

Dynamics of carbon dioxide release from insects infected with entomopathogenic nematodes

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Abstract

The quality of an insect as a host to an entomopathogenic nematode infective juvenile depends in part on whether or not the insect is already infected and on the stage of that infection. Previous research has shown that nematode response to hosts can change after infection and that, for uninfected hosts, CO₂ can be an important cue used by infective stage juveniles during attraction. We hypothesized that CO₂ production from an insect changes after it is infected, and that these changes could influence nematode infection decisions. Changes in CO₂ released by two insect species (*Galleria mellonella* and *Tenebrio molitor*) after infection by one of four nematode species (*Steinernema carpocapsae*, *Steinernema feltiae*, *Steinernema glaseri*, or *Steinernema riobrave*) were measured. Measurements were taken every 2 h from time of initial exposure to nematodes up to 224 h after infection. Dead (freeze-killed) and live uninfected insects were used as controls. Infected *G. mellonella* showed two distinct peaks of CO₂ production: one between 20 and 30 h and the other between 70 and 115 h after exposure to the nematodes. Peaks were up to two times higher than levels produced by uninfected insects. Infected *T. molitor* showed only one peak between 25 and 50 h. We found differences in peak height and timing among nematode and insect species combinations. The influence of these changes in CO₂ production on IJ attraction and infection behavior remains to be determined.

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1. Introduction

Entomopathogenic nematodes in combination with their mutualistic bacteria are lethal endoparasites of insects (Kaya and Gaugler, 1993; Gaugler, 2002). After infection, entomopathogenic nematodes liberate mutualistic bacteria that help overcome the immune system and modify the environment to make it more favorable for nematode development. If the infection is successful, nematodes resume development and start feeding. One to three generations

can occur inside the host and over the course of infection conditions become crowded and the nutritional quality of the resource diminishes (Adams and Nguyen, 2002; Kaya and Gaugler, 1993). As conditions decline, infective juveniles (IJs) are produced that leave the insect to seek new hosts. The IJ is the only free-living stage and it actively seeks hosts using a species-specific strategy along a continuum from ambush to cruise foraging (Campbell and Gaugler, 1993, 1997; Campbell and Kaya, 2002; Grewal et al., 1994). The use of a certain foraging strategy has implications for other aspects of parasite ecology, behavior, physiology, and morphology and thus influences how parasites interact with hosts (Campbell and Lewis, 2002). Several cues have been proposed to be important in host finding and recognition.

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Carbon dioxide has been demonstrated to be used during host attraction (Gaugler et al., 1980; Gaugler and Campbell, 1991; Lewis et al., 1993), and may be involved in other steps in the infection process. Other potential cues include temperature (Byers and Poinar, 1982) and host feces or fecal components (Grewal et al., 1993; Schmidt and All, 1979). Inter-specific differences in responses to contact and volatile cues by entomopathogenic nematode infective stages have been found (Campbell and Kaya, 2000; Lewis et al., 1992).

The majority of research on host finding and infection has focused on nematode response to uninfected hosts, but IJs can encounter hosts that are already infected by other natural enemies, including hosts infected by conspecific or heterospecific entomopathogenic nematodes. Entomopathogenic nematode species distributions in soil are often clumped (Campbell et al., 1998; Glazer et al., 1996; Stuart and Gaugler, 1994), and, when given a choice of multiple hosts, infective juveniles tend to infect in a pattern resulting in a clumped distribution among hosts (Campbell et al., 1999). Therefore the potential for encounter with an insect already infected by conspecifics is potentially high. The fitness consequences of infecting these hosts will depend on the species already established and the time after initial infection. Infection decisions after encountering an infected host have a major impact on fitness since these decisions are typically irreversible and host quality strongly influences survival and reproduction. There are risks associated with infection: e.g., overcoming the host immune system and, for *Steinernema* spp., not finding potential mates inside the host. Infecting a host already infected with conspecifics may reduce these associated risks, but only during specific time periods and within a certain range of nematode densities (Selvan et al., 1993).

The costs and benefits associated with infecting hosts that are already infected could lead to natural selection for the ability to distinguish between infected or uninfected hosts. Such host discrimination has been observed in other parasites: e.g., some parasitoid species avoid parasitized hosts (Goubault et al., 2004) or change their sex ratio in response to host cues (Shuker and West, 2004). There is evidence that entomopathogenic nematodes are attracted to hosts that have already been infected by conspecifics early in the infection, but attraction diminishes as infection progresses (Grewal et al., 1997). Some steinernematid species show a reduction in infection rate after a host had been injected with IJs, which could be attributed to the host giving off a substance in response to pathogenesis (Glazer, 1997). Exudates from infected hosts more than 48 h post infection reduced conspecific infection by *Steinernema glaseri* (Kunkel et al., 2006).

Carbon dioxide has been demonstrated to be an important cue used by entomopathogenic nematodes in finding uninfected insects, and it appears likely that the processes of host death and bacteria and nematode growth within the host would impact CO₂ production. Therefore, we hypothesized that CO₂ release from insects will change during the

course of an infection, and that these changes will influence infective juvenile behavior. Specifically, we were interested in determining if changes in CO₂ production might correspond with changes in host quality and therefore these changes might be useful in IJs making infection decisions. Here, we measured CO₂ production from two different insect species over the course of an infection following exposure to one of four different species of entomopathogenic nematode. This data will provide the foundation for further research into the behavioral response of IJs to infected hosts.

2. Materials and methods

2.1. Nematode species

Four entomopathogenic nematode species: *Steinernema carpocapsae* (Weiser) (All strain), *Steinernema feltiae* (Filipjev) (SN strain), *S. glaseri* Steiner (NC strain), and *Steinernema riobrave* Cabanillas, Poinar, and Raulston (TX strain) were tested. These four species have a range of foraging strategies from ambusher (*S. carpocapsae*) to cruiser (*S. glaseri*) with two species (*S. feltiae* and *S. riobrave*) exhibiting intermediate strategies. The nematodes were originally obtained from Harry K. Kaya at the University of California, Davis. They were reared in *Galleria mellonella* L. (Lepidoptera: Pyralidae) following the techniques described in Kaya and Stock (1997). Different nematode infection batches were used for each experimental block and only IJs less than two weeks old were used in experiments.

2.2. Host species

Two host species from two different orders were used in experiments: *G. mellonella* and *Tenebrio molitor* L. (Coleoptera: Tenebrionidae). Larvae of the greater wax moth, *G. mellonella*, have been used as a model organism for the study of entomopathogenic nematodes behavior and infection, but it is unlikely that entomopathogenic nematodes will interact with this insect in nature. We also tested the yellow mealworm, *T. molitor*, which inhabits areas more favorable for nematodes, is distributed worldwide, and is considered a stored-product pest. *Tenebrio molitor* is less susceptible to the nematode species tested than *G. mellonella* (Caroli et al., 1996).

2.3. CO₂ measurements

Carbon dioxide release was measured with a Micro-Oxy-max version 5.12 respirometer (Columbus Instruments International Corporation, Columbus, OH). This system measures percent O₂ and CO₂ gas levels in the closed test chamber. Measurements were taken every 2 h from 0 h after exposure to IJs up to 224 h in *G. mellonella* L. and 190 h with *T. molitor*, based on data from preliminary experiments.

Late instar larvae of *G. mellonella* (0.2–0.25 g) and *T. molitor* (0.1–0.15 g) were exposed to 100 IJs at the beginning of the experiment. Individual larvae were exposed to the corresponding nematode species in centrifuge tubes (1.5 ml) with 16 holes (approximately 0.6 mm diameter) on the sides (4 columns of 4), three on top and one on the bottom to allow air exchange. Nematodes were applied in 50 μ l of water to a 3.5 \times 1.5 mm piece of filter paper (Grade 360: Baxter Inc, McGaw Park, IL) inside the tube and then the insect larva was added. Live uninfected and dead uninfected (freeze killed) larvae were used as controls and also placed individually into tubes with filter paper and 50 μ l of water.

An individual centrifuge tube was placed inside a 50 ml sample chamber of the respirometer and placed in a water bath (25 °C). For each insect species, three blocks with two replicates of each nematode species and each control were performed for *S. carpocapsae*, *S. glaseri*, and *S. riobrave* (total of 10 sample chambers per block). Measurements were performed in blocks of 10 replicates, because there was a maximum of 10 chambers that could be processed at a time. For each insect species, two blocks with three replicates each were used for *S. feltiae* and the controls for a total of nine chambers each time. Infected larvae were dissected at the end of the experiment to confirm nematode presence.

2.4. Statistical analysis

Data are presented as the means \pm standard error of the mean. Differences between CO₂ release and time of release for nematode species were analyzed using the General Linear Models Procedure and *t*-test Procedure in the SAS program v. 8 (SAS Institute, 2001).

3. Results and discussion

Infected *G. mellonella* larvae showed two peaks in CO₂ production. These peaks varied from approximately 4–8 μ l/min depending on the nematode species (Fig. 1). The first peak occurred approximately 20–30 h after exposure to the nematodes for all four nematode species tested. There were differences in peak height among the nematodes: ranging from a mean of 5.6 μ l/min for *S. glaseri* to 7.6 μ l/min for *S. riobrave* infected hosts (Table 1). There were also differences among nematode species in the time after infection when the first peak of CO₂ production occurred. The peak occurred later in *S. feltiae* (27.3 h after infection) than in the other species tested (Table 1). The height and timing of the second peak varied more among species compared with the first peak. It occurred earliest in *S. glaseri*, and it was higher for *S. carpocapsae* and *S. riobrave* compared with that for the other species (Table 1).

Larvae of *G. mellonella* infected with *S. carpocapsae* had a greater CO₂ release than the dead insect controls at all time points and greater release than the live insect controls between 12 and 168 h after infection (General Linear

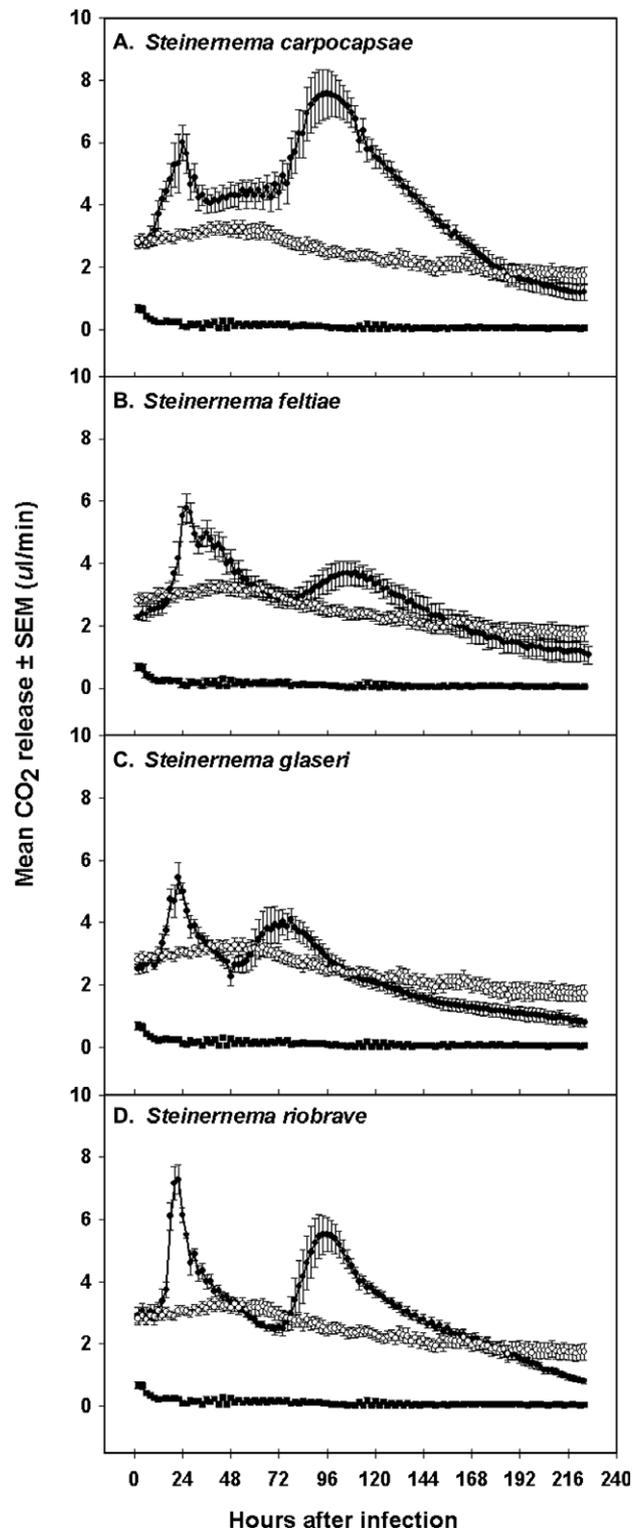


Fig. 1. Mean CO₂ measurements (\pm SEM) for infected larvae (solid line, filled circle), live, uninfected larvae (dashed line, empty circle), and dead, uninfected larvae (dotted line, filled square) of *Galleria mellonella* through the infection process of four entomopathogenic nematode species, *Steinernema carpocapsae*, *S. feltiae*, *S. glaseri*, and *S. riobrave*.

Models procedure; $P < 0.05$). For the other species, infected larvae were different from the live controls during the first and second CO₂ peaks (20–48 and 92–130 h post-infection

Table 1

Mean (\pm SEM) CO₂ peak height and time of peak for the two insect species *Galleria mellonella* and *Tenebrio molitor* infected with each of four nematode species, *Steinernema carpocapsae*, *S. feltiae*, *S. glaseri*, and *S. riobrave*

Insect species	<i>Steinernema</i> species	First CO ₂ peak height (μ l/min)	Timing of 1st CO ₂ peak (h after infection)	Second CO ₂ peak height (μ l/min)	Timing of 2nd CO ₂ peak (h after infection)
<i>Galleria mellonella</i>	<i>S. carpocapsae</i>	6.8 \pm 0.5 ab	22.7 \pm 1.1 b	7.8 \pm 0.7 a	97.0 \pm 3.0 a
	<i>S. feltiae</i>	6.0 \pm 0.4 b	27.3 \pm 0.7 a	4.1 \pm 0.4 c	111.3 \pm 5.3 a
	<i>S. glaseri</i>	5.6 \pm 0.4 b	22.7 \pm 0.4 b	4.6 \pm 0.5 bc	74.3 \pm 2.9 b
	<i>S. riobrave</i>	7.6 \pm 0.4 a	21.7 \pm 0.6 b	6.4 \pm 0.4 ab	97.7 \pm 3.1 a
<i>Tenebrio molitor</i>	<i>S. carpocapsae</i>	3.5 \pm 0.3 a	28.3 \pm 3.7 b	NA	NA
	<i>S. feltiae</i>	3.4 \pm 0.3 a	36.3 \pm 2.4 b	NA	NA
	<i>S. glaseri</i>	NA	NA	NA	NA
	<i>S. riobrave</i>	4.2 \pm 0.2 a	49.3 \pm 3.3 a	NA	NA

Different letters in the same column for each insect species represent significant differences between nematode species (General Linear Models Procedure and Tukey's test, $p < 0.05$). NA = no data.

for *S. feltiae*; 16–32 and 70–90 h for *S. glaseri*, and 16–44 and 82–150 h for *S. riobrave*). There was a drop in CO₂ production for *S. riobrave* that was significantly lower than the live control from 66 to 70 h. Carbon dioxide production by live, uninfected larvae was always greater than CO₂ produced by dead uninfected larvae ($P < 0.05$). Dead, uninfected *G. mellonella* controls were always lower than live uninfected and infected larvae ($P < 0.05$).

Tenebrio molitor infected with entomopathogenic nematodes showed a different pattern in CO₂ production than *G. mellonella* (Fig. 2). There was generally more variability in levels of CO₂ production and only one peak (corresponding to the first peak in *G. mellonella* infected with nematodes) was observed. In some cases there was a suggestion of a second peak, but it was generally low and broad. The CO₂ peak occurred later in insects infected with *S. riobrave* than in those infected with *S. feltiae* and *S. carpocapsae* (Table 1). Data for *S. glaseri* are not shown because only two insects were successfully infected. Carbon dioxide release for all nematode species was greater than for the live controls, with the difference showing up from 20–146, 26–150, and 14–140 h for *S. carpocapsae*, *S. feltiae*, and *S. riobrave*, respectively.

There were differences in CO₂ production between the insect species. Larvae of *T. molitor* used in this study are smaller than *G. mellonella*, so a difference between live controls may be expected. *Galleria mellonella* larvae produced more than twice the amount of CO₂ than *T. molitor* larvae (2.45 \pm 0.05 versus 0.82 \pm 0.02 μ l/min), and their corresponding peaks were also about twice the size in height. However, the changes relative to the baseline were greater in infected *T. molitor*, with peaks 63%, 70%, and 75% higher than the live insect for *S. carpocapsae*, *S. feltiae*, and *S. riobrave*, respectively, than in infected *G. mellonella* (*S. carpocapsae* (49%), *S. feltiae* (47%), and *S. riobrave* (58%)). The amount of CO₂ released by infected insects during peaks was higher for *S. riobrave* infecting both insect species. The first peak also tends to occur later in *T. molitor* than in *G. mellonella*.

Carbon dioxide production was relatively constant in dead and live, uninfected insects over time (Figs. 1 and 2). Dead insects produced levels of CO₂ that were close to zero

throughout the course of the experiment. Adding 100 IJs to a chamber without an insect host did not result in any detectable level of CO₂ compared with empty chambers (data not shown). In addition, the levels of CO₂ production decline close to zero at the end of the experiments, even though dissections of insects indicated that all those previously producing peaks of CO₂ had nematode infections and were full of IJs. This suggests that IJs are not producing detectable levels of CO₂.

The first peak of CO₂ production occurred before nematode reproduction occurred within the host and often the insects were still alive. The first peak could be caused by changes in the host due to infection and/or the growth of bacteria occurring at this time. Studies suggest that at this point the bacteria are undergoing exponential growth (Götz et al., 1981; Walsh and Webster, 2003). The second peak in *G. mellonella* occurs late in the infection and could be due to high numbers of nematodes respiring and to the presence of the secondary form of the *Xenorhabdus* bacteria. Stationary secondary forms are able to maintain higher levels of respiratory enzymes than primary forms (Smiglielski et al., 1994). Dissections of infected *G. mellonella* at this time point showed high numbers of juveniles and in *S. carpocapsae* second generation adults were found after peak reduction (Ramos-Rodríguez et al., unpublished data). Further research is needed to determine the mechanisms causing these peaks.

These changes in CO₂ production could be used to influence entomopathogenic nematode IJ infection behavior. Since IJs respond to CO₂ (Gaugler and Campbell, 1991; Gaugler et al., 1980; Lewis et al., 1996), the increased levels of CO₂ production may increase IJ response. However, it is unknown how variation in levels of CO₂ production influences nematode attraction. The first peak of CO₂ production is correlated with time early in an infection when the host immune system likely has been compromised and nematodes are developing into adults. It would appear that the fitness benefits of infecting at this time would be greater than in an uninfected host. However, CO₂ is a nonspecific cue, and infections by all of the nematode species caused this peak, so CO₂ alone would not provide information

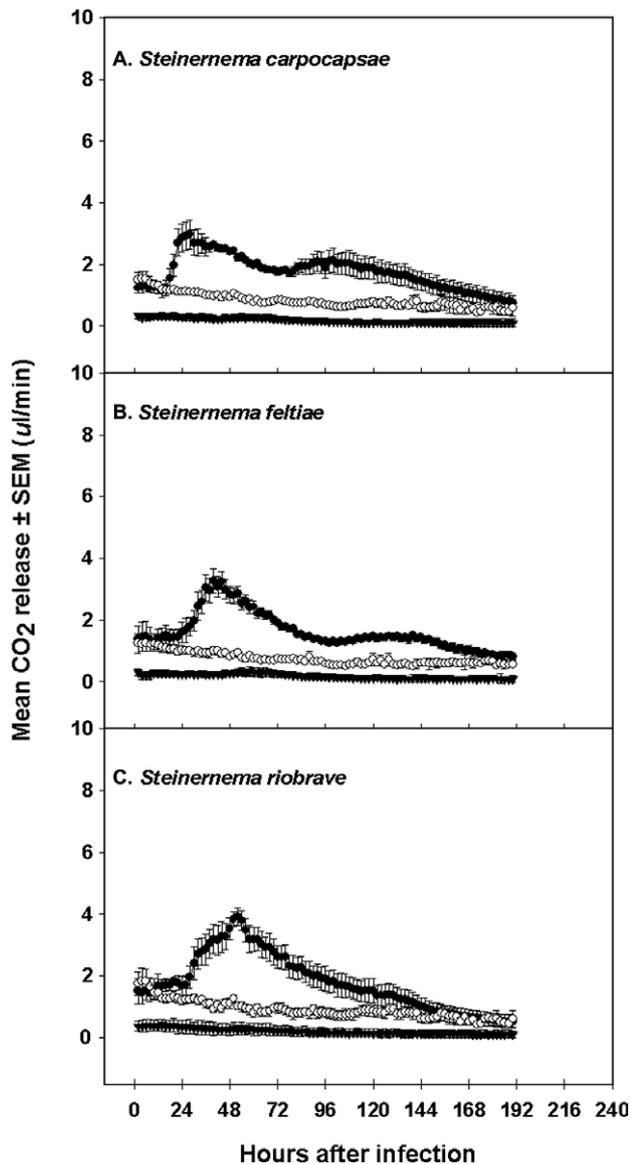


Fig. 2. Mean CO₂ measurements (\pm SEM) for infected larvae (solid line, filled circle), live, uninfected larvae (dashed line, empty circle), and dead, uninfected larvae (dotted line, filled square) of *Tenebrio molitor* through the infection process of three entomopathogenic nematode species, *Steinernema carpocapsae*, *S. feltiae*, and *S. riobrave*.

about the species causing the infection. In addition, species and stages of insects vary in their levels of CO₂ production. Therefore, it is unclear if the levels of CO₂ caused by nematode infection are greater than the normal range of production from different insect species.

The second peak of CO₂ production in nematode-infected *G. mellonella* corresponded with high numbers of juveniles. At this point resources are depleted and entering the host could result in mortality or reduced reproduction, with the possible exception of the situation in *S. carpocapsae* where a second generation of adults was available after the elevated CO₂ levels (Ramos-Rodríguez et al., unpublished data). Therefore, elevated CO₂ production did not always correlate with ideal conditions for

infection. Host species also impacted the level of CO₂ produced. If nematodes are to use CO₂ levels to make infection decisions, it would have to be used in combination other more specific information. Contact cues also play a role in host selection (Lewis et al., 1992, 1996), and IJs could be using CO₂ as a long-range attractant and then making infection decisions based on more specific contact cues. Behavioral assays that measured host attraction and infection at time points post-infection where changes in CO₂ production are observed could provide insight into this process. Ramos-Rodríguez et al. (in press) evaluated attraction using these same host species, nematode species and times after infection, but found no change in long-range attraction to hosts that corresponded with the peaks of CO₂ produced from infected hosts reported here. This suggests that any influence of these changes in CO₂ production is likely to be during the short-range attraction associated with finding routes of entry or with the process of host acceptance. Christen et al. (submitted manuscript under revision) found infection by *S. riobrave* continued out to 72 h post-infection, but at 24 h, corresponding with the first CO₂ peak, this species showed a preference for infected over uninfected hosts. While these changes in the level of CO₂ production from infected hosts are large, their impact on IJ behavior may be moderated by other changes in cues occurring at the same time and/or by a limited ability to detect or respond to changes in CO₂.

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