Attraction behaviour of three entomopathogenic nematode species towards infected and uninfected hosts

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SUMMARY

Entomopathogenic nematode infective juveniles are likely to encounter both uninfected and infected insects and host quality depends on the stage of the infection. We hypothesized that nematode response to infected hosts will change over the course of an infection. Here, we tested this hypothesis by focusing on the influence of host infection status on long-range attraction to host volatile cues. The attraction response of 3 nematode species (*Steinernema carpocapsae, S. glaseri* and *S. riobrave*) with different foraging strategies to infected and uninfected insects (*Galleria mellonella* and *Tenebrio molitor*) was tested at 24 h intervals from start of infection to emergence of infective juveniles from depleted host. As expected, based on their foraging strategies, *S. carpocapsae* was not very responsive to hosts, *S. glaseri* was highly responsive and *S. riobrave* was intermediate. Generally, the level of attraction did not change with time after infection and was similar between infected and uninfected hosts. An exception was *S. glaseri* infected *T. molitor*, which tended to be less attractive to *S. glaseri* than uninfected hosts. These results suggest that any influence of host infection status on infection behaviour is occurring at subsequent steps in the host-infection process than host attraction, or involves non-volatile cues.

Key words: *Steinernema carpocapsae*, *Steinernema glaseri*, *Steinernema riobrave*, *Galleria mellonella*, *Tenebrio molitor*, infection behaviour, host attraction, entomopathogenic nematodes.

INTRODUCTION

Entomopathogenic nematodes belonging to the families Steinernematidae and Heterorhabditidae are lethal parasites of insects. They have one free-living stage, the infective juvenile (IJ), which actively searches and infects hosts (Campbell and Lewis, 2002). The steinernematid IJ enters the host through natural openings and releases symbiotic bacteria (*Xenorhabdus* spp.) into the haemocoel. The host dies of septicaemia or toxaemia by 24–48 h after infection. Nematodes feed on the bacteria and host tissues, develop, and reproduce until resources are depleted (1–3 generations can be completed within a host). As nutrients become depleted, IJs are produced and they exit the host in search of another insect to infect. Steinernematid infective juveniles use a variety of foraging strategies to find hosts. These range from ambush to cruise foraging, with many intermediate types (Grewal *et al*. 1994; Campbell and Gaugler, 1997; Campbell and Kaya, 2002). Adoption of a certain foraging strategy is correlated with other aspects of parasite ecology, behaviour, physiology and anatomy and, thus, influences how parasites interact with hosts (Campbell and Lewis, 2002).

Host selection by parasitic nematodes follows a hierarchical series of steps that include search for host habitat, host finding, host acceptance, and host suitability (Doutt, 1964). The specific behaviours and cues used by entomopathogenic nematode IJs to search for and find hosts varies depending on the nematode species. Typically, ambushers that use a 'sit and wait' foraging strategy respond to host volatile chemical cues from the host when in a standing posture by changing their behaviour in ways to enhance contact with a passing insect (Campbell and Kaya, 2000), but are not attracted to host volatile cues while crawling on the substrate, unless they have already contacted a host (Lewis *et al*. 1995). In contrast, cruise foragers that actively search for hosts readily respond to volatile chemicals cues encountered by crawling toward the source of the cues (Lewis *et al*. 1992, 1993). Interspecific differences in response to host-associated contact and volatile cues have been described (Lewis *et al*. 1992, 1993; Campbell and Kaya, 2000). Some factors that have been shown to influence infective juvenile

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behaviour include CO_2, temperature gradients, and host feces (Gaugler *et al*. 1980; Byers and Poinar, 1982; Grewal *et al*. 1993; Lewis *et al*. 1993).

The distribution of nematodes in soil is often clumped (Stuart and Gaugler, 1994; Glazer *et al*. 1996; Campbell *et al*. 1998). Therefore, IJs have the potential to encounter hosts that are already infected. The decision about whether or not to invade an already-infected host has important fitness consequences for the individual infective juvenile because survival and reproduction are determined by the host environment, and infection decisions are irreversible (i.e. they cannot leave to find a more suitable host). For a nematode infective juvenile encountering an insect, prior conspecific infection may facilitate host exploitation because the host immune system may already be compromised and potential mates may be present. Alternatively, infecting an already infected host, particularly at time-points late in the infection process, may lead to increased competition for diminished resources or even the lack of sufficient nutrients in a host cadaver to complete development (Selvan *et al*. 1993). In addition to hosts infected by conspecifics, nematodes may also encounter hosts infected by heterospecific individuals. These costs and benefits could lead to selection for the ability to distinguish between infected or uninfected hosts, and among hosts at different time-points after infection. We hypothesize that early in an infection by conspecific nematodes, a host should be of higher quality, and therefore more attractive to an infective stage, than a host late in the infection process which is predicted to produce cues that suppress nematode infection. There is some evidence that waste products from hosts late in an infection repel nematodes (Kunkel *et al*. 2006).

The available evidence is mixed on whether or not infective juveniles respond differently to conspecific and heterospecific infected hosts and most studies have looked at a limited number of time-points post-infection (Lewis *et al*. 2006). Results of previous studies suggest that entomopathogenic nematodes are attracted to hosts that have already been infected by conspecifics early in the infection process, but that attraction diminishes as infection progresses (Grewal *et al*. 1997). This study evaluated responses up to 24 h after infection, but infective juveniles could encounter hosts at later stages of infection and response to cues after the 24 h period could be important. Lewis and Gaugler (1994) found increased attraction by *Steinernema glaseri* Steiner infective juveniles (4 days old) to parasitized hosts (4–6 h after nematode exposure) when compared to unparasitized hosts. Differences in infection behaviour have also been reported. Using *Steinernema feltiae* (Filipjev), *S. riobrave* Cabanillas, Poinar and Raulston, and *S. carpocapsae* Weiser, Glazer (1997) found a significant decrease in infection rate 6–9 h after injecting a host with conspecifics. Campbell and Lewis (2002) reported that *S. feltiae* showed significant infection preference for a previously parasitized host (24 h) compared to an unparasitized host. The cues triggering these changes in behaviour are unknown, but Ramos-Rodríguez *et al*. (2006) found differences in CO_2 production between infected and uninfected insects. Two distinct peaks were detected in *G. mellonella* larvae infected with *S. carpocapsae*, *S. feltiae*, *S. glaseri*, and *S. riobrave* while 1 peak was reported for infected *T. molitor* larvae. The first peak occurred early in the infection (21–49 h depending on the species) while the second appeared after 74 h. Because CO_2 has been shown to be an important cue used in host attraction (Gaugler *et al*. 1980, 1991), these peaks in CO_2 from infected hosts could influence nematode infection decisions by presenting information on the status of the host. However, it is also likely that other unknown quantitative and qualitative changes in volatile and non-volatile cues are occurring as well.

In this study, we focused on one of the steps in the host infection process (i.e. host attraction) and on one of the types of cues used in host attraction (i.e. volatile chemical cues) as part of a broader research approach involving breaking the host infection process into its different behavioural components and investigating the influence of host infection status on these behaviours. Here, we determined the long-range attraction response of 3 entomopathogenic species with different foraging strategies – *S. carpocapsae* (ambusher), *S. glaseri* (cruiser), and *S. riobrave* (intermediate) – to infected and uninfected *Galleria mellonella* (L.) (Lepidoptera: Pyralidae) and *Tenebrio molitor* L. (Coleoptera: Tenebrionidae). Unlike previous studies, we measured changes in IJ response to an insect over the whole course of an infection, from 24 h after start of infection to a point immediately prior to IJ emergence from a depleted host. We hypothesized that the level of attraction would change as the quality of the host as a resource changed over time from initial infection. Based on previous research measuring CO_2 production from infected hosts (Ramos-Rodríguez *et al*. 2006), we hypothesized that attraction to host would be greatest at the time-points when the distinct peaks of CO_2 production were reported. We also hypothesized that there would be differences among nematode species, due to differences in their foraging strategy, in terms of change in attraction to infected hosts over time from initial infection.

MATERIALS AND METHODS

Entomopathogenic nematodes and hosts

Cultures of *S. carpocapsae* All strain, *S. glaseri* NC strain and *S. riobrave* TX strain were first obtained from Harry K. Kaya (University of California, Davis) and reared in the laboratory using

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G. mellonella as the host, following techniques described by Kaya and Stock (1997).

Late instar larvae of 2 insect species, *G. mellonella* and *T. molitor*, were selected for these experiments. *Galleria mellonella* has been a model insect for entomopathogenic nematode studies, including many related with infection, even though it is not considered a natural host. *Tenebrio molitor* was included since it represents a different insect order and is commonly found at the soil surface in moist environments where entomopathogenic nematodes are also associated. Larvae of *G. mellonella* were obtained from Webster's Waxie Ranch (Webster, WI, USA) and *T. molitor* larvae from Southeastern Insectaries (Perry, GA, USA). *Galleria mellonella* larvae weighing between 0·2 and 0·25 g and *T. molitor* larvae weighing between 0·1and 0·15 g were selected for use in experiments.

Experimental arena

The arena used to measure nematode attraction was based on the one described by Gaugler *et al*. (1989). It consisted of 2 Plexiglass panels (10 × 13 × 1·2 cm). The upper one had a 1 cm diameter opening in the centre, which served as a nematode inoculation port, and 2 holes (4 mm diameter at the top, 2 mm at the bottom to keep pipette tips in place) opposite to each other at 2 cm from the centre. Each of these 2 holes held a 1000 μl pipette tip where 2 larvae of 1 host species or no larvae, depending on the treatment, were placed. To keep insects from blocking the pipette tip or from escaping, each pipette tip had steel wool inserted loosely in the tip and the top, and the top was sealed with Parafilm^{TM} (Pechiney Plastic Packaging, Menasha, WI). A silicone tube (6 mm o.d.) was glued around the perimeter of the interior portion of the bottom panel to hold agar and leave a space between the tips of the pipettes inserted in the lid and the agar surface. Two percent agar (30 ml) was poured into each arena and allowed to dry under ambient conditions for 1 h. After this time, the upper Plexiglass panel was placed on top of the tubing and held in place with rubber bands to make a tight seal. The 2 pipette tips with the respective treatment combination were set into holes and the inoculation port was sealed with transparent sticky tape.

After allowing 1 h to elapse so that a chemical gradient could form, IJs were separated from water using vacuum filtration and added to the agar surface with a fine brush inserted through the inoculation port. The port was resealed with tape and the arenas placed inside an insulated foam box and held in the dark at room temperature (25 ± 2 °C). Infective juveniles were left to move on the agar surface for 1 h, after which the agar was sliced into sections: (1) a size 5 cork borer (1 cm diameter) was used to remove the area under each pipette, (2) a centre section surrounding the nematode inoculation point (2 × 10 cm), and (3) the 2 remaining sides (6 × 10 cm each). Nematodes in the centre section were discarded to remove dead or non-viable individuals from the analysis. The nematodes from each of the remaining sections were rinsed into different Petri dishes (100 × 15 mm) and the number of infective juveniles in each section counted.

Treatments

The pipettes used in the attraction arena presented infective juveniles with a choice consisting of blank/blank and blank/live for the controls and blank/infected for the treatments. For the treatments, pipette tips contained insects at different times post-infection: 24-h intervals from time of infection until the time when infective juveniles emerged from the host. To set up these infections larvae of each host species were exposed to 100 IJs individually in plastic micro-centrifuge tubes (1·5 ml) with a small hole in the lid (approximately 0·6 mm diameter) to allow air exchange. Nematodes were added in 50 μl of water to a 3·5 × 1·5 mm piece of grade 360 filter paper (Baxter Inc, McGaw Park, IL) inside the tube. An individual host larva was then added to the tube and the tube sealed. A large number of tubes were set up at the start of the experiment and every 24 h larvae were selected at random for use in the experiments. If the host showed signs of IJ emergence the host was excluded and a new tube selected. This process continued until IJs had emerged from all the cadavers.

In the blank/blank treatment, the experimental arena contained 2 empty pipette tips. For the blank/live treatment, 2 live larvae of either *G. mellonella* or *T. molitor*, respectively, were placed in 1 of the pipettes inserted in the experimental arena, with the other side containing an empty pipette tip. The infected/blank treatment had one side of the experimental arena with an empty tip and the other side having a pipette tip with 2 infected larvae of 1 of the 2 insect species. At times 0 and 24 h, the treatment insects were alive, but at all later times they were dead. If infective juveniles emerged from the host cadaver during the course of the bioassay the replicate was discarded. The length of the experiment varied depending on the insect/nematode combination (from 96 to 168 h after infection). Four replicates were performed, with 2 replicates of each treatment combination (blank/blank, blank/live, and blank/infected) performed at each time-point after infection in each block and the experiment repeated twice (blocks).

Determination of host infection status

Infected treatment larvae from each time post-infection were dissected in Ringer's solution under a dissecting microscope to observe the status of

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infection. Nematodes inside the hosts were counted and classified as IJs (separated from other juveniles by adding liquid soap to the solution and counting the survivors), small (smaller than IJ) or large juveniles (same size or larger than IJ), and adults (males or females). When numbers were too high to count every individual, 4×25 μl samples were taken from the total suspension and the total number of nematodes of each type was estimated from this subsample.

Statistical analysis

The percentage of IJs under the pipette tip on the treatment side (infected, uninfected, blank) out of the total number of juveniles on the arena was calculated and used for the analyses. For some of the final time-points, we had only 1–3 replicates, due to other replicates having IJs emerging during the course of the experiment, and those time-points were removed from analysis. In initial analysis, General Linear Models (GLM) procedure (Version 8.2, SAS® Institute Inc.) was used with the factors nematode species, treatment and time included in the model, with a separate analysis for each insect species. For subsequent analyses, data were sorted by insect and nematode species and GLM procedures with treatment and time included in the model were performed. GLM procedures with Tukey's tests were used to determine significant differences among the treatments at each time-point for each insect and nematode species. A significance level of 0·05 was used for all comparisons. Data are presented as the mean ± standard error of the mean.

RESULTS

Response to host

As expected based on their foraging strategies, *S. carpocapsae* was not very responsive to hosts, *S. glaseri* was highly responsive and *S. riobrave* was intermediate (Fig. 1A–6A). While there were occasionally some differences between the infected and uninfected hosts, in most cases there was no discernable pattern to these differences. For *G. mellonella*, the overall model was significant ($F = 41{\cdot}4$, D.F. $= 53$, $P < 0{\cdot}0001$) with treatment ($F = 183{\cdot}5$, D.F. $= 2$, $P < 0{\cdot}0001$), time ($F = 18{\cdot}6$, D.F. $= 6$, $P < 0{\cdot}0001$), and nematode species ($F = 533{\cdot}3$, D.F. $= 2$, $P < 0{\cdot}0001$) being significant factors. There were also significant interactions between treatment and time ($F = 5{\cdot}1$, D.F. $= 12$, $P < 0{\cdot}0001$), treatment and nematode species ($F = 134{\cdot}1$, D.F. $= 4$, $P < 0{\cdot}0001$), and time and nematode species ($F = 2{\cdot}8$, D.F. $= 9$, $P = 0{\cdot}005$). For *T. molitor*, the overall model was significant ($F = 7{\cdot}9$, D.F. $= 68$, $P < 0{\cdot}0001$) with treatment ($F = 59{\cdot}5$, D.F. $= 2$, $P < 0{\cdot}0001$), time ($F = 2{\cdot}3$, D.F. $= 7$, $P = 0{\cdot}03$), and nematode species ($F = 88{\cdot}4$, D.F. $= 2$, $P < 0{\cdot}0001$) being significant factors. There were also significant interactions between treatment and time ($F = 2{\cdot}3$, D.F. $= 14$, $P = 0{\cdot}007$), and treatment and nematode species ($F = 33{\cdot}7$, D.F. $= 4$, $P < 0{\cdot}0001$).

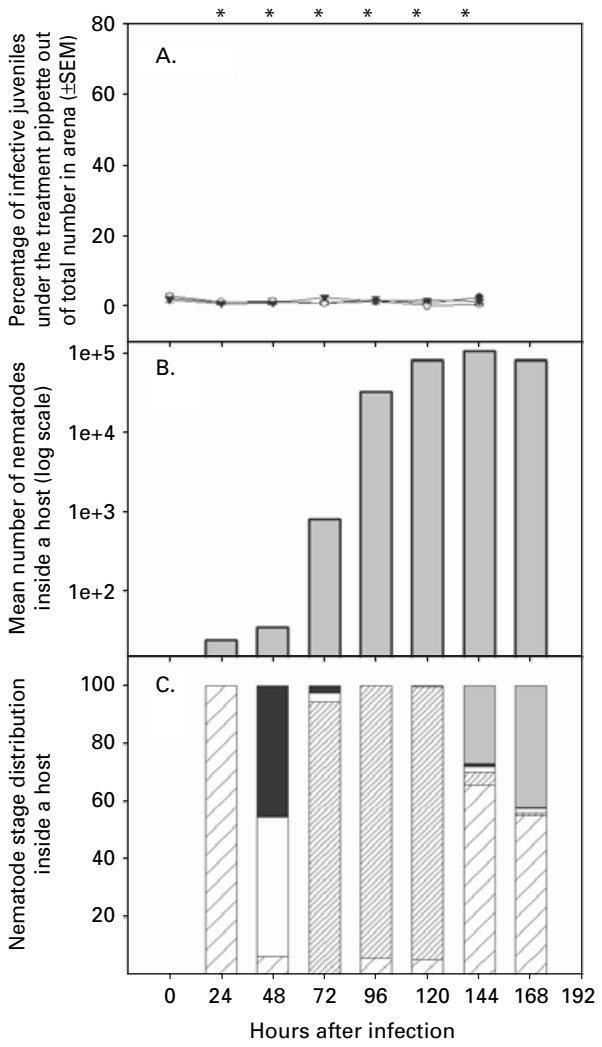

Fig. 1. Changes in (A) percentage of infective juveniles under pipettes with blank (filled circle), infected (empty circle) or uninfected (filled inverse triangle) hosts; (B) total number of nematodes inside *Galleria mellonella*; and (C) stage distribution of large juveniles (diagonal coarse line), small juveniles (diagonal fine line), infective juveniles (grey), females (white), and males (black) inside *G. mellonella* during infection by *Steinernema carpocapsae*. * Indicates time-points when CO_2 production was greater in infected *vs.* uninfected hosts (Ramos-Rodríguez *et al.* 2006).

The *S. carpocapsae* response to both *G. mellonella* (Fig. 1A) and *T. molitor* (Fig. 4A) was low at all times points tested. For *G. mellonella*, the GLM model was not significant ($F = 1{\cdot}4$, D.F. $= 20$, $P = 0{\cdot}16$), with neither treatment ($P = 0{\cdot}58$), time ($P = 0{\cdot}08$), nor time by treatment interaction ($P = 0{\cdot}27$) being significant factors. For *S. carpocapsae* response to *T. molitor*, the GLM model was significant ($F = 1{\cdot}68$, D.F. $= 23$, $P = 0{\cdot}05$), with a significant treatment

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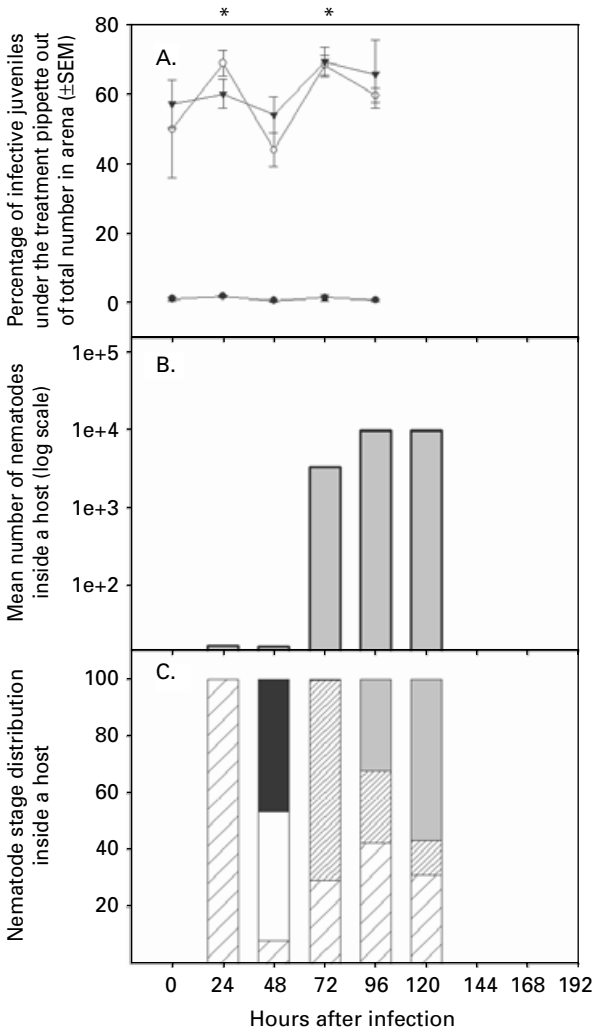

Fig. 2. Changes in (A) percentage of infective juveniles under pipettes with blank (filled circle), infected (empty circle) or uninfected (filled inverse triangle) hosts; (B) total number of nematodes inside *Galleria mellonella*; and (C) stage distribution of large juveniles (diagonal coarse line), small juveniles (diagonal fine line), infective juveniles (grey), females (white), and males (black) inside *G. mellonella* during infection by *Steinernema glaseri*. * Indicates time-points when CO_2 production was greater in infected *vs.* uninfected hosts (Ramos-Rodríguez *et al.* 2006).

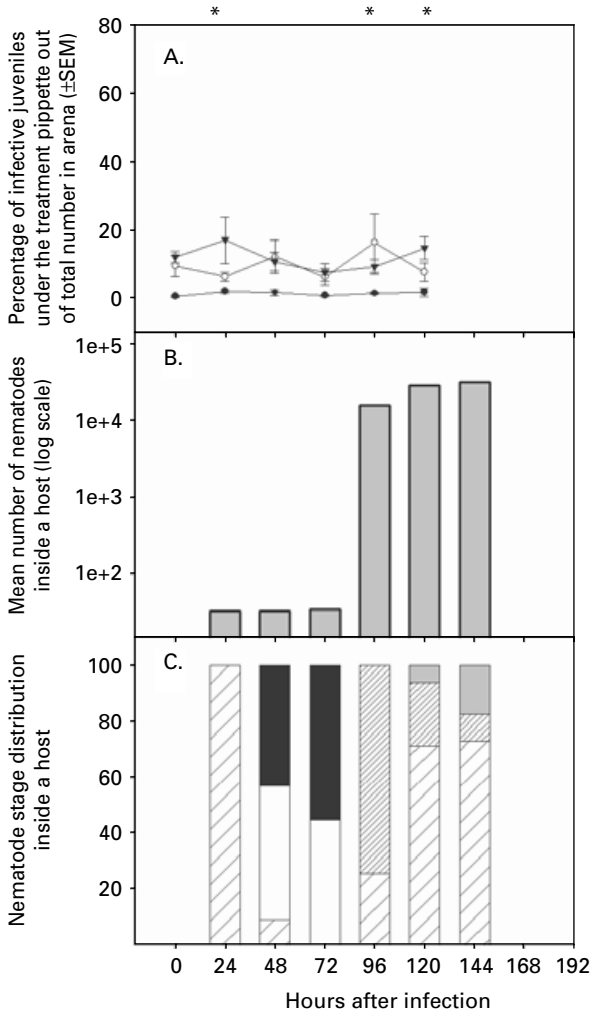

Fig. 3. Changes in (A) percentage of infective juveniles under pipettes with blank (filled circle), infected (empty circle) or uninfected (filled inverse triangle) hosts; (B) total number of nematodes inside *Galleria mellonella*; and (C) stage distribution of large juveniles (diagonal coarse line), small juveniles (diagonal fine line), infective juveniles (grey), females (white), and males (black) inside *G. mellonella* during infection by *Steinernema riobrave*. * Indicates time-points when CO_2 production was greater in infected *vs.* uninfected hosts (Ramos-Rodríguez *et al.* 2006).

factor ($P=0{\cdot}002$), but no significant time ($P=0{\cdot}32$) or time by treatment interaction ($P=0{\cdot}3$). Although attraction levels were low, there was lower total attraction towards infected hosts than either the blank or uninfected treatments. The only difference between treatments at individual time-points was found 168 h after infection when the response to the infected host was lower than the uninfected live host and the blank ($F=12{\cdot}05$, D.F. $=2$, $P=0{\cdot}003$), although the level of response was very low in all treatments and the difference is not visible in the graph.

For *S. glaseri* response to *G. mellonella*, the GLM model was highly significant ($F=28{\cdot}7$, D.F. $=14$, $P<0{\cdot}0001$), with treatment ($P<0{\cdot}0001$) and time ($P=0{\cdot}03$) being significant factors and time by treatment interaction ($P=0{\cdot}36$) not being significant. There was a higher attraction to the pipettes with both infected and uninfected *G. mellonella* hosts compared to blank pipettes ($P<0{\cdot}003$) and there was no apparent trend to the changes in response over time (Fig. 2A). At all individual time-points after infection, the response to the infected *G. mellonella* was not different from the uninfected, so the significant time factor was likely due to temporal variation not related to the changes in host infection status (lack of significant interaction between treatment and time). For *S. glaseri* response to *T. molitor* (Fig. 5A), the GLM model was highly significant

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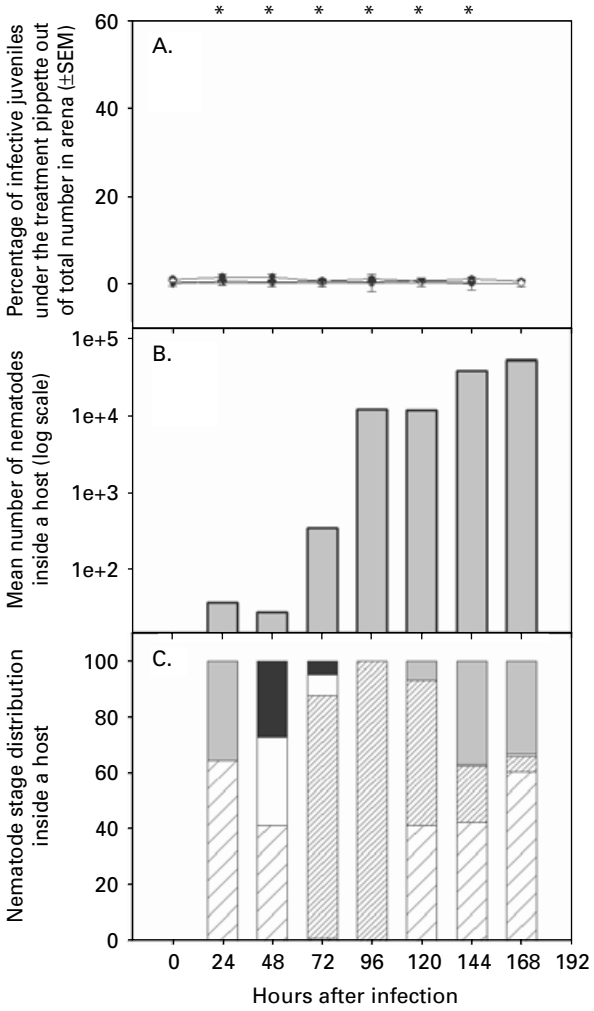

Fig. 4. Changes in (A) percentage of infective juveniles under pipettes with blank (filled circle), infected (empty circle) or uninfected (filled inverse triangle) hosts; (B) total number of nematodes inside *Tenebrio molitor*; and (C) stage distribution of large juveniles (diagonal coarse line), small juveniles (diagonal fine line), infective juveniles (grey), females (white), and males (black) inside *T. molitor* during infection by *Steinernema carpocapsae*. * Indicates time-points when CO_2 production was greater in infected *vs.* uninfected hosts (Ramos-Rodríguez *et al.* 2006).

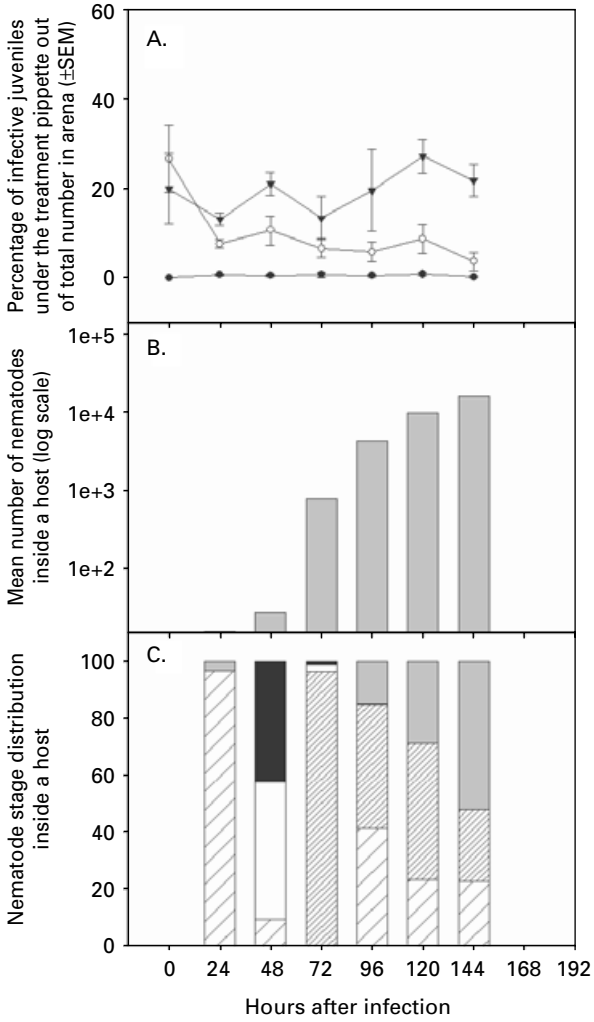

Fig. 5. Changes in (A) percentage of infective juveniles under pipettes with blank (filled circle), infected (empty circle) or uninfected (filled inverse triangle) hosts; (B) total number of nematodes inside *Tenebrio molitor*; and (C) stage distribution of large juveniles (diagonal coarse line), small juveniles (diagonal fine line), infective juveniles (grey), females (white), and males (black) inside *T. molitor* during infection by *Steinernema glaseri*.

(F = 6·22, D.F. = 20, $P < 0·0001$) with treatment ($P < 0·0001$) and time by treatment interaction ($P = 0·05$) being significant factors. However, time ($P = 0·07$) was not a significant factor. Uninfected and infected treatments were similar at 0, 72, and 96 h after infection. At 24 and 48 h, differences among all treatments were found (uninfected > infected > blank) ($P < 0·0007$); and at 120 and 144 h after infection response to infected hosts was similar to the blank and lower than that to uninfected hosts ($P < 0·0003$).

For *S. riobrave* response to *G. mellonella* (Fig. 3A), the GLM model was significant (F = 2·64, D.F. = 17, $P = 0·004$), with treatment ($P < 0·0001$) being a highly significant factor. Neither time ($P = 0·74$) nor the time by treatment interaction ($P = 0·5$) were significant. When looking at individual time-points, this treatment response was only significant at 0 h (GLM, F = 9·03, D.F. = 2, $P = 0·007$), after which no significant differences were found among treatments, even though the mean percentage under pipettes with hosts appeared higher than under the blank treatment (Fig. 3A). For *S. riobrave* response to *T. molitor* (Fig. 6), the GLM model was significant (F = 2·49, D.F. = 23, $P = 0·002$) (Fig. 6A), with treatment ($P < 0·0001$) highly significant and time ($P = 0·48$) and time by treatment interaction ($P = 0·3$) not significant. The only difference between treatments at individual time-points was found at 0 h (GLM, F = 15·53, D.F. = 2, $P = 0·001$) when the response to the larvae was higher than the blank.

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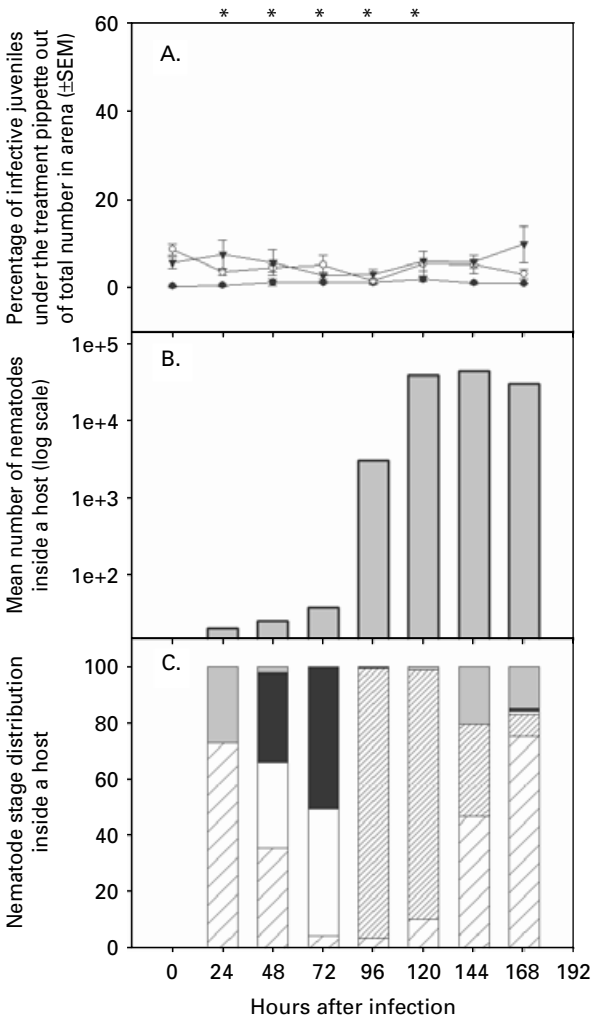

Fig. 6. Changes in (A) percentage of infective juveniles under pipettes with blank (filled circle), infected (empty circle) or uninfected (filled inverse triangle) hosts; (B) total number of nematodes inside *Tenebrio molitor*; and (C) stage distribution of large juveniles (diagonal coarse line), small juveniles (diagonal fine line), infective juveniles (grey), females (white), and males (black) inside *T. molitor* during infection by *Steinernema riobrave*. * Indicates time-points when CO_2 production was greater in infected *vs*. uninfected hosts (Ramos-Rodríguez *et al*. 2006).

Host infection status

The changes in numbers of nematodes present in infected hosts, and their age class distributions over time are shown in Figs 1–6, sections B–C. In all nematode species infecting *G. mellonella*, adults were observed 48 h after infection (Figs 1C, 3C, 6C) with a second generation in *S. carpocapsae* occurring after 144 h (Fig. 1C). Adults were observed in infected *T. molitor* after 48 h for all nematode species (Figs 2C, 4C, 5C). A second generation was observed for *S. carpocapsae* (Fig. 2C) and *S. riobrave* (Fig. 6C) at 144 and 168 h after infection, respectively. In *G. mellonella*, the total number of nematodes in a host plateaued at about 96–120 h (Figs 1B, 3B, 6B). In *T. molitor*, the total numbers of nematodes continued to increase over the infection for *S. carpocapsae* and *S. glaseri* infections (Figs 2B, 4B), but plateaued at 120 h for *S. riobrave* infections (Fig. 6B). Generally, the total number of nematodes in a host was less in *T. molitor* compared to *G. mellonella*. There was no apparent relationship between changes inside the host in terms of life-stages or total numbers and nematode attraction.

DISCUSSION

We hypothesized that the response to infected hosts would change over the course of an infection based on both proximate (changes in CO_2 production, build up of waste material) and ultimate mechanisms (changes in fitness benefits resulting from infection at different time-points). Ramos-Rodríguez *et al*. (2006) showed that 2 peaks of elevated CO_2 production, 1 early in the infection when the insect was still alive and 1 close to the time of IJs emergence, were produced from infected *G. mellonella*, while only 1 peak was observed early in the infection with infected *T. molitor*. In that study, for larvae infected with *S. carpocapsae* the CO_2 concentration was higher than from uninfected larvae from 24 to 144 h post-infection, while in *S. glaseri* and *S. riobrave* the time-period was more varied. As CO_2 is an important cue used for host attraction, we expected to see modified behaviour in response to changes in its concentration. However, no increased attraction was observed at peak times for elevated CO_2 production for any of the tested species (see asterisks in Figs 1–6, which denote when CO_2 production was higher than in an uninfected live insect).

We also hypothesized that response to hosts should decrease toward the end of an infection because of a presumed decrease in host quality and declining CO_2 production. Shapiro *et al*. (2000) reported that nitrogen production attracts *Heterorhabditis bacteriophora* infective juveniles to a host at the earlier stages of infection, but that later in the infection process when concentrations become higher, the nematodes are repelled. This pattern was not observed with the species tested here, except possibly for *S. glaseri*. The response of *S. glaseri* to infected *T. molitor* was lower than to uninfected hosts and toward the end was not significantly different from the blank control. In contrast, its response to *G. mellonella* was not significantly different between infected and live uninfected hosts at any time. Attraction to hosts late in the infection process does not appear to be adaptive. It remains to be determined whether the IJs actually infect these hosts at this time, or if other cues involved during close range attraction or after host contact might be involved in regulating infection. For example, exudates from the host could be used to assess host status after contact. Zhou *et al*. (2002) reported the response of ants to

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deterrent compounds produced by symbiotic bacteria of certain entomopathogenic nematodes and Glazer (1997) suggested the presence of a chemical substance, probably produced by the infected host in response to pathogenesis, which inhibits IJ invasion. Some of these compounds might affect behaviour leading to infection. If infective juveniles continue to infect and resume development in hosts even after they have become depleted, this suggests that under natural conditions strong selection against this behaviour does not exist or that there are constraints on the development of host recognition.

The positive response of *S. glaseri* IJs to live uninfected hosts was expected since it is a cruise forager. This species is highly mobile and searches for hosts by responding to cues such as CO_2 (Lewis *et al*. 1993). Changes in infection behaviour may arise at later steps in the host infection process than at long-range attraction, for example, contact with host exudates may have a negative effect even if long-range attraction is unaffected (Kunkel *et al*. 2006). There were differences when *S. glaseri* was exposed to infected *T. molitor*. The difference in host response to *T. molitor* versus *G. mellonella* may be related to the former having lower infection rates. This idea is supported by dissections, which showed lower numbers of founders, slower development, and lower nematode production of *S. glaseri* in *T. molitor* compared to *G. mellonella*.

Ramos-Rodríguez *et al*. (2006) documented peaks of CO_2 at different times during an infection, which occurred approximately at 22 and 72 h in *G. mellonella* infected by *S. glaseri*. Carbon dioxide data for *T. molitor* were not obtained because of a very low infection rate, which may be linked to the low susceptibility to *S. glaseri*. Although, in other studies, *S. glaseri* has been shown to be highly infective against *T. molitor* (Caroli *et al*. 1996). Lack of variation in response to hosts producing different levels of CO_2, may result from the presence of a threshold response to this cue, wherein CO_2 titres above a critical level do not elicit an increased response. It is also possible that the response to CO_2 occurs at very close range, perhaps involved in finding routes of entry such as spiracles. In this case, changes in CO_2 production in infected hosts may affect infection behaviour only within a very short spatial range. Although a measurable impact of infection status on infective juvenile host attraction could not be determined using our bioassay, further studies measuring behavioural response to host contact and impact on infection behaviour are ongoing.

We did not observe attraction of *S. carpocapsae* towards uninfected hosts at any time. Although the response to infected *T. molitor* was significantly lower than the other treatments at 168 h after infection, the response was very low for the treatments and the difference in level of response was too low, so that it is unlikely to be biologically meaningful. This finding suggests that although *S. carpocapsae* does not respond positively to the long-range host cues, it might be repelled by certain volatiles produced by a depleted host. However, the idea of repellency is not supported by the results for *G. mellonella*. Infective juveniles of *S. carpocapsae* and *S. glaseri* were attracted to hosts infected by conspecifics early in the infection (4 h), but attraction diminished after 24 h (Grewal *et al*. 1997). Glazer (1997) reported a decrease in invasion of insects that had been injected with *S. riobrave* and *S. carpocapsae* and exposed to IJs 6 h after injection. We did not observe a decrease in attraction, but we didn't test for it so early in the infection. The increase in response reported by Grewal *et al*. (1997) did not correspond to timing of increased CO_2 production reported by Ramos-Rodríguez *et al*. (2006). Ambush foragers, like *S. carpocapsae*, are not highly responsive to volatile cues when crawling, except after host contact, which suggests that they might be used for locating routes of entry into host (Lewis *et al*. 1995). Host volatile cues, probably at least in part CO_2, do trigger behavioural changes (i.e. jumping and waving behaviour) in standing *S. carpocapsae* (Campbell and Kaya, 2000). These data suggest that *Steinernema carpocapsae* could respond differently to changes in CO_2 levels if they first have host contact. When the first peak of CO_2 was observed, although hosts were still alive, they were becoming moribund and therefore would be less likely to encounter ambush foraging nematodes. After host encounter, however, infection rate might be greater for infected hosts than uninfected. This is an area that needs further research.

Infective juveniles from *S. riobrave* appeared to positively respond to host presence, but significantly greater attraction compared to blank was found only for the first time-point. *Steinernema riobrave* is considered an intermediate forager because it nictates or stands for brief periods (Campbell and Kaya, 2002), but also orientates to host cues (Grewal *et al*. 1994). As in the case of *S. glaseri*, it was predicted that this species would be attracted to the live hosts, although the level of response was not predicted to be as strong. For this species the lack of change in response to infected hosts over time suggests that they do not discriminate based on long-range attraction to a host.

The number of nematodes inside the host did not appear to correspond with peaks in CO_2 release reported by Ramos-Rodríguez *et al*. (2006). During the first peak the number of nematodes is low and all are in the juvenile stage. In the second peak observed in infected *G. mellonella*, the numbers are much higher and juveniles of different sizes are found. In *T. molitor* the CO_2 production was higher than for uninfected insects throughout most of an infection by *S. carpocapsae* and *S. riobrave*, and does not appear to be related to patterns in nematode number or stages. Results from the dissections suggest that *T. molitor* produces fewer nematodes than

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G. mellonella. This could be related to host size and host suitability. Caroli *et al*. (1996) reported lower host mortality and nematode penetration rates for some steinernematid species when exposed to *T. molitor* versus *G. mellonella*, suggesting differences in suitability between these insect species.

Based on the dissection data, attraction and subsequent infection of hosts at different time-points after infection are likely to have different fitness consequences for infective juveniles. Based on assessments of host quality and presence of potential mates, the putative best times to infect would be between 0 and 24 h, and in some cases up to 48 h. After 72 h, infective juveniles probably would not be able to reproduce successfully unless development of the F1 generation was timed so that their adults were present when the invading IJs became adults. However, second generations are not always successful in hosts. For example, the second generation of *S. riobrave* adults inside *T. molitor* occurred at 168 h, and IJs started emerging the next day. In this case, although adults were present they probably did not have sufficient nutrients and time to produce progeny that could develop into IJs. Infective juveniles responding to infection right before this time-point could potentially find mates, but this would likely be a dead end in the depleted host. Dissections of *G. mellonella* infected with *S. glaseri* late in the infection show high numbers of juveniles and no new adults developing, thus it doesn't appear to be beneficial for the IJs to infect at these later time-points. Further research measuring the levels of infection and the fitness consequences for invading nematodes and those already established in a host at different times after infection is needed.

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