Effects of infected insects on secondary invasion of steinernematid entomopathogenic nematodes

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SUMMARY

Factors affecting 'invasion efficiency' of steinernematid entomopathogenic nematodes into hosts were elucidated. The phenomenon that only part (10–40%) of the nematode population invades the target host has been recorded in many studies. It has been mainly ascribed to differences in the ability of individual nematodes to infect. In the present study the effect of an infected host, the wax moth *Galleria mellonella*, on subsequent infection of the entomopathogenic nematodes *Steinernema carpocapsae* Mexican, *S. riobravus* and *S. feltiae* was evaluated. The invasion rate of the 3 nematode species to a non-infected host was reduced by 40–60% after pre-exposure to infected hosts. These nematodes regained their full invasion potential after they were rinsed with water. Invasion into insects which were previously injected with nematodes was significantly reduced by 60–80% 6–9 h after injection. The reduction in subsequent invasion due to the initial infection was nematode species specific. This phenomenon was also observed with other lepidopteran pests (*Helicoverpa armigera* and *Spodoptera littoralis*). The data indicate that the initial infection by entomopathogenic nematodes induced the release of a substance which reduced the subsequent invasion. The chemical and biological characteristics of this substance are currently under investigation.

Key words: infectivity, entomopathogenic nematodes, host response, invasion inhibitory substance.

INTRODUCTION

Parasitoids and predators only use hosts as sources for development of offspring. Parasitoids distribute their eggs over resources that occur in discrete units of limited size, and so confine their offspring to a fixed amount of food (Alphen, Van & Visser, *et al*. 1990). In contrast, a predator's offspring can always hope to find more prey items when the present meal is insufficient to complete development (Alphen *et al*. 1990). However, any parasite which uses the host as a mating arena in addition to a nutritional source faces 2 contradictive requirements upon invasion (a) the need to encounter others from the opposite sex for mating and reproduction and (b) the need to reduce the numbers of individual parasites in each host to ensure the availability of host resources for development. Particularly interesting is the observed phenomenon among entomopathogenic nematodes from the families Steinernematidae and Heterorhabditidae of low 'invasion efficiency', i.e. only a proportion of applied infective juveniles (IJs) invade the host (Molyneux, 1986; Fan & Hominick, 1991; Glazer, 1991; Mannion & Jansson, 1993; Westerman, 1994; Caroli, Glazer & Gaugler, 1996; Ricci, Glazer & Gaugler, 1996). While steinernematids invade at a level of 10–40%, low levels of nematode invasion (1–5%) were recorded with the heterorhabditid species (Caroli *et al*. 1996; Epsky & Capinera, 1993; Ricci *et al*. 1996; Tahir, Otto & Hague, 1995).

Steinernematid and heterorhabditid nematodes are considered among the most promising alternative to chemical control of insect pests (Georgis & Manweiler, 1994). These nematodes are able actively to locate, parasitize and kill a wide range of insect species. Both *Steinernema* and *Heterorhabditis* pass through 4 juvenile stages before maturing. Only the 3rd-stage infective juvenile (IJ) can survive outside the insect host and move from one insect to another. Insect mortality, due to nematode infection, is caused by a symbiotic bacterium (*Xenorhabdus* spp. for steinernematids and *Photorhabdus* spp. for heterorhabditids) which the IJs carry in their intestines and release in the insect haemocel (Akhurst & Boemare, 1990; Boemare, Akhurst & Mourant, 1993). Invasion occurs through natural openings (spiracles, mouth, anus) or, in some cases, directly through the cuticle of certain insects (Bedding & Molyneux, 1982; Peters & Ehlers, 1994). The bacteria cells proliferate and eventually kill the insect host (usually within 24–72 h). However, fundamental knowledge concerning interactions among the nematode, the host and the environment is still lacking.

Bohan & Hominick (1995) attributed the low 'invasion efficiency' phenomenon to differences between infectious or non-infectious individual IJs within one population. In this study it was assumed

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that the 'transmission coefficient', which is the average probability of host infection/nematode/host/unit time, is independent of host and IJ density, as the hosts and IJs are mixed randomly. However, host reaction to the initial invasion of nematodes and the subsequent influence of an infected host on the nematodes at its close vicinity was not taken into consideration.

The objective of the present study was to ascertain the effects of an infected host on secondary invasion by 3 steinernematid species: *Steinernema feltiae* (strain UK), *S. riobravus* and *S. carpocapsae* (strain Mexican).

MATERIALS AND METHODS

Organisms cultured

Insects. The greater wax moth *Galleria mellonella* was reared in the laboratory according to the procedure of Woodring & Kaya (1988). Last instars of the African bollworm *Hellicoverpa* (*Heliothis*) *armigera* and *Spodoptera littoralis* were obtained from the laboratory of A. Navon (The Department of Entomology, Volcani Center, Israel), where they were reared on artificial media according to the method described by Glazer & Navon (1990).

Nematodes. *Steinernema feltiae* (strain UK), *S. riobravus* and *S. carpocapsae* (strain Mexican) were reared in last instars of *G. mellonella* according to the method of Woodring & Kaya (1988) at 25 °C. Nematodes were stored at 10 °C for 7–14 days before use. The nematodes in the water suspensions were allowed to acclimatize at ambient room temperature (21–23 °C) for 24 h prior to the exposure to insects.

Bioassays

Nematodes were exposed to the insects in 5 cm diameter Petri dishes padded with 2 filter paper discs (Whatman no. 1). The host effect on nematode invasion was measured by counting the number of individual nematodes which penetrated into the insect haemocele. Two days after exposure, hosts were dissected to enable examination of developing nematodes (Caroli *et al*. 1996). Each treatment consisted of 12 replicates/insects.

To determine the effect of different nematode concentrations on invasion level 1 *G. mellonella* larva was exposed to IJs of the different nematode species. The nematodes were transferred to each dish in a volume of 500 μl of distilled water. Controls received water only. The dishes were then incubated at 25 °C in the dark. Forty eight hours after inoculation insect mortality was recorded and the number of penetrated nematodes was determined in each host.

The effect of multiple exposure of the same group of nematodes to a series of non-infected hosts on the invasion rate was evaluated by replacing *G. mellonella* larvae in the Petri dishes which contained IJs of one of the species at a concentration of 1800 IJs/dish. Larvae were replaced every 24 h for 96 h. A control consisted of continuous exposure of larvae for 96 h. Insects which were removed from the dishes in the multiple exposure assay were incubated for an additional 72 h prior to determining their invasion rate as described above.

To elucidate the effect of time from the initial infection on the secondary invasion rate, *G. mellonella* larvae were injected with IJs and then exposed to nematodes in Petri dishes (1800 IJs/dish) 0, 3, 6 and 9 h after injection. The nematodes were injected in the following procedure. Approximately 30 surface-sterilized IJs (5 min exposure to 0·5% chlorax followed by rinsing 3 times in sterile saline solution) were injected directly into the haemocoel of the *G. mellonella* larvae in 25 μl using a 1 ml sterile syringe (Becton-Dickinson & Co., Rutherford, NJ, USA) with a 0·4 × 20 mm size needle (Terumo Europe N.V., Leuven, Belgium). The invasion level was recorded 48 h after exposure in the Petri dishes. The following treatments were used as a control: non-injected insects, saline-injected insects, insects injected with dead (heat killed) nematodes, insects injected with approximately 30 2nd-stage juveniles of the root-knot-nematode *Meloidogyne javanica*. The treatments were exposed to 1800 IJs in Petri dishes. In addition, non-injected insects and saline injected insects were incubated in nematode-free Petri dishes containing moist filter paper.

The effect of different hosts on the secondary invasion was determined with last instar larvae of *H. armigera* and *S. littoralis* by repeating exposure and the injection treatments as described above for *G. mellonella*.

The possibility that a particular substance is released by the host that influences the surrounding nematodes was also investigated. Ten larvae of *G. mellonella* were exposed to IJs of *S. riobravus* (1800 IJs/dish). Twelve hours after exposure the infected insects were taken from the dish, rinsed in distilled water to remove nematodes from the cuticle and placed in a new nematode-free dish padded with moist filter paper. After an additional 24 h the insects were removed from the Petri dish. Fresh *S. riobravus* IJs (1800/dish) were added to this dish in 100 μl of water and allowed to acclimatize for 6 h before a single fresh *G. mellonella* larva was placed in the dish. The invasion level of these larvae was determined after 48 h. Control treatments included fresh nematodes as well as nematodes which were exposed continuously to *G. mellonella* larvae.

To determine if the effect of the host on the secondary invasion is reversible, IJs of the different nematode species (3000 IJs/dish) were exposed for 48 h to *G. mellonella* larvae before they were re-suspended and rinsed 3 times in distilled water. To

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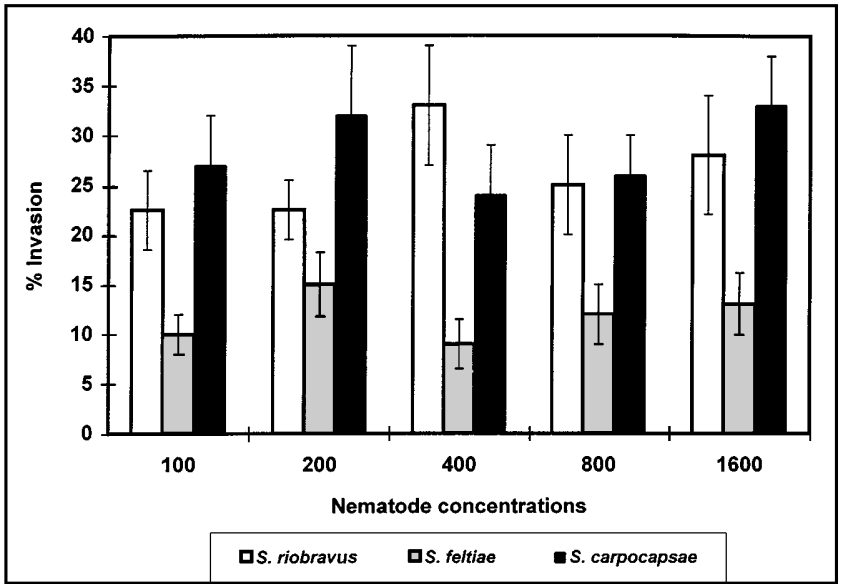

Fig. 1. The average proportion of nematodes which invaded the cadaver of *Galleria mellonella* larvae following 48 h exposure to different concentrations of infective juveniles of the species *Steinernema riobravus*, *S. feltiae* and *S. carpocapsae*.

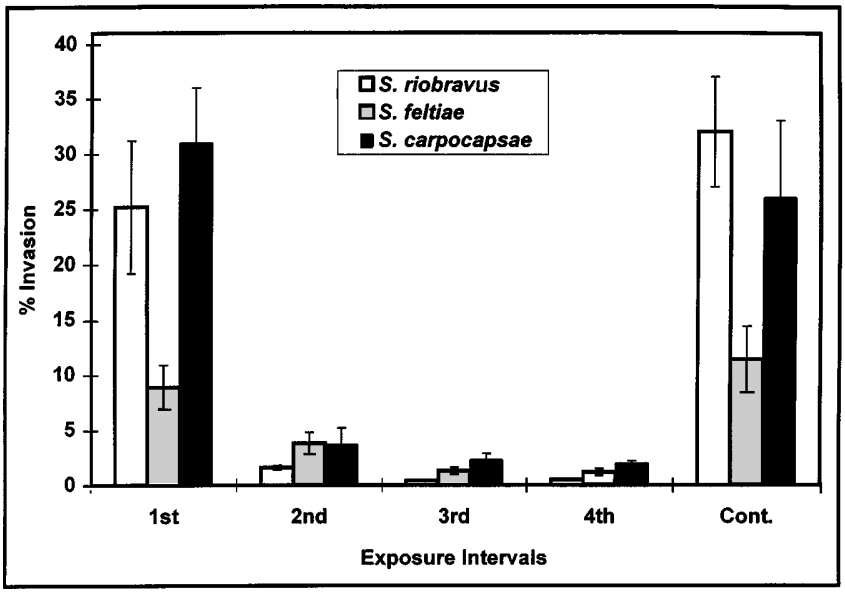

Fig. 2. The average proportion of nematodes which invaded the cadaver of *Galleria mellonella* larvae following multiple versus continuous exposure. Infective juveniles of *Steinernema riobravis*, *S. feltiae* and *S. carpocapsae* were placed on moist filter paper in 5 cm diameter Petri dishes (1800 IJs/dish). The insect larvae were replaced every 24 h for 96 h. Insects which were removed from the dishes were incubated for an additional 72 h prior to determining their invasion rate. A control consisted of continuous exposure of larvae for 96 h.

avoid agitation of the exposed IJs, which may increase their infectivity, rinsing was done by allowing the nematodes to settle gently in the distilled water. Then the rinsed IJs were re-exposed (1800 IJs/dish) to fresh insect larvae for an additional 48 h. The invasion rate was recorded with each insect. A control treatment consisted of non-rinsed nematodes.

The influence of the symbiotic bacteria on nematode invasion was resolved by exposing IJs of the different nematode species (1800 IJs/dish) to *G. mellonella* larvae which were treated as follows. (a) Cells of the primary form of the *Xenorhabdus* sp. (symbionts of the 3 nematode species) were injected as described above in 20 μl suspension. The bacteria were isolated, cultured and prepared for injection as described by Glazer, Galper & Sharon (1990). Nine hours after injection the treated larvae were exposed to the different nematode species. (b) Similarly to the treatment with *X. nematophilus*, insect larvae were injected with *Escherichia coli* (strain OP50). (c) Control treatments included water-injected and non-injected larvae. The invasion level was determined after an additional 48 h.

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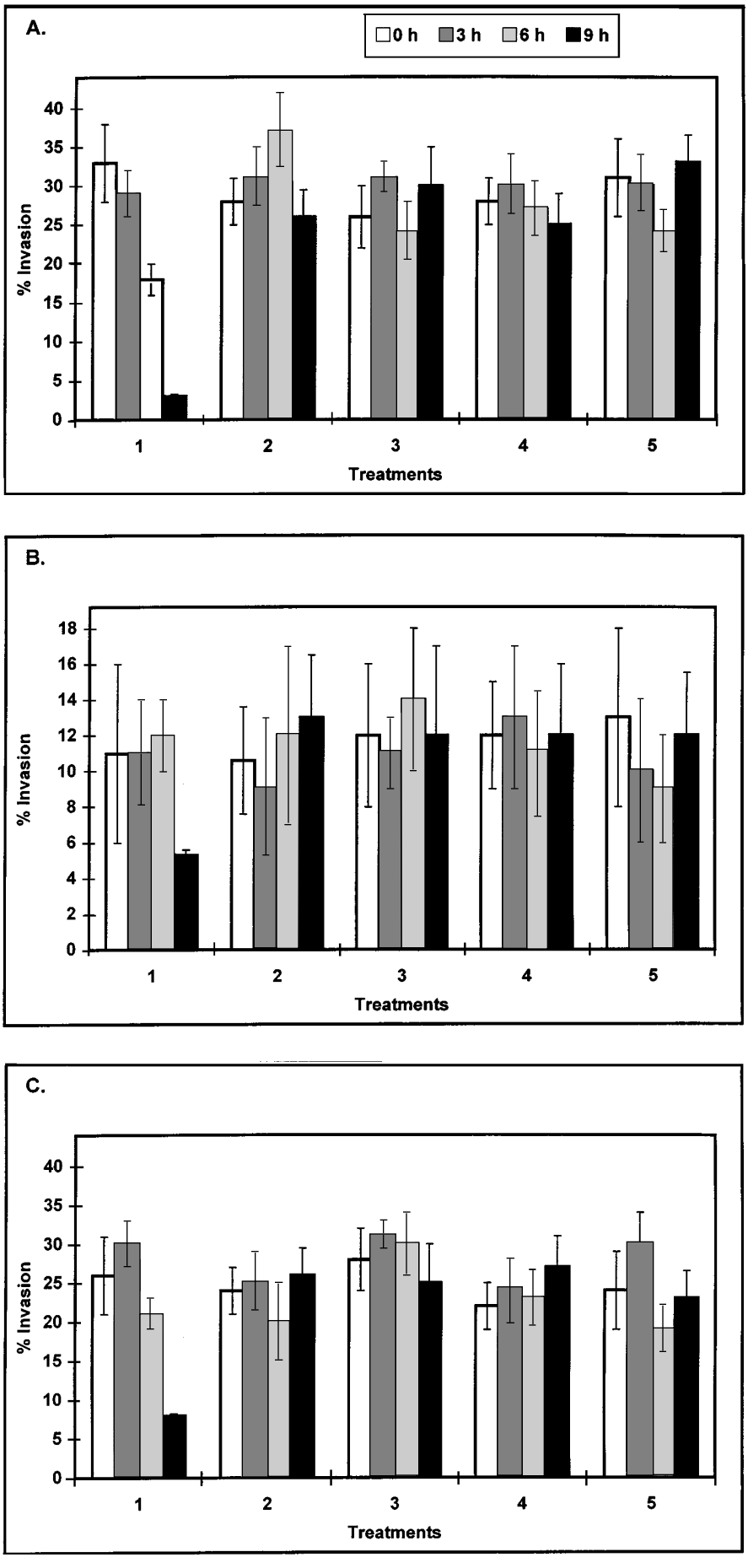

Fig. 3. For legend see opposite.

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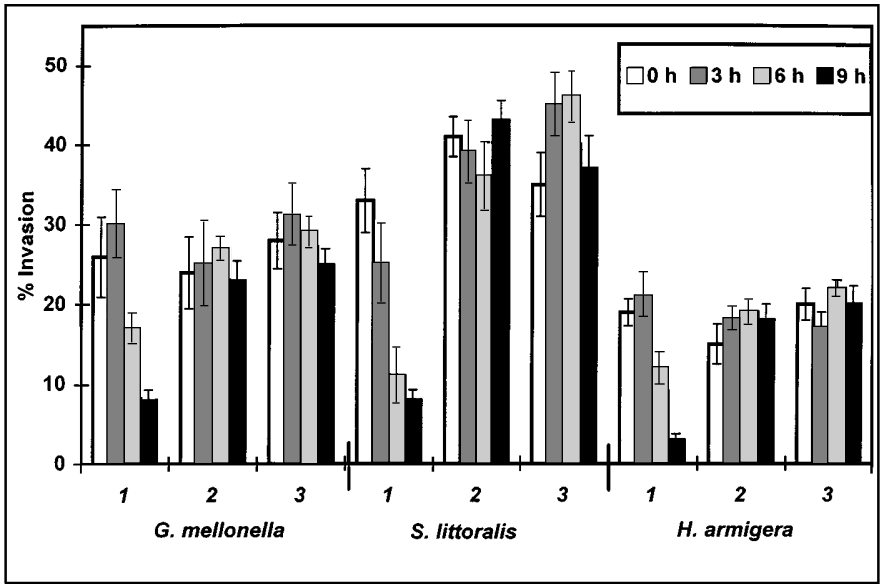

Fig. 4. Effect of time from the initial infection on the secondary invasion rate into different insect species. The larvae were injected with IJs of *Steinernema riobravus* and then exposed to nematodes in Petri dishes (1800 IJs/dish) 0, 3, 6 and 9 h after injection. The invasion level was recorded 48 h after exposure in the Petri dishes. Treatments: IJ-injected insects (1), saline-injected insects (2), non-injected insects (3).

Statistical analysis

An arcsin transformation was used on the invasion level data which are presented in percentages. The data were then subjected to ANOVA. If significant differences were detected among treatment effects they were separated using Tukey's multiple range test at $P < 0{\cdot}05$.

RESULTS

When fresh nematodes were exposed to *G. mellonella* larvae similar levels of invasion, with no significant difference ($P > 0{\cdot}05$) were recorded in all dosages for each of the nematode species (Fig. 1). While comparable levels of invasion were recorded for *S. riobravus* and *S. carpocapsae*, ranging between 23 and 33 %, relatively lower levels of invasion were characteristic for the *S. feltiae* nematode (Fig. 1). These levels of invasion were observed throughout the present study in treatments where the insects were exposed to the various nematode species at least for 48 h. The proportion of individuals which invaded the *G. mellonella* in the dose–response assay, in the present study, is similar to previous studies with these nematode species (Caroli *et al*. 1996; Ricci *et al*. 1996).

In the multiple exposure test the vast majority of nematodes invaded the first insect larvae (Fig. 2), within the first 24 h after exposure. The invasion levels of *S. riobravis* and *S. carpocapsae* in the subsequent exposures was reduced 10 to 15-fold. As for *S. feltiae*, the invasion level was reduced only 2-fold in the second exposure and 7-fold in the following exposures (Fig. 2). The cumulative percentage invasion in the multiple exposure treatment (28, 14 and 38 % for *S. riobravus*, *S. carpocapsae* and *S. feltiae*, respectively) was not significantly different from the invasion level which was recorded in the continuous exposure treatment.

Injection of live entomopathogenic nematodes significantly ($P < 0{\cdot}05$) reduced the number of nematodes which invaded the *G. mellonella* larvae with *S. riobravus* and *S. carpocapsae*, when the insects were exposed to the infective juveniles 6 h after injection (Fig. 3A, C). In the treatment with *S. feltiae* significant reduction in the invasion level was recorded only after 9 h from injection (Fig. 3B). No other treatments affected the invasion level of the various nematode species (Fig. 3A–C). Injection of *S. riobravus* IJs to larvae of different insect species resulted in significant ($P < 0{\cdot}05$) reduction in invasion level following exposure of the insects to fresh nematodes 6 h after injection (Fig. 4).

Fig. 3. Effect of time from the initial infection on the secondary invasion rate. *Galleria mellonella* larvae were injected with IJs of *Steinernema riobravus* (A), *S. feltiae* (B) and *S. carpocapsae* (C) and then exposed to nematodes in Petri dishes (1800 IJs/dish) 0, 3, 6 and 9 h after injection. The invasion level was recorded 48 h after exposure in the Petri dishes. Treatments: IJ-injected insects (1), saline-injected insects (2), non-injected insects (3), insects injected with dead (heat killed) IJs (4), insects injected with approximately 30 2nd-stage juveniles of the root-knot-nematode *Meloydogyne javanica* (5).

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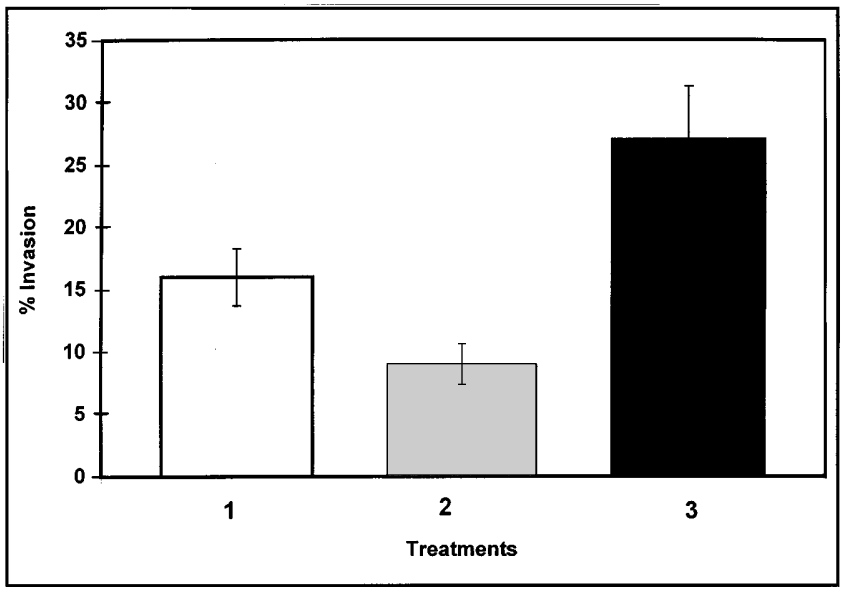

Fig. 5. Indirect effect of infected host on invasion rate of *Steinernema riobravus* IJs Ten infected *Galleria mellonella* larvae were placed in a nematode-free Petri dish padded with moist filter paper. After 24 h the insects were removed from the Petri dish. Fresh *S. ribobravus* IJs (1800/dish) were added to this dish in 100 μl of water and allowed to acclimatize for 6 h before fresh *G. mellonella* larvae were placed in the dish (treatment 1). The invasion level to these larvae was determined after 48 h. Control treatments included fresh nematodes (2) as well as nematodes which were exposed to *G. mellonella* larvae continuously (3).

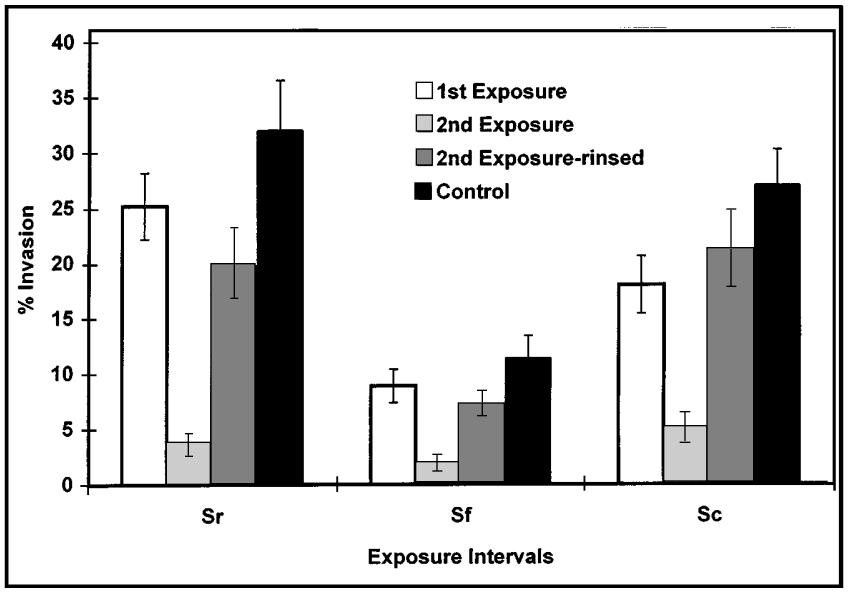

Fig. 6. Effect of water rinsing treatment on secondary invasion rate of IJs which were pre-exposed to the host. The IJs of the different nematode species (3000 Is/dish) were exposed for 48 h to *Galleria mellonella* larvae before they were re-suspended and rinsed 3 times in distilled water. Then the rinsed IJs were re-exposed (1800 IJs/dish) to fresh insect larvae for an additional 48 h. The invasion rate was recorded with each insect. A control treatment consisted of non-rinsed nematodes. Sr = *Steinernema riobravus*; Sf = S. feltiae; Sc = S. carpocapsae.

Acclimatization of fresh nematodes in a Petri dish which previously had been inhabited by an infected insect reduced the invasion level by 40% as compared to control treatment. Invasion to larvae after direct exposure to infected insects was 65% lower than the control (Fig. 5).

Pre-exposed nematodes which were washed with distilled water invaded a second host at a level similar to the first ones (Fig. 6) but the invasion level of the unwashed suspension was reduced significantly ($P < 0{\cdot}05$). The cumulative percentage invasion in the multiple exposure treatments (1st and 2nd exposure) was similar to the invasion level which was recorded in continuous exposure treatment with no significant differences ($P > 0{\cdot}05$).

Injection of the different bacteria did not reduce the level of invasion as compared to water-injected or untreated controls. In all treatments the ranges of

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the invasion levels were 24–31%, 9–15·5% and 26–34% for *S. riobravus*, *S. feltiae* and *S. carpocapsae*, respectively.

DISCUSSION

The data presented here indicate that the dynamics of an ongoing nematode infection have profound effects on the invasion rate of IJs. The findings in the present study showing a substantial reduction in invasion following injection of live infective juveniles indicate that the host influences the activity of the exterior infective juveniles within hours from the initial invasion. It was further demonstrated here that this effect is caused by different steinernematid species in different lepidopteran insects. The findings of Bohan & Hominick (1996) indicate that no changes in the non-infectious proportion, probability of infection or time-delay occurred as the host become ever more infected. They proposed a mathematical model for the infection processes based on a set of assumptions which were exclusively related to the nematode population. The assumptions embodied in this model were (1) the proportion of the non-infectious individuals is constant throughout the infection process, (2) the infection probability/nematode/unit time is constant throughout the infection process, (3) high mixing of host and infective juveniles, (4) a time-delay for infection which is constant throughout the infection process. However, this model excluded the effect of the infection on the host on the subsequent invasion rate. The present finding showing a change in behaviour on behalf of the nematodes when exposed to novel, previously infected hosts, provides complementary evidence to improve the predictive power of the Bohan & Hominick (1995) infection model.

Another model that described the infection dynamics of *S. feltiae* was recently proposed by Hay & Felon (1995). In this study it was demonstrated that nematode establishment was not related to application density in a linear fashion, as initial infection facilitated secondary colonization. The data suggested that 3 subpopulations may be distinguished by their infection behaviour: a first group of individuals which initiate infection in unparasitized insects, a second that only invaded infected hosts, and a third group of non-invaders. Although in first sight our data provide contradicting evidence on the secondary infection it is difficult to compare between the two studies. Hay & Felon (1995) used the sciarid fly *Lycoriellia solani* as the model insect and tested very low nematode concentrations (1–10 IJs/insect). The results obtained in that study may reflect a specific situation. Our data are comparable to other infectivity studies (Caroli *et al.* 1996; Epsky & Capinera, 1993; Fan & Hominick, 1991; Glazer, 1991; Mannion & Jansson, 1993; Molyneux, 1986; Ricci *et al.* 1996; Westerman, 1994) and particularly to Bohan & Hominick (1995, 1996). However, to take into consideration host effect on the infection process it is essential first to determine the source of this effect. It could be due to the host's previous exposure modifying either the rate of nematode penetration, or a second possibility is that the proportion of non-infectious nematodes increases. Further direct testing is necessary to determine which of these hypotheses is correct.

Since neither dead entomopathogenic nematodes nor other organisms which were injected into the haemoceol caused a decrease in infection, this phenomenon seems to be specific for live steinernematids. The cause for this response is not known. Among different possibilities it may be induced either by substances which are released from the infective juveniles upon invasion (Burman, 1982; Simões, Brehelin & Laumond, 1992) or host interaction with the nematode cuticle. If the latter was the case we would expect to see an effect also in the treatments with the dead nematodes, unless the heat treatment distorted these particular substances on the cuticle surface.

The fact that the inhibition effect on invasion remained after the infected larvae were removed from the dish suggests that this effect is caused by a chemical substance which remained in the arena and influenced the fresh nematodes. The ability to reverse the inhibition effect by washing the nematode surface indicates that this substance is water soluble. The source of this substance and its chemical composition are still unknown. The likelihood that the direct source of this substance is from the nematodes is low. More probable is that the host gives off the substance in response to the pathogenesis.

It has been well demonstrated among parasites, particularly parasitoids, that invaders will signal their host as 'occupied' to prevent over-crowding of the developing niche (Alphen *et al.* 1990; Driessen & Visser, 1993). We demonstrate here that in the case of entomopathogenic steinernematids the 'signal', which causes a reduction in invasion levels, occurs within 6–9 h of the initial penetration. Prior to this period a substantial number of infectious individuals invade the host. Their number is proportional to the nematode concentration when the insect is exposed to hundreds of nematodes (Fig. 1 in present study; Bohan & Hominick, 1995*b*; Caroli *et al.* 1996). However, at low (1–20 IJs) or high (> 3000 IJs) nematode concentrations this linear proportion is disrupted (Hay & Felon, 1995; Selvan, Campbell & Gaugler, 1993).

In conclusion this study indicates that an infected host may have an important effect on the surrounding nematodes, reducing their potential activity against other, non-infected target hosts. Additional studies are needed to evaluate the impact of this phenomenon on nematode activity in the field.

Furthermore, elucidation of the causes for this inhibitory effect will presumably allow the development of realistic models for the likelihood of field control.

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