



Two steps to suicide in crickets harbouring hairworms

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The hairworm (Nematomorpha) *Paragordius tricuspidatus* has the ability to alter the behaviour of its terrestrial insect host (the cricket *Nemobius sylvestris*), making it jump into the water to reach its reproductive habitat. Because water is a limited and critical resource in the ecosystem, we predicted that hairworms should adaptively manipulate host behaviour to maximize parasite reproductive success. Our results supported the hypothesis that the host manipulation strategy of hairworms consists of at least two distinct steps, first the induction of erratic behaviour and then suicidal behaviour per se. Hairworms secured mating by starting to manipulate their host before being fully mature. Once induced, the cricket's suicidal behaviour was maintained until the host found water but the fecundity of worms decreased over time. As expected, the fecundity of worms was better in crickets with suicidal rather than erratic behaviour.

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Parasites are capable of altering a large range of phenotypic traits in their host to favour the continuation of their life cycle (Poulin 1998; Moore 2002; Thomas et al. 2005). From an evolutionary point of view, these alterations are usually seen as illustrations of the extended phenotype concept (Dawkins 1982), in which genes in one organism (i.e. the parasite) have phenotypic effects on another organism (the host). Although there are many impressive examples of host manipulation by parasites, few studies have examined how the ecological factors of the host shape the evolution of this parasite exploitation strategy (Ponton et al. 2006).

Hairworm (Nematomorpha)-induced suicide is one of the clearest examples of the adaptive manipulation of

host behaviour by parasites (Thomas et al. 2002; Biron et al. 2006). As juveniles, hairworms are mostly parasites of terrestrial arthropods but become free-living adult worms in aquatic environments such as rivers, streams and lakes (May 1919) where they mate and produce eggs. Infected hosts harbouring mature hairworms commit suicide by jumping into water (Thomas et al. 2002). We examined whether selection has shaped the manipulation strategies of hosts prior to the crucial transmission event. Given that the probability of successful transmission varies because of environmental heterogeneity experienced by the host, hairworms infecting crickets should adaptively manipulate host behaviour to maximize parasite reproductive success. We predicted that because of the scarcity of suitable ponds during the hot summer months in native forests in the south of France, coupled with the short reproductive period of hairworms (Thomas et al. 2002), selection should favour worms that induce water-seeking behaviour before they are mature enough to reproduce.

Thus, our aim in this study was to test (1) whether parasites induce suicide by altering the host's behaviour in a way that increases the encounter rate with water, and (2)

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whether suicide timing maximizes parasite reproductive success.

Specifically, we predicted (1) that not all crickets with altered behaviour (compared to uninfected individuals) are induced to commit suicide at capture time, (2) these manipulated (but not suicidal) individuals, when kept for a few days in the laboratory, should finally show the typical suicidal behaviour, and (3) once induced, the suicidal behaviour should be maintained even though this comes at a cost for the subsequent fecundity of the parasite. The existence of manipulation prior to the crucial transmission event is important when considering what selection forces act at proximate and ultimate levels in shaping the manipulative effort of the parasites.

METHODS

Origin of Specimens

The host species considered in our study was the cricket *Nemobius sylvestris* and all specimens came from Avènes les Bains (43°45'N, 3°06'E, southern France) as in previous studies (Thomas et al. 2002). Uninfected *N. sylvestris* are exclusively found in the forest. Conversely, it is common to find specimens parasitized by the hairworm *Paragordius tricuspidatus* in atypical habitats surrounding the forest (paved areas such as car parks and beside buildings and near swimming pools). We collected crickets between 1000 and 0100 hours, following the same methodology as Thomas et al. (2002), in July 2005 (parasite fecundity experiment, see below) and July 2006 (behavioural test, see below). Although these sampling times are different, we assumed that no specific year effect influenced our findings. In addition, to avoid any potential bias caused by the date of capture (within July), specimens used for both experiments came from crickets exclusively collected on 3 consecutive nights in early July.

Behavioural Tests

Parasitized crickets found in unusual habitats show aberrant behaviour (unparasitized individuals are never found in unusual habitats). To test the hypothesis that these crickets show erratic behaviour before committing suicide, we recorded the rate of suicides of infected crickets starting from the date of capture. We predicted that a percentage of crickets, although found in atypical habitats, should not commit suicide (prediction 1), but that this percentage should increase through time (prediction 2). We randomly allocated captured crickets to four groups that were kept under laboratory conditions: 1, 2, 6 and >14 days (but less than 20 days) before being tested for their response to water (to jump in or not). During these periods, crickets were kept collectively in plastic tanks (30 × 25 cm and 16 cm high), at 25 °C and provided with ad libitum food (in equal proportions: cereals, fish food Tetra Ani Min, dry gammarids, and dry tubifex) and moist cotton (changed every week) that was placed in a standard water dish for caged birds. We placed the tanks in an undisturbed room with a 16:8 h light:dark photoperiod which mimics the natural photoperiod

during the capture period. For the behavioural test, each cricket was individually placed for 24 h in a separate terrarium (30 × 25 cm and 15 cm high) with sand 5 cm deep in which was inserted a container (8 cm diameter) filled with water. The water level was 3 cm from the rim and this ensured that once inside the water, crickets could not escape. A seminatural habitat was replicated by placing leaves from the forest inside the tank. At the end of the experiment, both the host and the parasite that had just emerged were preserved in alcohol (70%) for subsequent measurements, sex determination and morphological examination of the worm. To avoid confounding effects of size, we verified that there was no significant difference between worm size (length and diameter; ±1 mm) inducing the erratic versus the suicidal behaviour (Mann–Whitney *U* test: length: $U = 326.5$, $N_{\text{suicidal}} = 31$, $N_{\text{nonsuicidal}} = 22$, $P = 0.79$; diameter: $U = 546$, $N_{\text{suicidal}} = 41$, $N_{\text{nonsuicidal}} = 32$, $P = 0.22$). There was also no differences in cuticle thickness ($U = 509.5$, $N_{\text{suicidal}} = 41$, $N_{\text{nonsuicidal}} = 30$, $P = 0.22$) and fibre number ($U = 280.5$, $N_{\text{suicidal}} = 31$, $N_{\text{nonsuicidal}} = 19$, $P = 0.76$). For histology, short tissue pieces were taken from the midbody of each worm and fixed in 70% ethanol. The piece of tissue was dehydrated in an increasing ethanol series, transferred to 100% acetone and embedded in araldite. Series of semi-thin sections (1 µm) were cut with a Reichert ultramicrotome, stained with toluidine blue and investigated with a light microscope. Measurements were made from digital images. There was no significant difference in erratic versus suicidal behaviour when the crickets were infected with male versus female worms ($U = 313.5$, $N_{\text{suicidal}} = 31$, $N_{\text{nonsuicidal}} = 22$, $P = 0.56$).

Parasite Fecundity

To avoid the possible effects of host sex-specific factors and of multiple infections, only male crickets with one hairworm were used for this experiment. We collected infected crickets from unusual habitats and kept them in the laboratory as above. Worm emergence was experimentally induced by placing infected crickets in the water, at one of the following four moments: immediately after the cricket's capture (E0), and then 7 (E7), 14 (E14) and 21 (E21) days after that. The mean body length for both male and female worms was not significantly different between the four categories (E0–E21; Kruskal–Wallis ANOVA: males: $\chi^2_3 = 4.05$, $P = 0.26$; females: $\chi^2_3 = 5.92$, $P = 0.12$).

The worms emerging at the same time were paired together ($\delta\text{E0} \times \text{♀E0}$ ($N = 16$), $\delta\text{E7} \times \text{♀E7}$ ($N = 9$), $\delta\text{E14} \times \text{♀E14}$ ($N = 10$), $\delta\text{E21} \times \text{♀E21}$ ($N = 8$)) and then we measured their fecundity as the number of larvae they produced.

Pairs were maintained in small plastic cups (diameter 2 cm; height 5 cm) filled with constantly aerated freshwater. Males were removed after mating (15 days after pair formation). We added 70% ethanol to the cups 50 days after pair formation to kill the female worms and their larvae (based on previous experience most larvae will have hatched by 50 days). Then, we counted the larvae produced by each pair under a microscope (Leica DM LB) with a thoma chamber. Larval concentration was

estimated in 30 ml of 70% ethanol. We took 12 samples per cup to get a reliable estimate of larval quantity. The statistical tests followed Sokal & Rohlf (1981) and are two tailed.

RESULTS

Behavioural Tests

The analyses were based on 173 infected individuals and 158 uninfected ones. As expected, the rate of suicide was significantly higher among infected individuals than uninfected ones (infected: 81.50%; uninfected: 9.82%; Fisher's exact test: $P < 0.001$). The proportion of parasitized crickets committing suicide varied significantly over time (Fisher's exact test on table $r \times k$: $P = 0.0002$; Fig. 1a). A closer examination of the data (and after a Bonferroni correction) revealed that this difference was due to the lower proportion of parasitized crickets that committed suicide on the first date compared to other dates (Fisher's exact test between days 1 and 2: $P = 0.0001$; other comparisons: NS; Fig. 1a).

Parasite Fecundity

The timing of hairworm emergence had a significant effect on fecundity (larvae produced by pairs between the four categories of worms; Kruskal–Wallis ANOVA: $\chi^2_3 = 18.20$, $P = 0.0004$; Fig. 1b). Pairs that emerged immediately after cricket capture (E0) produced the fewest larvae

(post hoc comparison: $P < 0.05$; Fig. 1b). Pairs of worms that emerged 7 days after the crickets' capture produced the most larvae (post hoc comparison: $P < 0.05$; Fig. 1b). The number of larvae then significantly decreased over time (post hoc comparison, E7, E21: $P < 0.05$). One explanation for the observed difference in fecundity between worm pairs established on different days could be differences in the proportion of pairs that successfully initiated reproduction. All pairs from the E7 category reproduced successfully (i.e. 100%, 9/9) while only 62% of pairs (10/16) did in the E0 category, 80% (8/10) in the E14 category and 60% (6/10) in the E21 category. These values were not significantly different (Fisher's exact test on table $r \times k$: $P = 0.87$). However, we verified that our observations regarding differential fecundity across the four categories were not changed when we analysed data only from successful pairs (Kruskal–Wallis ANOVA: $\chi^2_3 = 15.55$, $P = 0.001$; mean number of larvae \pm SE: E0: $39\,217 \pm 15\,748$; E7: $144\,250 \pm 15\,748$; E14: $94\,375 \pm 17\,607$; E21: $41\,972 \pm 20\,331$).

DISCUSSION

The ability to fine tune changes in host behaviour to the moment when parasites are ready to be transmitted has been proposed as a convincing piece of evidence supporting the hypothesis that host manipulation may have evolved to enhance parasite transmission (Bethel & Holmes 1973; Poulin 1995). Parasites, just like the hosts they infect, must contend with adverse and often unpredictable environmental conditions. This requires an adaptive synchrony of key developmental stages with favourable conditions (reviewed in Tinsley 2005). There is currently no information about the exact longevity of *P. tricuspidatus* once emerged, but they are assumed to die soon after mating and egg laying. In addition, a strong temporal segregation between *P. tricuspidatus* and a second hairworm, *Spiniochordodes tellini* (reproducing in July and August, respectively) in the streams of the study area (Thomas et al. 2002), suggests that competitive interactions between them place strong pressure on rapid reproduction by the former in July. Given the narrow temporal window for *P. tricuspidatus* hairworms to reproduce successfully, we predicted that evolving adaptations to cope with environmental constraints of this period should be a part of the wider strategy of hairworms for the completion of their life cycle. Specifically, we predicted that infected crickets should reach water (through manipulation) at an optimal time for parasite reproduction.

We found that a high percentage of parasitized crickets were not induced to commit suicide even though the behaviour of the host was changed such that their was abnormal behaviour (i.e. they were found in an atypical habitat). We were also unable to detect any significant size and/or ultrastructural differences between worms inducing the erratic or the suicidal behaviour (see Methods). These findings are in accordance with observations made by Thomas et al. (2002) in a field experiment: of 41 infected *N. sylvestris* collected in an atypical habitat, only 20 (i.e. 48.7%) entered the water. A second finding of the present study is that the proportion of crickets showing suicidal behaviour increased significantly and then was maintained through time. The host manipulation

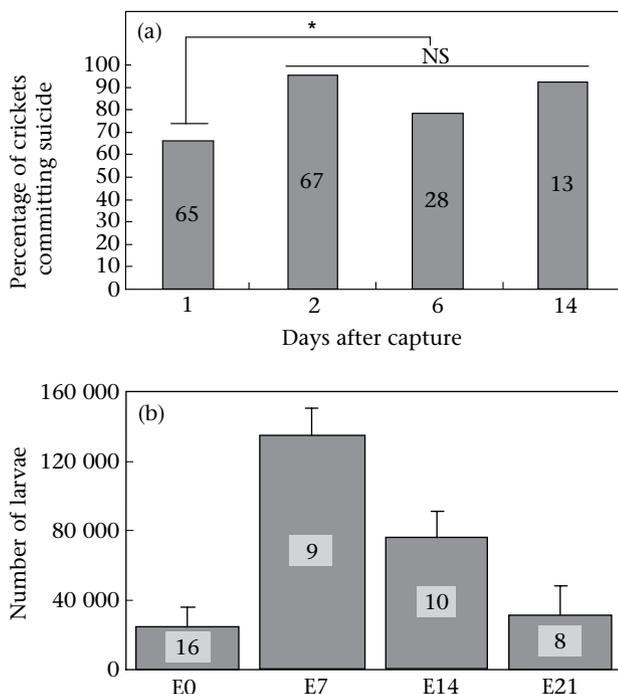


Figure 1. (a) Percentage of crickets committing suicide 1, 2, 6 or 14 days after capture. * $P < 0.001$. (b) Mean \pm SE number of larvae produced by pairs of hairworms emerging immediately (E0) or at 7 (E7), 14 (E14) or 21 (E21) days. Sample sizes are given within the bars.

strategy of hairworms thus seems to consist of at least two distinct steps, first the induction of erratic behaviour and then suicidal behaviour. The transition from one stage (abnormal behaviour in unusual habitats) to the other (suicide) seemed to be unidirectional, as we did not find a decrease in the rates of suicide over long periods after the first week. Given that individuals were randomly allocated to the different groups, there is no a priori reason to think that variables other than length of the manipulative period changed between groups.

We also found that the fecundity of hairworms was highest not when the cricket was captured but a week later. This is unlikely to be caused by better food conditions in the laboratory set-up since a significant fecundity decrease was observed after this peak. It is reasonable to assume that hairworm fecundity should be highest when the host has moved from its forest habitat and therefore likely to encounter water and mates. The delay that we observed seems paradoxical but given the very patchy distribution of pools, coupled with the narrow window that exists to find mates, we believe hairworms have evolved a strategy where hosts are manipulated behaviourally before being ready to enter water and reproduce. This optimization process is in line with the behavioural observations above. Comparisons with data from areas where ponds are historically more common would be necessary to confirm the influence of habitat (scarcity of ponds) as a selection pressure that favours the early erratic behaviour. Similarly, further field information would be required to conclude that the behavioural run-up to suicidal behaviour is adaptive, and not simply a prelude to suicide as systems within the cricket break down. For instance, one would expect potentially high costs to be associated with the erratic behaviour (e.g. predation). Thus, although our findings are in accordance with predictions, they could also illustrate a developmental pattern of behavioural change without invoking an adaptationist hypothesis.

Theory predicts that for parasites there should be a trade-off between manipulative and reproductive effort (Poulin 1994). Hairworms must find water to reproduce so it is undoubtedly better to maintain manipulation of the host at a high level even if this leads to a reduction in fecundity, rather than decreasing the manipulative effort to keep a potential fecundity that would be useless in the absence of water. Our results support this scenario because while the manipulative effort was maintained until crickets found water, the resulting energy expenditure was apparently at the expense of the total number of offspring produced. We cannot yet quantify manipulative effort in this (or any other) system, so for the time being no evidence for a trade-off between manipulative and reproductive effort exists. Alternative explanations to manipulative costs are possible (i.e. age, increasing exposure to cricket defences). Similarly, hairworm fecundity

may decrease not because manipulative costs increase, but just because energetic resources become limited. Further studies in controlled conditions would be necessary to confirm the existence and nature of such an interesting trade-off.

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