhynchus laevis* is a trophically transmitted parasite that uses freshwater fish as definitive hosts (Kennedy 2006). In the fish intestine, the adult parasite sexually reproduces and eggs are released in the environment with fish faeces. Freshwater amphipods (e.g., *Gammarus pulex*) become infected by ingesting eggs. Larvae then enter the host's hemocoel and grow, reaching successively several larval stages: the acanthella stages (noninfective to definitive host) and the cystacanth stage (infective to definitive host). *Pomphorhynchus laevis* cystacanth induces a range of behavioral alterations in its gammarid host: it reverses the natural photophobia of infected *G. pulex* (Franceschi et al. 2008) and lowers the level of refuge use in the presence of a predator (Kaldonski et al. 2007). Gammarids in-

fectured by cystacanth are more preyed upon than uninfected ones (Kaldonski et al. 2007; Lagrue et al. 2007), in agreement with the “predation enhancement” criterion of parasite manipulation (Parker et al. 2009). But does acanthella change the behavior of the intermediate host in ways increasing protection and hence “predation suppression”? Franceschi et al. (2008) showed that gammarids infected with acanthella were as photophobic as uninfected ones. However, because the photophobia was maximal for uninfected gammarids (they already spend all their time in the dark), this protective behavior could not be stronger in animals infected by acanthella. A recent study nevertheless showed that an acanthella coinfecting a *G. pulex* with a conspecific cystacanth partially prevents or delays the manipulation of phototaxis (Dianne et al. 2010). The acanthella stage is therefore not just a “passive passenger” in a coinfecting host, but another criterion than host phototaxis is needed to evidence its putative protective effect when alone.

Using experimental infections of *G. pulex* by *P. laevis*, to control for parasite stages, we compared levels of refuge use between uninfected gammarids, gammarids infected by *P. laevis* acanthellae, and gammarids infected by cystacanths, using a microcosm experiment. Then, we carried out predation tests to compare the relative susceptibility to predation of acanthella-infected gammarids compared with uninfected ones, and of cystacanths-infected gammarids compared with uninfected ones. Following the model of Parker et al. (2009), we predict that *P. laevis* acanthellae should enhance the anti-predatory behavior of *G. pulex* and make gammarids less vulnerable to predation than uninfected ones. Once the cystacanth stage is reached, there should be a switch from strong anti-predatory behavior to low anti-predatory behavior and from predation suppression to predation enhancement.

Material and Methods

BIOLOGICAL MATERIAL AND EXPERIMENTAL INFECTION

Experimentally infected *G. pulex* were used in both the refuge use experiment and the predation test in microcosm. Uninfected *G. pulex* were collected in a small tributary of the Suzon River, Burgundy, eastern France (47°24'13.91"N; 4°53'00.54"E). Yearling brown trout (*Salmo trutta fario*, 10–15 cm) from a commercial fishery were used to provide olfactory cues in refuge use experiments, and as predator in predation experiments. This fish was recorded as a suitable definitive host for *P. laevis* (Kennedy 2006). Before experiments, trout were fed regularly with uninfected *G. pulex*.

Ten gravid *P. laevis* females were collected in intestine of chubs (*Leuciscus cephalus*), from naturally infected fish caught in the river Vouge, Burgundy (47°9'34.36"N; 5°9'2.50"E).

Molecular identification of parasites and exposure of *G. pulex* to parasite eggs were performed following the procedure of Franceschi et al. (2008). A total of 2515 *G. pulex* (1574 males and 941 females) were exposed to parasite eggs. *Gammarus pulex* unexposed to parasite eggs, but handled and maintained under the same conditions as the exposed ones, were used as controls. Uninfected gammarids thereby provided controls matching the age of acanthella-infected gammarids and, later, of cystacanth-infected gammarids. About six weeks after exposure, the acanthella could be spotted through host cuticle (translucent light-orange and shapeless larval stage). A first series of behavioral experiments was made within 24 h of acanthella detection. Some infected gammarids were kept until parasite grown to the cystacanth stage, for a second series of behavioral experiments serving as positive controls for parasite manipulation (Kaldonski et al. 2007, 2009). These last experiments were performed two weeks after the cystacanth stage had been reached.

BEHAVIORAL MEASUREMENTS

Refuge use

Refuge use was recorded in a microcosm, with or without olfactory cues from a fish predator. Trout-conditioned water was obtained by isolating individually three trout for 24 h in a small aquarium (24 × 16 × 15 cm) filled with 250 mL of treated tap water per fish centimetre (Kaldonski et al. 2007). The experimental arena for refuge use consisted of an aquarium (32 × 20 × 20 cm) filled with 8 L of treated tap water and surrounded by opaque screens. The refuge consisted of a piece of air-brick (21.5 × 10 × 5 cm) where gammarids could shelter, following Kaldonski et al. (2007). For each trial, 10 gammarids of similar infection status (acanthella-infected, cystacanth-infected, or uninfected) were introduced in the aquarium. After a 10-min acclimatization period, the number of individuals outside the refuge (from 0 to 10) was recorded every 30 sec for 5 min. The 10 numbers were then summed, to get a total score for the experiment ranging from 0 (high refuge occupancy) to 100 (no refuge use). This first sequence gave the ‘basal level’ of refuge use by gammarids. We then added 100 mL of trout-conditioned water (by mixing 3 × 33 mL of water conditioned by individually isolated trout) in half of trials, and 100 mL of control water in the other half. After a 5-min acclimatization period, the number of individuals seen outside the refuge was recorded as described above. Controls using age-matched uninfected gammarids (see above) were run at the same time as infected ones.

Predation tests

The selectivity of trout toward infected or uninfected gammarids was assessed in a microcosm, providing the same refuges as the ones used for ‘refuge use’ experiment, following the procedure of Kaldonski et al. (2007). Predation tests were performed

with 40 male gammarids in two independent sets of experiments: ‘acanthella trials’ (14 replicates, with 14 acanthella-infected and 26 uninfected gammarids, the latter being more prevalent in the field than the former, Lagrue et al. 2007) and ‘cystacanth trials’ (10 replicates, with 14 cystacanth-infected and 26 uninfected gammarids). The duration of cystacanth trials was 90 min (following Kaldonski et al. 2007) whereas the duration of acanthella trials was increased to 180 min, because we noticed in preliminary experiments that the number of eaten gammarids was about three times lower in trials involving acanthellae than in trials involving cystacanths. At the end of each trial, remaining gammarids of each type (infected or uninfected ones) were counted. To be able to analyze predation bias statistically, only replicates where more than six prey and less than 25 prey were eaten were kept for analyses (Manly 1974; Chesson 1978; Kaldonski et al. 2007).

STATISTICAL ANALYSES

Scores of refuge use (the cumulative proportion of gammarids seen outside the shelter) were arcsine-transformed to meet normality and homoscedasticity conditions. We performed a repeated measure analysis of variance (ANOVA) to compare scores of acanthella-infected and uninfected gammarids before and after water injection. Because of a small number of replicates, comparisons of levels of refuge use between cystacanth-infected and uninfected gammarids were realized using two-tailed Mann–Whitney U-tests and Wilcoxon signed-rank tests. We used Cohen’s *d* effect statistics to quantify the ‘parasite stage’ size effect on refuge use in the presence of predator cues. Ninety-five percent confidence intervals (hereafter named 95% CI) of Cohen’s *d* values were calculated (Nakagawa and Cuthill 2007).

Following Kaldonski et al. (2007, 2009), predation bias toward one of the two types of prey (acanthella-infected vs. uninfected or cystacanth-infected vs. uninfected gammarids) was estimated using Manly’s selectivity index for variable prey population (α_i), which is appropriate when prey cannot be replaced (Manly 1974; Chesson 1978). Index values could range from 0 (when only uninfected prey are eaten) to 1 (when only infected prey are eaten). We assessed selective predation on infected or uninfected prey using a one-tailed *t*-test to the specified value of $\alpha = 0.5$ (meaning no predation bias toward one type of prey or another) (Sokal and Rohlf 1995). Bootstrapped 95% CI were calculated to provide additional information concerning the significance of the effect. In addition, because predation rate appeared to vary with the type of trial run (‘acanthella’ vs. ‘cystacanth’), we compared the number of prey eaten per unit of time between these two types of trials using a *t*-test (Sokal and Rohlf 1995).

Statistical analyses were performed using JMP software version 7.0.1 (SAS Institute, Cary, NC, USA), except bootstrapped 95% CI that were calculated using R software version 2.11.1 (R Development Core Team, www.r-project.org).

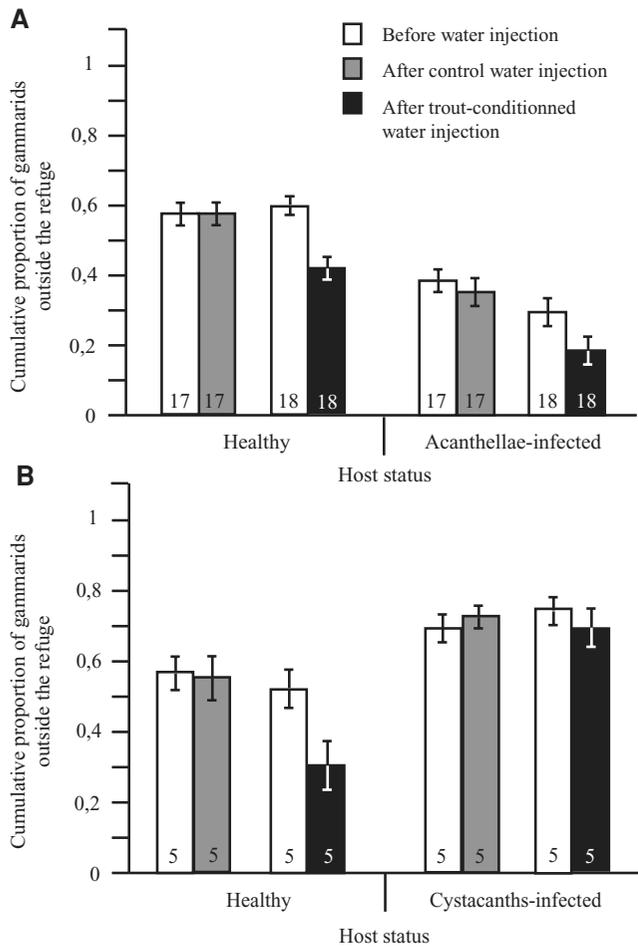


Figure 1. Effects of host status and type of injected water on the level of refuge use by *Gammarus pulex* infected by *Pomphorhynchus laevis acanthella* (A) or cystacanth (B) compared to uninfected hosts (means \pm SEM). Level of refuge use is represented by the cumulative proportion of gammarids seen outside the refuge. Numbers in the bars refer to number of replicates in each category (each replicate was pooling 10 gammarids).

Results

REFUGE USE

Acanthella-infected gammarids showed a greater level of refuge use than uninfected gammarids (Fig 1A and Table 1). Moreover, gammarids that received trout-conditioned water in their aquarium presented a greater level of refuge use than gammarids that received control water (Fig 1A and Table 1). Finally, the cumulative proportion of gammarids seen out of the refuge was higher for males than for females (males: mean \pm SEM = 0.47 ± 0.03 , $N = 40$; females: mean \pm SEM = 0.36 ± 0.04 , $N = 30$; Table 1). None of the interactions between host status, injected water status, and host sex was significant (Table 1). Gammarids responded to the injection of trout-conditioned water by significantly enhancing refuge use, whereas their behavior

Table 1. Results of the repeated measures ANOVA analyzing the intensity of refuge use in acanthella-infected and uninfected hosts. Nonsignificant interactions have been retrieved from the model. The analyses were made on arcsine-square root transformed data.

	<i>F</i>	<i>df</i>	<i>P</i> -value
Between subjects			
All between	19.24	3,66	<0.0001
Host status (acanthella-infected vs. uninfected)	41.42	1,66	<0.0001
Injected water status (trout-conditioned vs. control)	7.29	1,66	0.008
Host gender (male vs. female)	8.48	1,66	0.004
Intra subjects			
All intrainteractions	8.38	3,66	<0.0001
Reaction to water injection (before vs. after)	26.68	1,66	<0.0001
Reaction to water injection \times host status	1.31	1,66	0.256
Reaction to water injection \times injected water status	21.47	1,66	<0.0001
Reaction to water injection \times host gender	2.86	1,66	0.095

was not significantly changed by the injection of control water (Table 1 and Fig. 1A). Acanthella-infected and uninfected *G. pulex* displayed the same pattern of refuge use after water injection (Table 1 and Fig 1A). The hiding behavior of gammarids after the addition of water did not change according to host gender (Table 1).

Despite the small number of replicates, we found an opposite pattern of refuge use by cystacanth-infected gammarids, in agreement with Kaldonski et al. (2007). Cystacanth-infected gammarids used significantly less the refuge than uninfected gammarids, both before and after injection of trout-conditioned water (Mann–Whitney U-tests: $\chi^2 = 4.84$, $P = 0.03$, and $\chi^2 = 5.31$, $P = 0.02$ respectively; Fig. 1B). Moreover, the cumulative proportion of cystacanth-infected gammarids seen outside the refuge was not significantly reduced after injection of trout-conditioned water, in contrast to uninfected hosts (Wilcoxon signed-rank test: $P = 0.08$ and $P = 0.01$ respectively; Fig 1B). The magnitude of acanthella-induced and cystacanth-induced behavioral alteration, estimated by Cohen's *d* effect statistic, was similar although in opposite direction ($d = -5.01$, 95% CI = -6.38 to -3.64 for acanthella-infected gammarids; $d = 4.81$, 95% CI = 2.04 – 7.59 for cystacanth-infected gammarids).

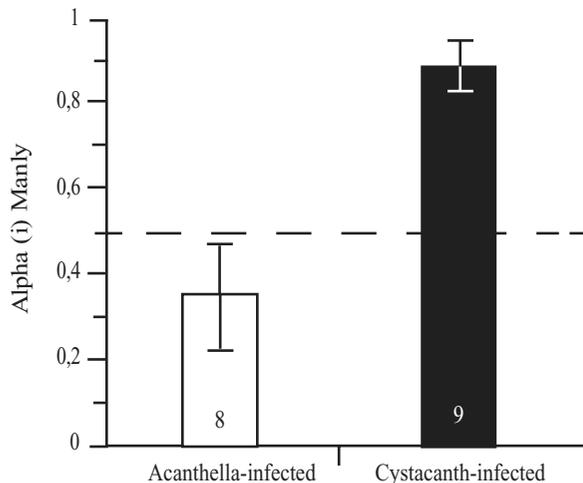


Figure 2. Manly's alpha (true means \pm 95%CI) representing selective predation of brown trout on *G. pulex* infected by *P. laevis* acanthella versus uninfected *G. pulex* (white bar), and cystacanth-infected *G. pulex* versus uninfected *G. pulex* (black bar). The dotted line indicates no predation bias toward one of the two types of prey. A value above the dotted line means selective predation on infected prey, whereas a value below means selective predation on uninfected prey. The number of replicates in each type of trial is indicated at the bottom of the bars. [Correction made June 1, 2011 after Online publication: In the first version of this article, legend of Table 1 was incorrectly appended at the end of the legend of Figure 2. This has been corrected.]

PREDATION TESTS

Acanthella-infected gammarids were significantly less predated upon by trout than uninfected ones (t -test to a specified value: $N = 8$, $t_7 = -2.13$, $P = 0.035$; Fig. 2). By contrast, cystacanth-infected gammarids were more predated upon than uninfected ones (t -test to a specified value: $N = 9$, $t_8 = 11.5$, $P < 0.0001$; Fig. 2). These significant results were complemented by bootstrapped 95% CI that did not cross the " $\alpha = 0.5$ " line, neither in acanthella trials (95% CI = 0.22–0.47) nor in cystacanth trials (95% CI = 0.82–0.95). The number of total gammarids eaten per unit of time in acanthella trials was significantly lower than in cystacanth trials (mean \pm SEM: 3.75/h \pm 0.65 vs. 12.15/h \pm 0.62, respectively) (t -test: $t_{15} = 9.28$, $P < 0.0001$). Consequently, only eight trout of 14 ate more than six prey in acanthella trials, whereas all the 10 trout consumed more than six prey in the cystacanth trials.

Discussion

Any strategy diminishing the mortality risk of intermediate host is expected to be selected in parasites before they reach transmission stage, because it increases their own survival and time frame for growth (Parker et al. 2009). As predicted by this "protective

strategy," gammarids infected with *P. laevis* acanthella were found more often under refuge than uninfected gammarids. This was true regardless of the presence of predator olfactory cues. In addition, acanthella-infected gammarids and uninfected ones responded similarly to the olfactory cues from a predator by enhancing refuge use. Thus, acanthella tend to reinforce a protective behavior in its intermediate host—sheltering under a refuge— independently of predation risk. Moreover, acanthella-infected gammarids were significantly less preyed upon by trout than uninfected ones when both types of prey were offered. By contrast, cystacanth-infected gammarids displayed a lower level of refuge use and experienced a higher predation rate than uninfected ones, in agreement with a previous study (Kaldonski et al. 2007). Therefore, there was a switch from enhanced to decreased refuge use, and from decreased to increased susceptibility to predation, between the acanthella and the cystacanth stages infecting *G. pulex*. This switch coincided with a life-history switch from investment in growth and survival to investment in transmission (Parker et al. 2009). Following Poulin (2010), we can thus consider this parasite strategy of "protection first then facilitation" as optimal in terms of parasite growth and transmission, hence fitness. Interestingly, the magnitude of changes in antipredatory defenses induced by acanthella or cystacanth was similar (although in opposite directions). The acanthella-induced "protective strategy" should thus be considered with as much importance as cystacanth-induced facilitation of predation. In showing that the acanthella is affecting host behavior, this study complements previous observation that *P. laevis* acanthella diminishes or delays the reversal of phototaxis induced by a coinfecting cystacanth (Dianne et al. 2010). Supposedly protective behavior has already been reported in other nonmature parasites (Anderson et al. 1999; Koella et al. 2002; Hammerschmidt et al. 2009). However, this is the first study, to our best knowledge, showing that such behavioral changes do indeed increase parasite survival, a necessary criterion to assign an adaptive value to this protective strategy (by analogy to facilitation by the infective stage, Cézilly et al. 2010).

The existence of a "predation suppression" strategy in *P. laevis* acanthellae may have several ecological and/or evolutionary outcomes. From an ecological point of view, the role of manipulative parasites in community ecology is more and more emphasized, in particular by strengthening trophic links (Lefèvre et al. 2008). Our study and previous ones (Hammerschmidt et al. 2009; Parker et al. 2009) call for a full consideration of the entire parasite life cycle in modeling the impact of trophically transmitted parasites on community network and food chain. From an evolutionary point of view, the net fitness effect of infection by a manipulative parasite on its intermediate host fitness should be reconsidered by including the acanthella-induced

predation suppression. Costs of cystacanth-induced behavioral manipulation on host fitness are obvious because it increases predation probability (Kaldonski et al. 2007; Lagrue et al. 2007). More balanced costs and benefits may arise from the protective strategy of the acanthella on its intermediate host. Increasing survival to predation risk is a shared interest between *P. laevis* acanthella and its host, and could compensate the physiological costs of parasite growth on host survival (Franceschi et al. 2010). However, we showed that the refuge use by acanthella-infected gammarids is higher than the refuge use by uninfected ones, regardless of predation risk. This reflects a departure from an optimal activity level, which could potentially impact the host fitness, by decreasing for example food provisioning and/or mating success. In crustaceans, reduced activity in the presence of predator olfactory cues reduces the food uptake (Short and Holomuzki 1992) and the probability of breeding pair formation (Dunn et al. 2008). In agreement with such a trade-off between sheltering and searching for mates, we found that refuge use by *G. pulex* males was lower than refuge use by females. *Pomphorhynchus laevis* parasites may therefore indirectly suffer from their host's suboptimal activity. Whether such potential indirect costs are outweighed by the benefits from predation suppression awaits further investigation.

This work brings novel perspectives to behavioral manipulation studies by demonstrating that a manipulative parasite protects its intermediate host from predation, as long as it is noninfective for definitive host. Uninfected *G. pulex* display behavioral plasticity according to predation risk, as shown by their context-dependent sheltering behavior. *Pomphorhynchus laevis* acanthella seems to be able to exploit this plasticity and shift its host optimal response toward its own optimal balance. According to Parker et al. (2009), the host protective strategy should evolve in noninfective stages of trophically transmitted parasites more readily than facilitation by infective stages through behavioral manipulation. Indeed, a protective strategy does not need to specifically target a suitable next host to benefit the parasite, as much as facilitation by host behavioral manipulation does (see however Seppälä and Jokela 2008). Despite this apparent readiness to evolve, our study is one of the rare studies that has addressed this topic yet, and little is known about the ubiquity of protective strategies in trophically transmitted parasites (Parker et al. 2009; Chubb et al. 2010).

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