

Impact of olfactory non-host predator cues on aggregation behaviour and activity in *Polymorphus minutus* infected *Gammarus pulex*

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Abstract Parasites with a complex life cycle are supposed to influence the behaviour of their intermediate host in such a way that the transmission to the final host is enhanced, but reduced to non-hosts. Here, we examined whether the trophically transmitted bird parasite *Polymorphus minutus* increases the antipredator response of its intermediate host, the freshwater amphipod *Gammarus pulex* to fish cues, i.e. non-host cues ('increased host abilities hypothesis'). Aggregation behaviour and reduced activity are assumed to decrease the predation risk of gammarids by fishes. Uninfected *G. pulex* are known to aggregate in the presence of a fish predator. In the present study, gammarids were allowed to choose either to join a group of conspecifics or to stay solitary (experiment 1) or between two groups differing in

infection status (experiment 2), both in the presence or absence of fish odour. The perception of the groups was limited to mainly olfactory cues. Contrary to the 'increased host abilities hypothesis', in infected gammarids of experiment 1, fish cues induced similar aggregation behaviour as in their uninfected conspecifics. In experiment 2, uninfected as well as infected gammarids did not significantly discriminate between infected and uninfected groups. Although only uninfected gammarids reduced their activity in the presence of predator cues, infected *G. pulex* were generally less active than uninfected conspecifics. This might suggest that *P. minutus* manipulates rather the general anti-predator behaviour than the plastic response to predation risk.

Keywords Shoaling · Host-parasite interaction · Invertebrate · Crustacean · Gammarid · Group · Stickleback · Parasitic manipulation · Acanthocephala

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Introduction

In parasites having a complex life cycle the transmission from one host to the next is of fundamental importance. Often, the intermediate host must be eaten by the parasite's final host (trophic transmission). The necessity of trophic transmission creates a strong selective pressure on the parasite's capacity to increase

the probability of predation of the intermediate host by the final host (Moore, 2002). Increased predation probability might be achieved by altering the anti-predator behaviour, e.g. by inducing preference for predator cues or the cryptic appearance of the intermediate host (for reviews see Thomas et al., 2005; Lefèvre et al., 2009), that is parasitic manipulation. However, the parasitic manipulation should not increase the predation risk of the intermediate host by non-host predators. Concerning non-host predators the interests of the intermediate host and parasite appear to coincide at first glance. However, the so-called ‘increased host abilities hypothesis’ suggests that the parasite induces a stronger anti-predator response than it is optimal for the host (Médoc & Beisel, 2008). A disproportionate anti-predator response might, for instance, considerably reduce the encounter rate of potential mating partners or food intake of the host. Thus, even concerning non-host predators the optimal trade off between different behaviours might be different between host and parasite. Both, increased affinity to host predators as well as extreme anti-predator behaviour can be regarded as a form of parasitic manipulation. Whereas parasitic manipulation inducing increased predation by host predators is well examined (see Thomas et al., 2005 for a review; recent studies: Baldauf et al., 2007; Perrot-Minnot et al., 2007), manipulation inducing stronger anti-predators behaviour, i.e. ‘increased host abilities hypothesis’, has received less attention (see Médoc & Beisel, 2009; Médoc et al., 2009).

Prey animal species have evolved a wide range of anti-predator strategies; one of them is aggregation behaviour (Welty, 1934; Brock & Riffenburgh, 1960; Magurran, 1990; Pitcher & Parrish, 1993; Krause & Ruxton, 2002). Many vertebrates form larger groups in the presence of predators (Pitcher & Parrish, 1993; Krause & Ruxton, 2002). Recently, it has been shown that in the presence of chemical cues of a predator fish, uninfected individuals of the amphipod *Gammarus pulex* prefer to stay close to conspecifics, whereas they show no significant aggregation behaviour when there is no predation risk (Kullmann et al., 2008). These results suggest that *G. pulex* aggregate in order to reduce predation risk. For an individual, the probability of predation is decreased in a group, because (i) groups are more effective at detecting predators (Roberts, 1996), (ii) predators might be confused by a large number of prey (Krakauer, 1995;

Frommen et al., 2009), or (iii) of dilution effects (Krause & Ruxton, 2002). Besides aggregation, another factor leading to predator-confusion increase is phenotypic uniformity (Landeau & Terborgh, 1986; Theodorakis, 1989). It is known that individuals within a group differing in size, coloration or else, so called odd individuals, suffer a higher risk of being predated (e.g. Theodorakis, 1989). Consequently, individuals prefer to aggregate with conspecifics of similar phenotypes in many animal species (e.g. Krause & Godin, 1994; Krause et al., 1996).

Parasitisation can affect grouping behaviour in several ways. An individual might avoid groups containing parasitised conspecifics to prevent infection with directly transmitted parasites. For example, healthy three-spined sticklebacks, *Gasterosteus aculeatus*, avoid grouping with conspecifics infected with the microsporidian parasite *Glugea anomala* (Ward et al., 2005). Furthermore, parasite infection might also affect the decision of individuals to aggregate. Parasites might alter their intermediate host behaviour in their interest (manipulation hypothesis). For example, satiated sticklebacks infected with the trophically transmitted cestode parasite *Schistocephalus solidus* spend significantly more time outside a group than uninfected conspecifics (Barber et al., 1995). Thus far, studies concerning the influence of parasitisation on grouping behaviour of invertebrates are underrepresented. One exception is the study of Jakobsen & Wedekind (1998), which shows that uninfected copepods *Macrocyclops albidus* preferred the odour of conspecifics whereas infected individuals avoided the group under the threat of predation.

A well-described example for parasite-induced changes in host behaviour is the acanthocephalan-amphipod system (e.g. Bakker et al., 1997; Maynard et al., 1998; Médoc et al., 2006; Kaldonski et al., 2008; Benesh et al., 2009), where the amphipods serve as intermediate hosts that have to be ingested by birds or fishes, the parasite’s final hosts (Crompton & Nickol, 1985). Fish parasites of the genus *Pomphorhynchus* are known to manipulate the photophobic behaviour (Bethel & Holmes, 1973; Kennedy et al., 1978; Bakker et al., 1997), the response to predator fish cues (Baldauf et al., 2007; Perrot-Minnot et al., 2007) as well as the activity (Dezfuli et al., 2003) of their amphipod intermediate hosts, and make them more conspicuous as the yellow–orange cystacanths are clearly visible through their cuticle (Bakker et al.,

1997). The life cycle of *Polymorphus minutus* includes water birds as definitive hosts. *P. minutus* is easily noticeable as orange–red cystacanths within its intermediate amphipod host. It alters the geotactic behaviour of the gammarids, probably facilitating the transmission to its definitive host (Cézilly et al., 2000; Bauer et al., 2005). Médoc & Beisel (2008) suggest that *P. minutus* might not only manipulate the anti-predator behaviour of its intermediate host in order to reach its definitive host but also the anti-predator response towards non-hosts in order prevent inappropriate transmission ('increased host abilities hypothesis'). This hypothesis is partly supported by empirical data. Médoc & Beisel (2008) found that *G. roeseli* infected with *P. minutus* have a higher swimming speed than uninfected individuals and that infected individuals spend generally more time in a refuge and at the surface (Médoc et al., 2009). In contrast, Kaldonski et al. (2008) found no significant influence of the parasite on refuge use in *G. pulex*. However, *P. minutus* infected *G. pulex* are less susceptible to predation by water scorpions (Kaldonski et al., 2008). A clear support of the 'increased host abilities hypothesis' would be a stronger tendency to aggregate by infected than by uninfected individuals.

The present study had a twofold aim: First, we aimed to elucidate whether aggregation behaviour of *G. pulex* is influenced by the infection with *P. minutus*. To answer this question we conducted two experiments. First, we tested whether increased aggregation behaviour in *P. minutus* infected *G. pulex* is induced by cues of a non-host fish predator. In the second experiment, we investigated whether infected or uninfected *G. pulex* prefer to associate with groups consisting of either infected or uninfected gammarids. Here, uninfected individuals are predicted to avoid the infected group because of increased oddity and the general higher visual conspicuousness of that group. For infected individuals, a prediction might be difficult because the positive effect of reduced oddity might be traded off by the generally increased conspicuousness of the infected group. The second aim was to elucidate the relationship between *P. minutus* infection and *G. pulex* activity. In amphipods, reduced activity is a strategy to reduce predation by fishes (Andersson et al., 1986; Wooster, 1998; Dezfuli et al., 2003; Wellnitz et al., 2003). Consequently, the 'increased host abilities hypothesis' predicts a stronger reduction of activity in infected gammarids.

Methods

Uninfected as well as *Polymorphus minutus* infected specimen of *Gammarus pulex* were sampled from the brook Katzenlochbach (50°41'59.03"N, 7°4'54.27"E) near Bonn, Germany, and transferred to the laboratory, where they were kept in tanks filled with aerated tap-water under standardised summer conditions (light/dark: 16/8 h, temperature $15 \pm 1^\circ\text{C}$). The tanks were equipped with dead leaves taken from the natural habitat to provide nutrition and shelter. The infection status of gammarids could easily be determined visually, because in case of infection, the orange–red cystacanth in the body cavity is visible through the cuticle. The determination of the parasite species was confirmed by a DNA analysis (unpublished data).

In all experiments, the odour of three-spined sticklebacks (*Gasterosteus aculeatus* L.), a fish present in the Katzenlochbach drainage system, was used as a predator cue. Three-spined sticklebacks prey both on infected and uninfected *Gammarus* (Mazzi & Bakker, 2003). Sixteen fish were taken from a pond located in the garden of the Institute for Evolutionary Biology and Ecology, Bonn using minnow traps, transferred to a tank (60 cm × 30 cm × 30 cm) filled with aerated tap-water and equipped with clay pots as hiding places. Temperature and light regime were the same as for the gammarids described above. Each evening after the last experiment, fish were fed with frozen *Chironomus* larvae. In order to avoid confounding effects of decaying larvae on predator odour, larvae which were not consumed within 30 min were removed. Immediately before the start of each predator trial, stickleback-conditioned water was taken from the holding tank and added to the test tank (see below). After 1 week, sticklebacks were replaced by 16 new individuals.

Experiment 1: Aggregation behaviour of infected individuals

Several hundred *G. pulex* were sampled on 18th and 20th June 2008. Experiments took place from 23rd June 2008 to 7th July 2008 between 10 a.m. and 6 p.m. under daylight conditions. The experimental set-up was identical to the one described in Kullmann et al. (2008). In a test aquarium (30 cm × 20 cm × 20 cm), a tea ball (diameter 3.5 cm) consisting of a

fine metal grid was placed in the middle of the left and right side wall one cm above the bottom. Ten uninfected randomly chosen gammarids were placed into one tea ball, whereas the second tea ball remained empty. Precopulatory pairs were discarded. The metal grid allowed olfactory exchange with the surrounding tank water, while visual and tactile contact was limited. The side on which the group was presented alternated between trials to avoid side effects. All gammarids were only used once.

Two lines drawn on the bottom divided the tank into three equal-sized compartments (10 cm × 20 cm). The outer compartments containing the tea balls served as association zones, the middle compartment served as neutral zone. Tanks were filled with one-day-old tap-water to a height of 10 cm. In the experiments, 200 ml stickleback-conditioned water (called ‘predator treatment’, $N = 92$) or 200 ml tap-water (called ‘no predator treatment’, $N = 72$) was added. Ten minutes after the tea balls were introduced to the tank, a haphazardly chosen infected test gammarid was put into a clear plastic cylinder (diameter: 3.5 cm) placed in the middle of the tank. As soon as the test gammarid started to move the cylinder was lifted. Thirty-seconds after lifting the cylinder it was scored whether the test gammarid was located in the association zone with or without the group of conspecifics or in the neutral zone by observations from above the tank. This observation was repeated at 30 s intervals for a period of 5 min, recording 10 positions for each individual. The observer was unaware of the predator regime of the trials. After each trial, the test tank was rinsed using tap-water.

Experiment 2: Impact of group infection status

For this experiment, several hundred *G. pulex* were sampled on the 9th October 2008. Experiments took place from 13th to 31st October 2008 between 10 a.m. and 5 p.m. under daylight conditions. The experimental procedure was similar to that of experiment one except for both tea balls containing a group of gammarids. One group consisted of 10 infected gammarids; the other group consisted of 10 uninfected specimens. As test individuals, in total 160 individuals were used (80 infected and 80 uninfected gammarids). In half of the trials they were exposed to a ‘predator treatment’ ($N_{\text{infected}} = 40$, $N_{\text{uninfected}} = 40$), in the

other half to a ‘no predator treatment’ ($N_{\text{infected}} = 40$, $N_{\text{uninfected}} = 40$). Test gammarids were used only once, while gammarids of the groups were used several times. However, gammarids of the groups were never used twice a day or in the same combination. Again, positions of the test gammarids were scored in 30 s intervals for a period of 5 min.

Activity

Activity of the test gammarid in both experiments was measured as the number of changes between the zones recorded by the scans every 30 s. The maximum activity value was 10 when the test gammarid had always switched the zone between two consecutive observation intervals. Minimum activity was 0 when the test gammarid was in the same zone in all observation intervals.

Statistical analysis

In order to test whether parasitism alters aggregation behaviour, data of infected gammarids from experiment 1 were compared with the data of uninfected gammarids described in Kullmann et al. (2008). Because of the proportional character of the data (number of visits at the side with group relative to the number of visits at the side without group) a generalised linear model (GLM) was conducted with binomial or, in the case of overdispersion, quasibinomial error distribution and logit link function (R Development Core Team, 2009). Overdispersion was assumed when the value of the residual deviance was greater than the degrees of freedom. In cases of quasibinomial error distribution F values and in the case of binomial distribution χ^2 values are provided. We created a dependent variable for the proportional data using the R function *cbind* (Crawley, 2005), thereby combining the number of visits at the side with group and the number of visits at the side without group. As fixed factors, ‘infection status’ (infected or uninfected) of the test gammarid and ‘predation regime’ (fish odour present or absent) were added to the model. Furthermore, to test whether the aggregation behaviour differed between infected and uninfected individuals dependent on the predation regime the interaction between ‘infection status’ and ‘predation regime’ was added.

Group-preferences in experiment 2 were analysed using a GLM with the preference for infected or uninfected group as response variable (see above). As fixed factors ‘infection status’ and ‘predation regime’ and the interaction between both factors were entered into the model.

To compare the activity levels of infected and uninfected individuals, we combined the activity data from experiment 1 and 2 and the data from Kullmann et al. (2008). The activity levels of infected and uninfected gammarids were compared using a GLM with poisson or, in the case of overdispersion, quasipoisson error distribution and log link function. The influence of the fixed factors ‘infection status’ of the test gammarids and ‘predation regime’ on gammarids activity was tested. In order to test whether infected and uninfected individuals differed in their response to predator cues the interaction between ‘infection status’ and ‘predation regime’ was added. In order to correct for differences in the activity levels of gammarids in experiment 1 and 2, the factor ‘experiment’ were entered into the model. However, as this had no significant effect (GLM, $F = 0.651$, $P = 0.420$), the factor was excluded from further analyses.

Likelihood ratio tests (‘LRT’) assessed whether the removal of a variable caused a significant decrease in model fit. Hence, degrees of freedom always differed by one. Non-significant ($P > 0.05$) factors and interactions were removed from the analysis (Engqvist, 2005). P values refer to the increase in deviance when the respective variable was removed. All given test probabilities are two-tailed throughout. All analyses were performed using R 2.8.1 statistical package (R Development Core Team, 2009).

Results

Experiment 1

The predation regime, i.e. the presence or absence of fish odour, significantly predicted aggregation behaviour (GLM, $F_1 = 6.17$, $P < 0.001$, Fig. 1a). In the presence of fish odour, the gammarids significantly preferred the proximity of the group (GLM, intercept, $T_{145} = 3.64$, $P < 0.001$, Fig. 1a), whereas without fish odour they showed no significant tendency to

aggregate (GLM, intercept, $T_{126} = 0.05$, $P = 0.96$, Fig. 1a). Infection status did not significantly predict the general aggregation behaviour, i.e. infected and uninfected gammarids did not differ significantly in the time spent near the group (GLM, $F_1 = 1.26$, $P = 0.26$, Fig. 1a). Furthermore, infection status did not significantly predict the response to predator cues, i.e. infection with *P. minutus* did not increase the tendency to aggregate in response to predator cues in *G. pulex* (interaction ‘infection status \times predation regime’, GLM, $F_1 < 0.001$, $P = 0.99$, Fig. 1a).

Experiment 2

Gammarids did not significantly prefer groups consisting of infected individuals or groups consisting of uninfected individuals (GLM, intercept, $T_{155} = 0.02$, $P = 0.99$, Fig. 1b). Furthermore, neither predation regime ($F_1 = 0.15$, $P = 0.7$, Fig. 1b) nor infection status had a significant impact on choice behaviour ($F_1 = 0.5$, $P = 0.5$, Fig. 1b). The choice of infected and uninfected gammarids did not significantly differ between predation regimes (interaction ‘infection status \times predation regime’, GLM, $F_1 = 0.004$, $P = 0.95$, Fig. 1b).

Activity

The interaction between predation regime and infection status significantly explained variation in activity (GLM, $F_1 = 3.943$, $P = 0.047$). Post-hoc tests revealed that uninfected gammarids were generally more active than infected ones (GLMs, all $F_1 > 21.45$, all $P < 0.001$, Fig. 1c). Uninfected gammarids reduced their activity in the presence of predatory cues (GLM, $\chi_1^2 = 5.085$, $P = 0.024$, Fig. 1c), whereas infected gammarids did not significantly (GLM, $F_1 = 0.29$, $P = 0.59$, Fig. 1c).

Discussion

The aim of the present study was to examine the ‘increased host abilities hypothesis’ with *Polymorphus minutus* infected *Gammarus pulex*. Although infected individuals of *G. pulex* generally had a lower activity, they did not show a significantly stronger response to non-host predator cues concerning

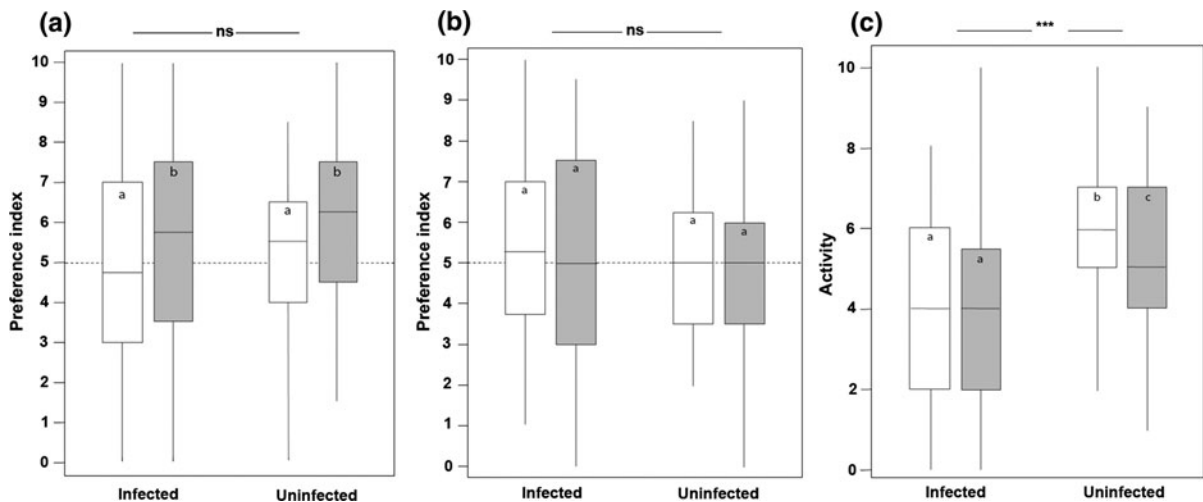


Fig. 1 **a** Preference indices of infected and uninfected gammarids choosing between joining a group and staying solitary either in the presence (*grey boxes*) or absence of fish odour (*white boxes*). The index is calculated by subtracting the number of visits at the side of the tank without conspecifics from the number of visits at the side with conspecifics. Positive values indicate a preference for the group, while negative values indicate avoidance of the group. **b** Preference indices of infected and uninfected gammarids choosing between a group of uninfected or infected conspecifics in the presence (*grey boxes*) or absence of fish odour (*white boxes*). The index is

calculated by subtracting the number of visits at the side of the tank with infected conspecifics from the number of visits at the side with uninfected conspecifics. Positive values indicate a preference for the uninfected group, while negative values indicate avoidance of the uninfected group. **c** Activity levels of infected and uninfected gammarids in the presence (*white boxes*) or absence of fish odour (*grey boxes*). Values are given as median (\pm quartiles, ranges). Upper statistics show differences between infection status (n.s. = nonsignificant $P > 0.05$). Different letters indicate statistically significant differences ($P < 0.05$)

aggregation behaviour and activity than uninfected conspecifics.

Aggregation behaviour

In the first experiment, infected *G. pulex* preferred to stay near the group when olfactory fish cues were present, whereas they showed no significant aggregation behaviour when fish cues were absent. Thus, infected gammarids adjust their grouping behaviour in the same manner as uninfected gammarids do (Kullmann et al., 2008). This result confirms previous studies, which showed that gammarids are capable of olfactory recognition of predators (Dahl et al., 1998; Baldauf et al., 2007; Perrot-Minnot et al., 2007), a capability that is not affected by parasitisation. Furthermore, it indicates that *P. minutus* does not decisively manipulate anti-predator aggregation behaviour in *G. pulex*. Unlike other behaviours that affect the probability to encounter a predator, e.g. refuge use or fleeing from predators (Bakker et al., 1997; Maynard et al., 1998; Dezfuli et al., 2003;

Wellnitz et al., 2003), aggregation behaviour seems to be still under the control of the host.

In experiment 2, both infected as well as uninfected *G. pulex* did not significantly choose between groups differing in their infection status. Infected individuals differ from uninfected group mates in coloration and behaviour and thus might be easier to detect by predator fish within a group of uninfected individuals. However, infected groups might be generally more conspicuous to predator fish. Thus, infected individuals may be equally likely to be detected by a predator fish in both groups and therefore did not choose one particular group. In contrast, the lack of significant preferences in uninfected individuals was surprising, because uninfected individuals were expected to avoid the infected group for two reasons. First, the infected group should generally be more conspicuous and thus be avoided. Second, an uninfected individual among infected individuals would be odd, more easily detectable and thus exposed to an increased risk of predation by fish (Theodorakis, 1989). A possible explanation for this

lack of discrimination between the groups might be the disability of *G. pulex* to recognise infected individuals. This conjecture is underlined by the results from a mate-choice experiment where male *G. pulex* did not differentiate between infected and uninfected females of similar size (Poulton & Thompson, 1987). Furthermore, due to our experimental set-up, visual and tactile communication between the test gammarid and the group was limited and thus potential assessment of the groups was based probably on merely olfactory cues. Although olfaction has been described to play a key role in the perception of the environment in amphipods (Baumgärtner et al., 2002; Krang & Baden, 2004; Baldauf et al., 2007; Kullmann et al., 2008), we cannot exclude that gammarids use tactile cues or general behavioural abnormalities to recognise parasitised conspecifics.

Activity

In the presence of predator fish cues, uninfected gammarids reduced their activity, which is in concordance with results from previous studies (Anderson et al., 1986; Wooster, 1998; Dezfuli et al., 2003; Wellnitz et al., 2003). Lowered activity reduces the risk of encountering a predator fish and, thus, reduces the risk of predation (Gerritsen & Strickler, 1977). *P. minutus* infected gammarids did not significantly alter their activity level in the presence of non-host predator cues, which disagrees with the predictions of the ‘increased host abilities hypothesis’. However, infected individuals generally showed a lower activity than uninfected ones. Our results are in accordance with two previous studies, which also found no significant differences in the response of *P. minutus* infected and uninfected gammarids to fish cues (Kaldonski et al., 2007; Médoc et al., 2009). In these studies, infected gammarids spent generally more time hiding than uninfected ones irrespective of the actual predation risk. Thus, one may argue that the parasitic manipulation rather influences the general than the plastic anti-predator response. Manipulation of general behaviours might be sufficient for the parasite in order to reduce the transmission to non-hosts. Alternatively, the differences between infected and uninfected gammarids could be a side-effect of the infection. Infected gammarids might suffer from a reduced food intake (McCahon et al., 1988; Brown &

Pascoe, 1989). Thus, lowered activity might be considered as an adaptive host response. The impact of an acanthocephalan infection on host activity is still ambiguous (for a review see Poulin, 1994). Further studies are required to disentangle manipulative and pathological effect of parasites.

Summarising, the results of our study indicate that olfactory cues of a non-host predator do not induce an increased anti-predator response in *P. minutus* infected *G. pulex* relative to uninfected ones. However, the general lowered activity of infected gammarids might be interpreted as parasitic manipulation in order to avoid predation by non-host predators.

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