Journal of Nematology 46(3):281–286. 2014.
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# Infectivity of *Steinernema carpocapsae* and *S. feltiae* to Larvae and Adults of the Hazelnut Weevil, *Curculio nucum*: Differential Virulence and Entry Routes

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*Abstract:* We investigated the existing susceptibility differences of the hazelnut weevil, *Curculio nucum* L. (Coleoptera:, Curculionidae) to entomopathogenic nematodes by assessing the main route of entry of the nematodes, *Steinernema carpocapsae* strain B14 and *S. feltiae* strain D114*,* into larvae and adult insects, as well as host immune response. Our results suggested that *S. carpocapsae* B14 and *S. feltiae* D114 primarily entered adult insects and larvae through the anus. Larvae were more susceptible to *S. feltiae* D114 than *S. carpocapsae* B14 and adults were highly susceptible to *S. carpocapsae* B14 but displayed low susceptibility to *S. feltiae* D114. Penetration rate correlated with nematode virulence. We observed little evidence that hazelnut weevils mounted any cellular immune response toward *S. carpocapsae* B14 or *S. feltiae* D114. We conclude the differential susceptibility of hazelnut weevil larvae and adults to *S. carpocapsae* B14 and *S. feltiae* D114 primarily reflected differences in the ability of these two nematodes to penetrate the host.
*Key words:* Curculionidae, entry routes, immune response, penetration rate, *Steinernema carpocapsae*, *Steinernema feltiae*.

Entomopathogenic nematodes (EPNs) in the families Heterorhabditidae and Steinernematidae are obligate insect parasites (Kaya and Gaugler, 1993) that have been extensively used in biological pest control (Georgis et al., 2006). These nematodes have evolved a mutualistic association with bacteria in the genera *Xenorhabdus* and *Photorhabdus*. *Photorhabdus* is associated with *Heterorhabditis* and is carried in the intestine (Bird and Akhurst, 1983; Silva et al., 2002). *Xenorhabdus* is associated with *Steinernema* and confined to a specific vesicle within the intestine of the infective juveniles (IJs). Nematodes locate their potential host by following insect cues (Lewis et al., 2006). After IJs locate either insect, they infect the host through either orifice such as the anus, mouth, and spiracles or by penetrating the cuticle. Once IJs enter the host, they shed their outer cuticle (Sicard et al., 2004) and begin ingesting hemolymph, which triggers the release of symbionts by defecation or regurgitation (Martens et al., 2004; Martens and Goodrich-Blair, 2005).

The developing nematodes then consume the bacteria and liquefied host tissues (Kondo and Ishibashi, 1988). The nematodes-bacteria complex kills the host within 24 to 72 hr (Dowds and Peters, 2002; Forst and Clarke, 2002) unless the hosts' immunity system can stop the infection. Hemocytes play a key role in the defense against nematodes by mounting a defense called encapsulation, which involves the binding of hemocytes to the surface of the nematodes to form an overlapping sheath of cells (Strand, 2008). In addition to the immune system, insects have evolved behavioral, morphological, and physiological barriers to nematode infection (Sicard et al., 2004). Grooming behaviors including rubbing and using the mandibles to scrape the cuticle can remove nematodes attached to the surface of the insect (Gaugler et al., 1994; Koppenhöfer et al., 2000). The morphology of orifices can also restrict the entry of nematodes into some insects (Ishibashi and Kondo, 1990).

EPNs are one of the most effective biological control agents of soil insect pests (Grewal and Peters 2005; Georgis et al., 2006) and consequently their virulence has been tested in the laboratory against many different insect species (Grewal et al., 2005). It has been widely reported that different stages of an insect species respond differently to EPNs (Shapiro-Ilan, 2001a, 2001b; Grewal et al., 2005; Lewis et al., 2006). Our preliminary laboratory studies indicated that EPNs kill the hazelnut weevil (HW), *Curculio nucum* (L.) (Coleoptera: Curculionidae) (Batalla-Carrera et al., unpubl. data) but efficacy varied with nematode species and weevil developmental stage. The aim of this study was to elaborate on these observations by assessing the basis for differential susceptibility of HW adults and larvae to the nematodes *Steinernema carpocapsae* (Weiser, 1955) and *S. feltiae* (Filipjev, 1934). In particular, we investigated the penetration ability of the nematodes and presence of encapsulations as a sign of immune response.

## Materials and Methods

*Source of insect and nematodes:* HW larvae and adults were field-collected from hazelnut orchards in the northeast portion of the Iberian peninsula. Insects were maintained in boxes filled with autoclaved soil at 25°C for 2 wk to remove diseased individuals before being used. *S. carpocapsae* B14 and *S. feltiae* D144 were reared at 25°C in last instar *Galleria mellonella* (L.) (Lepidoptera: Pyralidae) according to the method of Woodring and Kaya (1998). IJs that emerged from cadavers were recovered using modified White traps (Kaya and Stock, 1997). After storage at 7°C for a maximum of 2 wk, the

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This research was supported by a Spanish Ministerio de Educación y Ciencia Project (AGL2006-037228/AGR) and facilitated by the BE-DGR 2011 grant. Many thanks to Gonçal Barrios, Jordi Mateu, Anna Aymamí from the Servei de Sanitat Vegetal (Generalitat de Catalunya), and especially Frederic Mallol (ADV Associació pel Desenvolupament Rural de les Muntanyes de Prades) for all their efforts and assistance.
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This paper was edited by Axel Elling.

nematodes were acclimatized at room temperature for 3 hr before use and viability was checked by observation of movement under a stereomicroscope.

*Routes of entry used by nematodes:* Nematode routes of entry into HW larvae and adults were assessed as follows. To determine whether nematodes preferentially enter HW larvae through the anus or mouth, larvae were ligated between the sixth and seventh abdominal segment using a cotton thread, which prevented IJs entering from the anterior of the larva from migrating to the posterior and vice versa. Larvae were then individually exposed for 48 hr to 2,716 IJs (100 IJs/$cm^2$) of *S. carpocapsae* B14 or *S. feltiae* D114 in 5.5-cm petri dishes lined with two moistened filter paper discs. After the exposure period, all larvae were rinsed with sterile tap water followed by dissection in physiological saline (0.90% w/v of NaCl) to determine the number of nematodes present in the anterior and posterior domains. If no nematodes were found, we further confirmed the absence of endosymbiotic bacteria by culturing a drop of hemolymph in NBTA medium (NA + 0.0025% bromothymol blue + 0.004% triphenyltetrazoliumchloride) (Akhurst, 1980). We assumed that all nematodes in the anterior hemocoel penetrated via the mouth or one of the spiracles in the anterior portion of the larva whereas any nematodes in the posterior hemocoel penetrated through the anus or spiracles in the posterior portion of the larva. Controls consisted of ligated larvae with no IJs added. A total of 15 larvae per treatment were examined, and each treatment was replicated five times using different batches of nematodes.

Entry into adults was assessed using 1-ml Eppendorf tubes filled with sand. We pierced the cap of each tube and threaded the weevil's rostrum through the opening and then glued it in place using SuperGlue3 (Loctite, Henkel adhesives SL). For half of the samples the beetles were on the outside of the cap with the rostrum pointing to the inside, whereas for the other half the beetles were placed abdomen first into the tube with the rostrum oriented to the outside. We exposed only the mouth to nematodes by using beetles with the rostrum pointing to the inside of the tube. Nematodes were then added to the sand in the tube and then the tube was closed, which put the rostrum in contact with the sand. Reciprocally, we exposed only the anus and spiracles to nematodes using beetles inserted in the tube where with the rostrum pointed outward. Again, nematodes were added to the sand followed by closure of the tube. Nematodes were applied in tubes at a rate of 157 IJs (200 IJs/$cm^2$) of *S. carpocapsae* B14 or *S. feltiae* D114. We used two control treatments: (i) to measure whether gluing affected weevil survival, glued weevils were kept in the same conditions as above but with no IJs being added to the sand, and (ii) to compare mortality when all orifices were exposed to nematodes, weevils were placed completely inside the Eppendorf tubes surrounded by sand with the same application rate of IJs. Adult weevils were exposed to nematodes for a period of 3 hr. Weevils still glued to the Eppendorf caps were then rinsed and placed in petri dishes with filter paper. Mortality was assessed daily and dead weevils were dissected to determine the presence of nematodes. A total of 30 adults (15 males and 15 females) per treatment were assayed, and the experiment was repeated twice. Finally, scanning electron micrographs (SEM) studies were conducted on the mouth, anus and spiracles of larval and adult stage HW to determine the ultrastructure of the main entry routes used by the nematodes. In brief, samples were not treated but fresh exanimate specimens were examined using a Zeiss Evo ® MA 10.

*Virulence and encapsulation presence:* We assessed whether *S. carpocapsae* B14 and *S. feltiae* D114 IJs trigger an encapsulation response in HW larvae or adults by conducting laboratory assays. In brief, individual last-instar HW were exposed for 12 hr to a low (4.4 IJs/$cm^2$, 50 IJs total) or high (44.1 IJs/$cm^2$, 500 IJs total) application rate of *S. carpocapsae* B14 and *S. feltiae* D114 by filling cups with 45 g of loamy sand soil (85% sand, 10% silt, 5% clay; 2.7% organic matter; pH 6.1) at field capacity (14% w/w). After the exposure period, each larva was rinsed with sterile tap water to remove any external IJs and placed in a new cup containing soil without nematodes. For each nematode and concentration five individuals were randomly chosen (dead or alive) and dissected daily during the subsequent 4 d. For each dissected larva the following data were recorded: number of nematodes inside, whether the nematodes were dead or alive, whether the nematodes were encapsulated by hemocytes. Assays with each nematode species and application rate were repeated twice. Adults were similarly examined with the exception that assays were conducted in 5.5-cm petri dishes with 23-g soil at field capacity. After 12 hr, cohorts of five adults were dissected daily for 4 d with the same data collected as described for the larvae. Assays with each nematode species and application rate were also repeated twice.

*Statistical analysis:* To determine the effect of nematode treatment and application rate on larval and adult HW mortality independent crosstabs test were used. Differences in penetration rate between species were analyzed using a Mann-Whitney (M-W) nonparametric test. To compare insect mortality on partially exposed adults and larvae to nematodes, a crosstabs test was used and M-W was employed to differentiate the mean number of nematodes in each section of the insect. For all tests a level of significance of $P < 0.05$ was used. All data were analyzed using SPSS-PC v.19.0 (SPSS, 2007).

## Results

*Routes of entry used by nematodes:* In the absence of nematode infection, there was no mortality of ligated HW larvae over the duration of our assays. We thus concluded that ligation was a useful approach for assessing whether *S. carpocapsae* B14 and *S. feltiae* D114

preferentially enter the anterior or posterior of HW larvae. We visually observed *S. carpocapsae* B14 and *S. feltiae* D114 IJs around both the head and anus of HW larvae in our assay areas. However, our dissection results strongly indicated a much higher proportion of ligated HW larvae had *S. carpocapsae* B14 in the posterior hemocoel (91.7%) than in the anterior hemocoel (16.7%) ($\chi^2 = 14.22$, 1, $P < 0.05$). In two individuals, we observed nematodes in both the posterior and anterior. In no case did we detect symbiotic bacteria in the anterior or posterior hemocoel if no nematodes were present in the same domain.

Our dissection results also indicated that a higher proportion of ligated HW larvae contained *S. feltiae* D114 IJs in their posterior hemocoel (97%) than in their anterior hemocoel (44%) ($\chi^2 = 73.1$, 1, $P < 0.05$). The mean number of nematodes was also higher in the posterior (8.97 nematodes) than in the anterior (2.07 nematodes) (M-W test $P < 0.05$). In 11 of these larvae, nematodes were found simultaneously in the anterior and posterior portions of the insect. Similar to *S. carpocapsae* B14, no symbiotic bacteria were detected in the anterior or posterior hemocoel if *S. feltiae* D114 nematodes were absent in the same domain. Therefore, the ligation was effective in preventing the nematodes or the symbiotic bacteria from crossing anterior to posterior part or vice versa.

Gluing the rostrum of adult HW to the cap of Eppendorf tubes had no adverse effect on mortality relative to unglued controls ($\chi^2 = 3.26$; $P > 0.1$). Our results also showed that adult mortality approached 90% when beetles were fully exposed to *S. carpocapsae* B14. Exposure of the anus plus spiracles to *S. carpocapsae* B14 resulted in 55% mortality, whereas exposure of only the rostrum resulted 15% mortality ($\chi^2 = 7.033$, 1, $P < 0.05$). When using *S. feltiae* D114, weevil mortality was 20% in adults that were fully exposed to nematodes. In those adults with the anus + spiracles exposed, mortality was also 20%, whereas no mortality occurred when only the rostrum was exposed to *S. feltiae* D114 ($\chi^2 = 4.44$, 1, $P < 0.05$).

Examination of larvae by SEM showed the head capsule and mandibles of HW larvae are heavily sclerotized. Spiracles were biforous (having two small accessory chambers adjacent to the margin) on larvae with a peritreme surrounded by cuticular wrinkles. The anal orifice was simple with no external protective structures. Adult openings showed a mouth with a long-snout and small, saw-like terminal teeth. Spiracles presented swollen peritreme and spiral ridges of taenidia composed of many inwardly curved small spines. The anal orifice was Y shaped and lacked any apparent protective structures (Fig. 1).

*Virulence and encapsulation presence:* Differences in larval mortality because of application rate were not detected in either nematode species ($\chi^2 = 2.72$, 1, $P > 0.05$; $\chi^2 = 2.94$, 1, $P > 0.05$). Larval mortality using a low or high rate of *S. carpocapsae* B14 was significantly lower than the mortality associated with a low and high rate of *S. feltiae* D114 ($\chi^2 = 3.89$, 1, $P < 0.05$; $\chi^2 = 4.03$, 1, $P < 0.05$) (Table 1). The nematode penetration rate into larvae of HW after 12-hr exposure was low for both nematode species tested (Table 1). The *S. carpocapsae* B14 penetration rate at the low rate was 0.025 nematodes/larva and at high rate was 1.55 nematodes/larva. The penetration of *S. feltiae* D114 was 1.28 nematodes/larva and 7.13 nematodes/larva at the low and high rate, respectively. There was a significant rate effect for

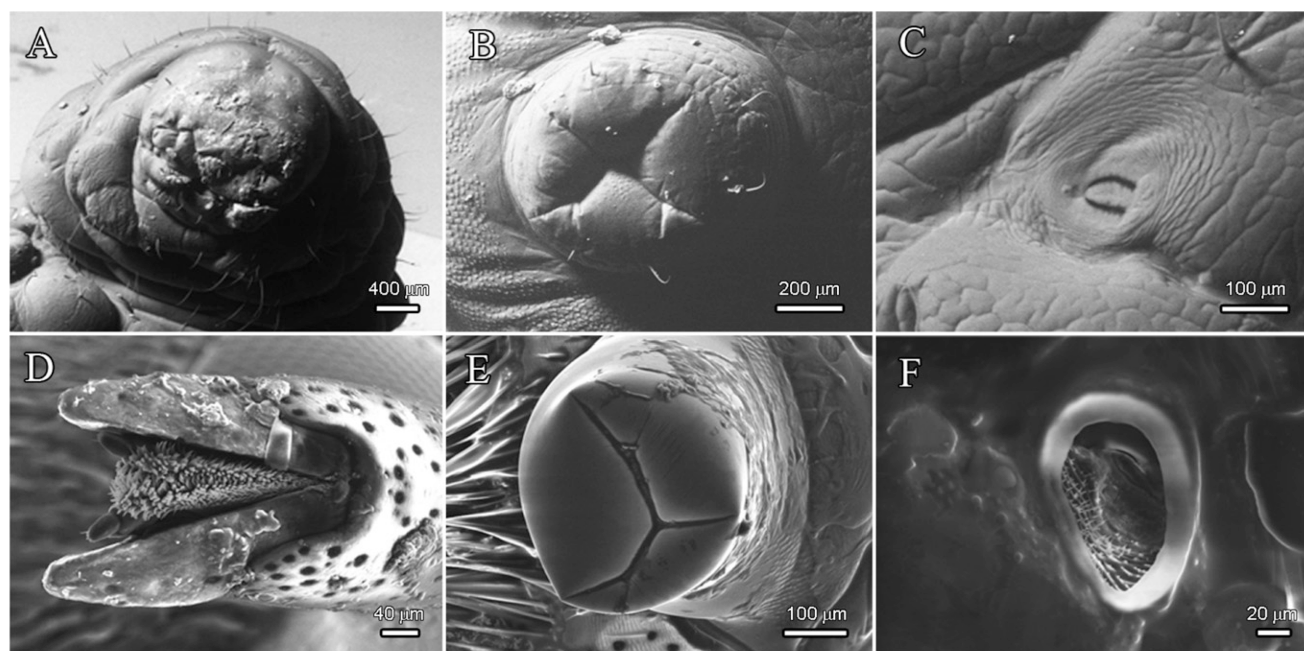


Fig. 1. SEM micrographs of hazelnut weevil (A) larval cephalic capsule, (B) larval anus, (C) larval spiracle, (D) adult mouth, (E) adult anus, and (F) adult spiracle.

TABLE 1. Percentage of larval mortality of hazelnut weevil and nematode penetration rate after 12-hr exposure to a *Steinernema carpocapsae* B14 (Sc) and *S. feltiae* D114 (Sf) using a high rate (HR), 500 IJs, and to a low rate (LR), 50 IJs.

| | | Sc | Sf |
|---|---|---|---|
| Larval mortality | LR | 5% | 15% |
| | HR | 5% | 20% |
| Penetration rate | LR | 0.05% | 2.6% |
| | HR | 0.31% | 1.4% |

penetration of *S. carpocapsae* B14 (M-W test, $P < 0.001$) but not for *S. feltiae* D114 (M-W test, $P = 0.516$). The *S. feltiae* D114 penetration rate was higher than *S. carpocapsae* B14 at low rate (M-W test, $P < 0.05$) but not at the high rate (M-W test, $P = 0.444$). No nematodes collected from the hemocoel of larvae were encapsulated.

In the case of adult insects, *S. carpocapsae* B14 caused higher mortality than *S. feltiae* D114 ($\chi^2 = 108.49$, 1, $P < 0.05$) (Table 2). Mortality was unaffected by application rate for both *S. carpocapsae* B14 ($\chi^2 = 3.11$, 1, $P = 0.08$) and *S. feltiae* D114 ($\chi^2 = 1.01$, 1, $P = 0.314$). *S. carpocapsae* B14 penetration rate into adult weevils was 2.53 nematodes/adult at the low rate and 19.58 nematodes/adult at the high rate. Penetration of *S. feltiae* D114 was 0 and 0.1 nematodes/adult at the low and high rate, respectively. There was a significant effect of application rate on the penetration of *S. carpocapsae* B14 (M-W test, $P < 0.001$) but not *S. feltiae* D114 (M-W test, $P = 0.056$). The penetration rate was also higher for *S. carpocapsae* B14 than *S. feltiae* D114 at both application rates tested (M-W test, $P < 0.05$). Inspection of *S. carpocapsae* B14 from the hemocoel of dissected adults showed only one encapsulated nematode from a total of ten adults dissected at 24 hr post-infection. This nematode was also still alive as evidenced by its ability to move inside the capsule. After 72 hr, two adult insects out of ten contained encapsulated nematodes. One of these insects had a single living nematode surrounded by hemocytes, and the other had two living nematodes with only one of them surrounded by hemocytes. No encapsulated nematodes were identified from dissected HW adults exposed to *S. feltiae* D114.

Comparing the susceptibility of larva and adult stage HW with EPNs, mortality ($\chi^2 = 1.54$, 1, $P < 0.05$) and penetration rate (M-W test, $P < 0.05$) was higher in larvae than adults for *S. feltiae* D114. In contrast, morality ($\chi^2 = 104.76$, 1, $P < 0.001$) and penetration rate was higher in adults than larvae for *S. carpocapsae* B14 (M-W test, $P < 0.05$).

TABLE 2. Percentage of adult mortality of hazelnut weevil and nematode penetration rate after 12-hr exposure to a *Steinernema carpocapsae* B14 (Sc) and *S. feltiae* D114 (Sf) using a high rate (HR), 500 IJs, and to a low rate (LR), 50 IJs.

| | | Sc | Sf |
|---|---|---|---|
| Adult mortality | LR | 77.5% | 0% |
| | HR | 92.5% | 2.5% |
| Penetration rate | LR | 0.51% | 0% |
| | HR | 3.9% | 0.02% |

## DISCUSSION

Differences in the susceptibility of larvae and adults to EPNs have been reported in studies with several species of insects (Shapiro-Ilan et al., 2002; Shapiro-Ilan et al., 2005; McGraw and Koppenhöfer, 2008). Overall, larvae tend to be more susceptible than adults (Boivin and Bélair, 1989; McGraw and Koppenhöfer, 2008; Morton and Garcia-del-Pino, 2009), although higher rates of adult susceptibility are also known (Schroeder et al., 1994; Shapiro-Ilan 2001a, 2001b; Loya and Hower, 2003; Shapiro-Ilan et al., 2005; Laznik et al., 2010). Collectively, these findings suggest the relationship between stage and susceptibility to EPNs is species specific. Studies by Shapiro-Ilan (2001a; 2001b) using an insect closely related to HW, the pecan weevil, *Curculio caryae* (Horn) (Coleoptera: Curculionidae), reported that larvae were equally susceptible to *S. feltiae* and *S. carpocapsae* but adults were particularly susceptible to *S. carpocapsae,* more so than *S. feltiae*. In the case of *C. nucum*, previous findings obtained by Batalla-Carrera et al. (*unpublished data*) that larvae were more susceptible to most of the EPN species infection than adults, although adults showed higher susceptibility to *S. carpocapsae* B14. Accordingly, in the present study we observed differential susceptibility of the two insect stages to the nematodes species tested. Although larval mortality was higher when exposed to *S. feltiae* than *S. carpocapsae*, the adult mortality was much higher because of *S. carpocapsae* exposure. Mechanism behind the differential susceptibility is elucidated herein.

Differential susceptibility among larval and adult stages of insects might be related to differences in the infection process among nematodes. First, differential attraction of EPNs to volatile cues emanating from the different host stages could be a factor (Lewis et al., 2006). However, because insects were confined in a very small arena, it is doubtful that host attraction had an impact on the observed differences in HW susceptibility.

Differential host susceptibility can also vary with IJ entry strategy (Cabanillas, 2003) or host behavior (Bedding and Moulyneux, 1982; Koppenhöfer et al., 2000). Our results clearly show that the anus is the main entrance for *S. carpocapsae* B14 and *S. feltiae* D114 into HW larvae. The anus is also the primary route of nematode entry for other species including *Liriomyza trifolii* (Burgess) (Diptera: Agromyzidae) (LeBeck et al., 1993) and *Musca domestica* (L.) (Diptera: Muscidae) (Renn, 1998). In contrast, Koppenhöfer et al. (2007) observed equal or higher penetration through the mouth than the anus when exposing four different species of white grub larvae (Scarabeidae) to *S. glaseri* (Steiner, 1929) and *S. scarabaei* (Stock and Koppenhöfer, 2003). The size of natural openings in HW larvae does not seem to be a prime determinant for the preference we observed because neither the mouth nor anus have morphological features that would impede nematode

penetration. Thus, HW behavior or differences between the mouth and anus in the cues used by nematodes to locate HW may account for the penetration preferences we identified.

Nematodes attached to the cuticle may be eliminated by insect grooming behavior (Gaugler et al., 1994; Koppenhöffer et al., 2000; Mankowski et al., 2005; Koppenhöffer et al., 2007). Insects can also kill nematodes with their mandibles when IJs enter through the mouth (Gaugler and Molloy, 1981). We observed that nematodes stimulated noticeable grooming behavior in HW larvae. Furthermore, the mandibles of HW larvae have hard sclerotized structures, which could crush nematodes entering the mouth. In contrast, we did not observe evasive behaviors like defecation by HW larvae to reduce the anal route of entry as has been observed in some insects (Bedding and Molyneux, 1982). Our results also suggest that HW larval gut fluids are not detrimental to nematodes as has been described for some other hosts (Wang et al., 1995).

We cannot rule out spiracles as a possible route of penetration for nematodes into the HW larvae. However, because the structure of spiracles in the anterior and posterior of the insect is essentially the same and the number of spiracles present in the anterior and posterior portions of the ligated insects was also equal, it is unlikely that spiracles account for the strong tendency for nematodes to infect the posterior hemocoel. The ultrastructure of the larval spiracles also showed they are protected by sieve plates, which allow the free exchange of oxygen and $CO_2$ but exclude invading nematodes. Similar structures have been reported from larval stage *Phyllophaga hirticula* (Knoch) (Coleoptera: Scarabaeidae) to avoid EPN infection (Forschler and Gardner, 1991). We also rule out direct penetration of steinernematids through the integument because this entry route is mainly observed in insects without an epicuticle (Peters and Ehlers, 1994). We thus conclude the anus is the main opening used by nematodes to enter HW larvae.

In adults, the higher penetration observed through anus could be related to orifice size because the anus is bigger than the mouth. Direct observations showed that adult weevils display evasive behavior toward nematodes; mobility increased when nematodes were present. However, this avoidance behavior does not explain the differences observed on the entry route. In the case of adults we also rule out the spiracles and direct penetration through the integument as entry routes because adults have spiracles firmly enclosed by the elytra, which provide difficult access for nematodes. This fact was also observed in other Curculionidae such as *Cosmopolites sordidus* (Padilla, 2003).

Hominick and Reid (1990) reported that IJ penetration rate could be a good indicator of nematode virulence. Our results demonstrate how penetration into HW depends more on life stage. Our results also indicate that increasing nematode dosage does not necessarily result in higher mortality. Similar results have been obtained by other authors who confirmed the lack of a correlation between penetration rate and host mortality (Gaugler et al., 1990; Epsky and Capinera, 1994; Morton and Garcia-del-Pino, 2007).

Finally, our results show that HW larvae and adults do not usually encapsulate *S. carpocapsae* B14 or *S. feltiae* D114 that enter the hemocoel. The lack of response could be attributable to a depression of hemocyte activity caused by the founding population of EPNs (Silva et al., 2002). Moreover, the observation of hemocytes inside dead adult insects imply that when the nematode penetrated the insect immune system was triggered, but the response came too late to interfere with the lethal action of symbiotic bacteria. Therefore, we can conclude that encapsulation was not responsible for the differential susceptibility among *C. nucum* stages and nematode species, but rather the differences appear to be related to differential penetration. As many factors may be involved in differential susceptibility to EPNs among insect stages, further studies are needed to deepen our knowledge of this subject and to allow us to optimize the efficiency of insect control efforts, e.g., by selecting the appropriate nematode species against each insect stage.

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