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Conflict of interest between a nematode and a trematode in an amphipod host: test of the “sabotage” hypothesis

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Abstract *Microphallus papillorobustus* is a manipulative trematode that induces strong behavioural alterations in the gamaridean amphipod *Gammarus insensibilis*, making the amphipod more vulnerable to predation by aquatic birds (definitive hosts). Conversely, the sympatric nematode *Gammarinema gammari* uses *Gammarus insensibilis* as a habitat and a source of nutrition. We investigated the conflict of interest between these two parasite species by studying the consequences of mixed infection on amphipod behaviour associated with the trematode. In the field, some amphipods infected by the trematode did not display the altered behaviour. These normal amphipods also had more nematodes, suggesting that the nematode overpowered the manipulation of the trematode, a strategy that would prolong the nematode's life. We hypothesize that sabotage of the trematode by the nematode would be an adaptive strategy for the nematode consistent with recent speculation about co-operation and conflict in manipulative parasites. A behavioural test conducted in the laboratory from naturally infected amphipods yielded the same result. However, exposing amphipods to nematodes did not negate or decrease the manipulation exerted by the trematode. Similarly, experimental elimination of nematodes from amphipods did not permit trematodes to manipulate behaviour. These experimental data do not support the hypothesis that the negative association between nematodes

and manipulation by the trematode is a result of the “sabotage” hypothesis.

Keywords Amphipod · Conflict of interest · Manipulation · Nematode · Trematode

Introduction

There is growing interest in studying the evolutionary and community ecology of manipulative parasites (Lafferty and Morris 1996; Thomas et al. 1997, 1998a, b; Poulin 1998; Poulin et al. 1998; Brown 1999; Lafferty 1999; Cézilly et al. 2000; Dezfuli et al. 2000; Lafferty et al. 2000). Depending on the ecology of the different parasite species co-occurring with the manipulator, clear, qualitative predictions exist for the adaptive transmission strategies that selection should favour (see Lafferty et al. 2000). For instance, when non-manipulative and manipulative parasites share the same intermediate and definitive hosts (i.e. shared interests) and when manipulation is costly to achieve, non-manipulative parasites should preferentially parasitize hosts already manipulated so as to increase their chance of transmission without having to invest in manipulation (i.e. “hitch-hiking” strategy; Thomas et al. 1997, 1998a). Conversely, conflicts of interest are likely to exist when one or several parasite species share an intermediate host with a manipulator but do not have the same definitive hosts (Lafferty 1999). There are at least three evolutionary solutions to such a conflict (Lafferty et al. 2000): (1) avoiding intermediate hosts already infected by a manipulator; (2) killing a manipulator; and (3) overpowering the manipulation of a manipulator. Lafferty et al. (2000) named the last strategy “hijacking”, in the case where the second parasite is also a trophically transmitted manipulator. Here, we introduce another strategic analogy, “sabotage”, for the case where the second parasite is not a manipulator, is not trophically transmitted, and benefits most from having the host behave normally. We evaluate this new hypothesis in a community of parasites in an amphipod in France.

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The parasitic community of the brackish-water crustacean, *Gammarus insensibilis* (Amphipoda), includes a manipulative trematode (*Microphallus papillorobustus*), three non-manipulative trematodes (*Maritrema subdolum*, *Levinseniella tridigitata* and *Microphallus hoffmanni*) (Helluy 1981) and a nematode (*Gammarinema gammari*) (Fauchier and Thomas, in press). Metacercariae of the trematode are located in the amphipod's brain and induce strong behavioural alterations in amphipods (positive phototaxis, negative geotaxis and an aberrant evasive behaviour), making them more vulnerable to predation by aquatic birds (definitive hosts) (Helluy 1981, 1984). The three non-manipulative trematode species have shared interests with the manipulator *Microphallus papillorobustus*, as they also finish their life-cycle in the same aquatic bird species (Rebecq 1964; Helluy 1981). Thomas et al. (1997) showed that *Maritrema subdolum* is a hitch-hiker parasite, as it favours its transmission to aquatic birds by preferentially infecting amphipods already manipulated by *Microphallus papillorobustus*. *Microphallus hoffmanni* and *L. tridigitata* are not positively associated with the manipulator; instead they infect hosts at random and only occasionally achieve higher transmission success by sharing an amphipod with *M. papillorobustus* (Thomas et al. 1998b).

The aim of this paper was to study the interaction between the manipulator *M. papillorobustus* and the nematode *Gammarinema gammari*. Indeed, a conflict of interest is theoretically expected between these two parasite species since the trematode uses amphipods as "vehicles" to be transmitted to birds, while the nematode uses amphipods as a habitat and source of nutrition (Fauchier and Thomas, in press). In accordance with the idea that the nematode and the trematode have a conflict of interest, a previous study showed that a negative relationship exists between the abundance of these two parasites inside amphipods (Fauchier and Thomas, in press). However, the co-occurrence of the nematode and the trematode may be sufficiently frequent for adaptations in addition to avoidance to evolve. The purpose of this study was to determine whether or not the nematode is able to sabotage manipulated amphipods by decreasing the efficiency of the manipulation exerted by the trematode. We first studied the behaviour of naturally infected amphipods in the field and laboratory. Then, we analysed the consequences of mixed infection on amphipod behaviour by manipulating nematode load in the laboratory.

Field study

Methods

To investigate the association between the parasite community and amphipod behaviour, we collected manipulated and normal *Gammarus insensibilis* during October 2000 in the brackish lagoon of Thau (Sète, southern France, 43°25'N, 3°35'E) and noted their parasites. If the nematode was able to sabotage the trematode's manipulation, we predicted that amphipods infected by both the trematode and the nematode would be less likely to occur at the

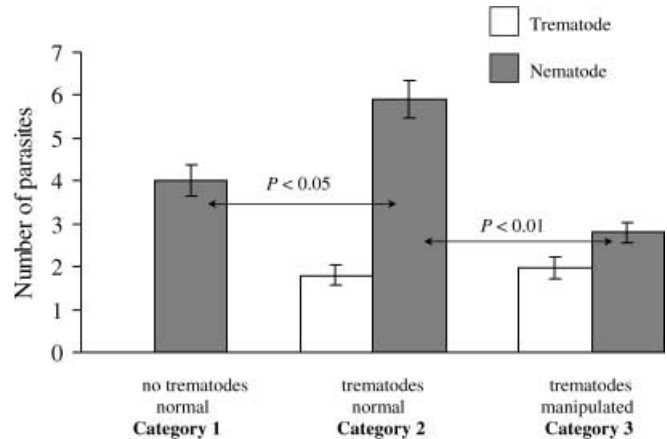


Fig. 1 Mean abundance (\pm SE) for the trematode and nematode in amphipods in the three categories (category 1: amphipods with no metacercariae and with normal behaviour; category 2: amphipods harbouring metacercariae but having normal behaviour; category 3: amphipods harbouring metacercariae and displaying altered behaviour). Only significant differences are indicated

surface of the water. We collected manipulated amphipods at the surface of the water (see Thomas et al. 1996) and normal amphipods under algae, just at the surface of the mud. All amphipods were preserved in alcohol (70%) to be sexed, measured for length (from head to tip of telson), and dissected for parasitic examination. Because the prevalence of the nematode is very low in female amphipods compared to males (Fauchier and Thomas, in press), only male amphipods were used ($n=100$). Metacercariae of the trematode form distinctive ovoid cysts (on average $270 \times 350 \mu\text{m}$; Rebecq 1964) in the amphipod's brain, and cannot be confounded with other trematodes infecting *Gammarus insensibilis* in this geographic area (see Helluy 1981). For the nematode *Gammarinema gammari*, we only considered adult worms (which are located around the mouth parts) because smaller larvae could be accidentally omitted (F. Thomas, unpublished observations). We compared the abundance of the trematode and the nematode from the two samples. All statistical tests were performed following Sokal and Rohlf (1981) and Siegel and Castellan (1988). All tests were two-tailed and results were considered significant at the 5% level.

Results

We found evidence consistent with our prediction of the sabotage hypothesis in the field. Three behaviour/trematode categories of amphipods occurred in the field: (1) normal amphipods without the trematode (category 1, $n=36$), (2) normal amphipods with the trematode (category 2, $n=14$), and (3) manipulated amphipods with the trematode (category 3, $n=50$). There were no manipulated amphipods without the trematode. The mean body length of amphipods was slightly different between the 3 categories [mean (mm \pm SD), category 1: 15.2 ± 1.1 ; category 2: 15.6 ± 1.2 ; category 3: 14.2 ± 1.9 , Kruskal-Wallis ANOVA, $H=8.72$, $df=2$, $P<0.01$]. Even though this difference is slight, it could represent a decrease in moult rate associated with trematode infection. There was no significant difference in the mean number of trematode metacercariae between manipulated and normal amphipods with the trematode (categories 2 and 3, Fig. 1,

Mann-Whitney *U*-test, $z=-0.42$, $P=0.67$). However, there was a significant difference in the abundance of the nematode between the 3 categories (Fig. 1, Kruskal-Wallis ANOVA, $H=22.4$, $df=2$, $P<0.001$). Thus, among amphipods with the trematode, the abundance of nematodes was higher in normal amphipods (category 2) than in manipulated amphipods (category 3). Nematode load between categories 1 and 3 was not significant (pair comparison, $P=0.06$, Fig. 1).

Thus, in accordance with the “sabotage” hypothesis, gammarids that display a normal behaviour despite the presence of the trematode also harbour more nematodes than other gammarids.

Experiment 1

Methods

To determine if sabotage could be observed in the laboratory, we noted the behavioural response of amphipods to an artificial stimulus. If the nematode is able to sabotage the trematode’s manipulation, we predicted that an amphipod infected by trematodes and nematodes would be more likely to respond normally to a stimulus than would an amphipod infected by trematodes alone. A new sample of amphipods was taken from Thau’s lagoon in February 2001. Amphipods were sampled in the aquatic vegetation and in water no more than 40 cm in depth. We attempted to obtain manipulated amphipods and amphipods with normal behaviour. Only male amphipods were returned to the laboratory and were maintained throughout the experiment in seawater (salinity 38 ppt). We observed each amphipod’s response to two standardized mechanical disturbances of 3 s (separated by an interval of 5 min) in a water column (length: 5 cm, width: 5 cm, height: 26 cm) filled with aerated seawater (salinity 38). A rotor fixed to an electric motor clipped the water column as it turned and generated a disturbance that all amphipods responded to. Amphipods manipulated by the trematode responded to the disturbance by swimming rapidly to the surface. Conversely, an amphipod was considered normal if it responded to the disturbance by staying below the middle of the column (i.e. within the first 13 cm). With this procedure, we distinguished amphipods displaying: (1) two altered responses, (2) one altered and one normal response, and (3) two normal responses. Each amphipod was then preserved in alcohol (70%) and, as before, measured for length (from head to tip of telson) and examined for parasites. We compared the abundance of the trematode and nematode in amphipods in the three behavioural categories.

Results

We found evidence consistent with predictions of the sabotage hypothesis in the laboratory. Among the 82 amphipods tested, 32 did not have the trematode. The mean body length (mean \pm SD) of these amphipods was 17.2 mm \pm 2.4 and the mean nematode load was 3.4 \pm 1.72. As expected, all these amphipods responded normally to the two mechanical disturbances. Among the 50 amphipods infected by the trematode, 14 swam to the surface after both disturbances (group +/+), 11 swam to the surface after only 1 of the two disturbances (group +/-), and 25 did not swim to the surface (group -/-). The mean body length of these three categories of infected amphipods was not significantly different [mean (mm \pm SD), group +/+: 17.4 \pm 2.1, group +/-: 17.8 \pm 2.3, group -/-:

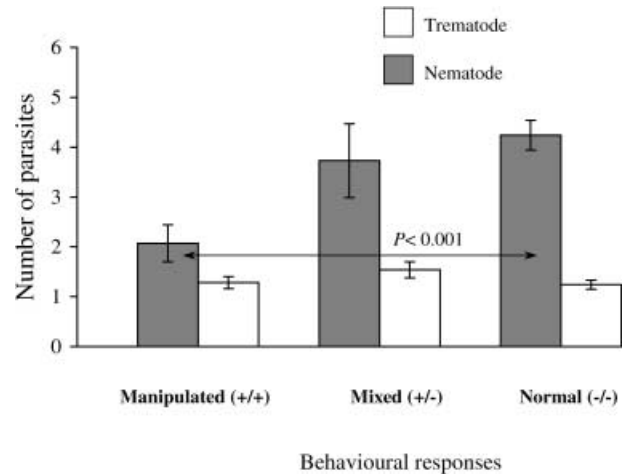


Fig. 2 Mean abundance (\pm SE) for the trematode and nematode in amphipods according to their behavioural responses. Only significant differences are indicated

17.8 \pm 2.5; ANOVA, $F_{1,47}=0.17$, $P=0.84$]. The mean abundance of metacercariae of the trematode was not significantly different between the three categories of amphipods (Fig. 2, Kruskal-Wallis ANOVA, $H=3.31$, $df=2$, $P=0.19$). However, there was a significant difference in mean nematode abundance (Fig. 2, Kruskal-Wallis ANOVA, $H=13.98$, $df=2$, $P<0.001$), indicating that, as before, normal amphipods infected by the trematode were more heavily infected with nematodes than were those amphipods with manipulated behaviour.

Thus, in accordance with the “sabotage” hypothesis (and the field study), this experiment conducted with naturally infected gammarids confirms in the laboratory that gammarids that display a normal behaviour despite the presence of the trematode also harbour more nematodes than other gammarids.

Experiment 2

Methods

To test if sabotage was experimentally inducible, we exposed amphipods to nematodes and observed the amphipod’s subsequent behaviour. If the nematode is able to sabotage existing behavioural alterations induced by the trematode, we predicted that an amphipod manipulated by trematodes would regain normal behaviour after experimental infection with nematodes. A new sample of amphipods was collected during March 2001. We only collected males at the surface of the water. We separated amphipods randomly and formed three groups (G1, G2 and G3). Nematodes were killed in amphipods from G1 and G2 by placing them in a tank containing fresh water for two 5-min periods (separated by an interval of 10 min in seawater). This osmotic shock serves as a very efficient method of killing nematodes without killing the gammarids or the trematodes (F. Thomas, unpublished observations). They were then returned to their initial seawater tank. We used the same behavioural test as before (see experiment 1) to verify that all amphipods exposed to fresh water responded to the two mechanical disturbances. Then, amphipods from G1 were experimentally infected with the nematode by placing them in a petri dish containing injured amphipods from G3. Nematodes in G3

amphipods rapidly left their dying host and penetrated the target amphipod (i.e. G1). All amphipods (G1 and G2) were kept for 1 week under standard conditions (15°C, salinity 38, natural photoperiod) and were fed with fish food (Tetra Ani Min). Then, we compared the behaviour of nematode-eliminated (G1) and nematode-reinfected (G2) amphipods in response to two mechanical disturbances in the water column (same procedure as in experiment 1). Each amphipod was then preserved in alcohol (70%), measured for length (from head to tip of telson), and dissected for parasitic examination.

Results

We did not find evidence that sabotage was experimentally inducible. We exposed 29 amphipods infected by the trematode to fresh water, killing their nematodes. Among these amphipods, 13 were subsequently infected with nematodes (group G1), while the others (group G2, $n=16$) were left uninfected. The mean body length of amphipods was not significantly different between these two groups [mean (mm SD), G1: 19.2 ± 2.0 ; G2: 18.3 ± 2.2 , unpaired t -test, $t=1.097$, $P=0.28$]. There was no significant difference between the mean number of trematode metacercariae in the two groups (mean \pm SD, G1: 6.85 ± 5.29 ; G2: 14.94 ± 22.1 , Mann-Whitney U -test, $z=0.15$, $P=0.88$). As expected, the mean abundance of nematodes found in amphipods after exposure to nematodes (mean \pm SD, G1: 5.31 ± 2.43) was significantly higher than that of amphipods exposed to fresh water only (mean \pm SD, G2: 0.12 ± 0.34) (Mann-Whitney U -test, $z=4.79$, $P<0.0001$). However, despite the efficiency of our procedure to kill and to add nematodes in amphipods, there was no significant difference between the behaviour of amphipods from the two groups. Indeed, only 1 amphipod (out of 13) in the nematode-infected group (G1) responded normally to the 2 mechanical disturbances. Other nematode-infected amphipods (G1), as well as nematode-uninfected amphipods (G2), had manipulated behaviour in response to the two mechanical disturbances. These proportions were not significantly different (Fisher exact test, $P=0.45$).

Thus, against the prediction made by the "sabotage" hypothesis, adding nematodes inside modified gammarids does not reduce the behavioural alterations induced by the trematode.

Experiment 3

Methods

To test if sabotage was reversible, we removed nematodes from amphipods and observed their subsequent behaviour. If the presence of nematodes causes trematode-infected amphipods to behave normally, we predicted that removing these nematodes would allow the trematode to manipulate the amphipod. A new sample of male *Gammarus insensibilis* was collected in April 2001 at Thau's lagoon. This time, we collected only amphipods living under algae (i.e. with a normal behaviour). In the laboratory, we placed all these amphipods in an opaque plastic tank (28 \times 22 cm) and exposed them collectively to a mechanical disturbance every 15 min for 3 h. During this period, we removed all manipulated amphi-

pods. We separated the remaining normal amphipods randomly to form two groups. Then, we eliminated nematodes from amphipods in the first group using fresh-water exposure. All amphipods (i.e. from the treated and the untreated groups) were kept 2 weeks in separated tanks in the laboratory and in standard conditions (see experiment 2). During this period, the two tanks were exposed to a mechanical disturbance twice per day (in the morning and afternoon) and we collected all the manipulated amphipods. Amphipods were then preserved in alcohol (70%), measured for length (from head to tip of telson), and dissected for parasitic examination.

Results

We did not find evidence that sabotage was experimentally reversible. While all amphipods had normal behaviour at the beginning of the experiment, dissection 2 weeks later revealed that 50 individuals were infected by at least 1 metacercaria of the trematode, and only 17 amphipods were not infected by the trematode. The fresh-water exposure killed nematodes in all 21 treated amphipods, while the mean nematode abundance in amphipods in the untreated group was 4.0 ± 2.7 (group B, $n=29$) (Mann-Whitney U -test, $z=-5.69$, $P<0.001$). There was no significant difference in the abundance of the trematode between the two groups (mean \pm SD, untreated group: 3.62 ± 2.72 , $n=29$, treated group: 4.05 ± 3.15 , $n=21$, Mann-Whitney U -test, $z=0.45$, $P=0.65$). The mean body length of amphipods in the untreated group [mean (mm \pm SD), 19.7 ± 2.38] was slightly larger than that of treated amphipods [mean (mm \pm SD), 17.9 ± 2.21] (unpaired t -test, $t=2.63$, $df=48$, $P=0.01$). It is possible that treatment slowed moulting rates.

During the 2 weeks, 37 amphipods out of the 50 infected by the trematode changed their behaviour from normal to altered. However, the proportion of amphipods displaying an altered behaviour was not significantly different between the untreated group and the treated group (i.e. exposed to fresh water). Indeed, in the untreated group, 21/29 amphipods (i.e. 72.4%) displayed an altered behaviour compared with 16/21 (i.e. 76%) in the treated group (Fisher exact test, $P=0.99$). There was no significant difference in the mean abundance of metacercariae between amphipods displaying a normal or an altered behaviour in the two groups (mean \pm SD, untreated group, normal behaviour: 3.62 ± 3.16 , $n=8$, altered behaviour: 3.62 ± 2.62 , $n=21$; treated group, normal behaviour: 2.0 ± 2.24 , $n=5$, altered behaviour: 4.69 ± 3.18 , $n=16$; Kruskal-Wallis ANOVA, $H=5.18$, $df=3$, $P=0.16$). Finally, among amphipods from the untreated group, there was no significant difference between the mean abundance of nematodes in individuals that stayed normal (mean \pm SD, 4.25 ± 2.66 , $n=8$) and those that became manipulated (mean \pm SD, 3.9 ± 2.41 , $n=21$) (Mann-Whitney U -test, $z=0.32$, $P=0.75$).

Thus, against the prediction made by the "sabotage" hypothesis, removing nematodes in normal-behaving gammarids that harbour the trematode does not induce the modified behaviour.

Discussion

Results from the experiments (2 and 3) in this study are not consistent with predictions of the sabotage hypothesis and do not provide an explanation for our observation that the abundance of nematodes in trematode-infected amphipods is higher in normal amphipods than in manipulated amphipods (field study and experiment 1). There are several potential reasons why our results were not consistent with our predictions. First, it is conceivable that the nematode is only able to decrease the manipulation of the trematode in naturally infected amphipods. For example, it is possible that the experimental infection decreased the nematode's future ability to counteract the trematode's manipulation. In addition, manipulation by the trematode might be irreversible (explaining the results of experiments 2 and 3), and sabotage might occur only if infection with nematodes precedes trematode infection. Testing this hypothesis would require sequentially exposing uninfected amphipods to both parasites in the laboratory.

Our results may indicate that the abundance of the nematode is more a consequence than a cause of the normal behaviour. Under particular circumstances or habitats, amphipods might display a normal behaviour despite the presence of the trematode in their brain. Several hypotheses can be proposed to explain this result. If the infective stages of the nematode are mainly present under algae, normally behaving amphipods would be more likely to be infected by nematodes than those displaying an altered behaviour at the surface. Under this hypothesis, we might also expect amphipods with an altered behaviour to have fewer nematodes than those uninfected by the trematode. Manipulated amphipods might also be a poor habitat for nematodes, so that nematodes avoid them or do not survive well in them. For example, by spending time at the surface, manipulated amphipods might expose nematodes to lethal conditions (e.g. low salinity). Although the field study did not indicate that nematode loads are significantly different between amphipods with an altered behaviour and those uninfected by the trematode (Fig. 1), further investigations would be necessary to clarify this point.

Because resistance to parasites usually has a cost for hosts, resistance to different parasites should vary with parasite pathogenicity (e.g. Thomas et al. 2000). In our system, there is more likely to be selection for resistance against the trematode than the nematode. This is because the trematode significantly reduces amphipod survival (Helluy 1984; Thomas et al. 1995a) and mating success (Thomas et al. 1995b, 1996) while the nematode seems not to affect male amphipods (Fauchier and Thomas, in press). Our findings are consistent with the idea that some amphipods could resist the manipulation exerted by the trematode since several individuals with metacercariae were not manipulated. It could, then, be possible that amphipods that direct resources to resist behavioural change, become more susceptible to nematodes. Although this hypothesis remains speculative without ex-

perimental infection, it is in accordance with the fact that the largest nematode load is observed for the amphipods that, a priori, resist the manipulation exerted by the trematode (Fig. 1).

More likely explanations stem from the possibility that nematodes might be more abundant in amphipods with metacercariae that are not yet mature enough to manipulate the host (manipulated behaviour is expected to be associated with parasite maturity). Behavioural alterations in amphipods only appear more than 15 days after infection by the trematode (Helluy 1981). If mortality is higher for amphipods infected with both nematodes and trematodes, double infections would be less likely to persist long enough for trematodes to mature to the point that they manipulate the amphipod's behaviour, thereby leading to fewer nematodes being present in manipulated amphipods with mature trematodes. The same pattern could arise if the presence of the nematode slows metacercarial maturation. Indeed, it seems that our collection initially contained numerous amphipods harbouring developing metacercariae. The proportion of amphipods that changed their behaviour from normal to altered in experiment 3 was unexpectedly large. A pulsed exposure of trematode cercariae could explain this result. During the spring, we usually observe a sudden increase in the number of manipulated amphipods (F. Thomas, unpublished observations), suggesting that parasitized snails release numerous trematode cercariae at this time.

There are several reasons why nematodes might not be able to sabotage the trematode's manipulation. First, sabotaging the host may simply be impossible for nematodes for physical or physiological reasons. It is difficult to speculate on this without knowing the mechanism by which the trematode alters behaviour. Another possible explanation is that, counter to our expectation expressed in the Introduction, there may be little selective pressure for sabotage to evolve. For instance, if the prevalence of the trematode is small in natural amphipod populations, selection for sabotage seems unlikely (see also Lafferty et al. 2000). In addition, even if prevalence is high, the nematode may be sufficiently successful at avoiding manipulated hosts that there may be little selective pressure for sabotage. Unfortunately, it is difficult to determine trematode prevalence as the behavioural alterations in infected amphipods systematically introduce a bias in samples (i.e. infected individuals are more likely to be collected than uninfected ones, Thomas et al. 1995a, b). Since little is known about the ecology of the nematode, further studies would be necessary to determine whether it is host specific to *Gammarus insensibilis*.

In conclusion, this study indicates that the resolution of apparent conflicts of interest is not always intuitive and that experimental studies are essential to confirm field observations, as well as experiments conducted with naturally infected individuals.

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