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Effect of Timber Condition on Parasitization of Pine Weevil (*Hylobius abietis* L.) Larvae by Entomopathogenic Nematodes under Laboratory Conditions

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*The large pine weevil (*Hylobius abietis *L.) is one of the most important pests in coniferous reforestation in Europe. Larvae develop in the stumps of recently felled trees; the emerging adults feed on the bark of seedlings and may kill them. The ability of the entomopathogenic nematodes* Heterorhabditis megidis *and* Steinernema carpocapsae *to invade pine weevil larvae in Sitka spruce (*Picea sitchensis*) buried in moist sand was evaluated. Overall, four times as many* H. megidis *as* S. carpocapsae *invaded pine weevil larvae. The two species of nematode differed in their response to timber condition. The number of* S. carpocapsae *invading pine weevil larvae was twice as high in billets inoculated with the wood-rotting fungus* Phlebiopsis gigantea *as in fresh timber, while the number of* H. megidis *invading was reduced by 25%. Invasion into non-feeding insects (larvae of the wax moth* Galleria mellonella*) contained in timber disks was also affected by timber quality, indicating that nematode behaviour was affected directly by the physical or chemical condition of the timber, though trophically mediated effects may also have been involved.*

Keywords: *entomopathogenic nematodes,* Heterorhabditis megidis*,* Steinernema carpocapsae*,* Hylobius abietis*,* Phlebiopsis gigantea*, forest protection, biological control, nematode behaviour*

INTRODUCTION

The large pine weevil, *Hylobius abietis* (L.) is the most common cause of insect related damage in young conifer plantations established on felled areas and is a serious threat to European forestry (Leather *et al*., 1999). Development takes place in the stumps and root system of dead coniferous trees. Adults feed on the bark of newly planted trees

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causing seedling mortality, stem deformation, and reduced growth (Eidmann *et al*., 1996). In the absence of any control, the mortality of new trees due to the feeding of adults can be very high. Alternatives to reliance on chemical control are being investigated, including the use of biological control agents as a component in an integrated pest management programme.

Entomopathogenic nematodes (*Steinernema* spp. and *Heterorhabditis* spp.) are currently used for the control of several insect pests in Europe, the US and elsewhere. Both adult and larval pine weevils are susceptible to these nematodes (Pye & Burman, 1978; Pye and Pye, 1985; Collins, 1993). Results of field trials in Scotland indicate that up to 70% of *H. abietis* larvae in pine stumps can be killed with a single application of commercially available nematodes (*S. carpocapsae*) (Brixey, 1997). Nematode infective juveniles (IJs) applied to soil actively disperse in search of insect hosts. In laboratory experiments, they have been shown to respond to volatile and contact cues associated with insects (Grewal *et al*., 1993; 1997), and to insect-damaged roots (van Tol *et al*., 2001), but the identity and importance of cues used by the nematodes for host-finding in complex natural environments are unknown. Entomopathogenic nematodes applied for control of pine weevil larvae will be exposed to volatiles emanating from the conifer stumps, which may affect their ability to locate an insect host.

A number of wood-rotting Basidiomycetes grow rapidly under the bark of stumps and roots. The saprotrophic fungus *Phlebiopsis gigantea* (Fr.) Jül. is a strong competitor of *Heterobasidion annosum* (Fr.) Bref., the causative agent of root and butt rot. *P. gigantea* has been commercialized and is applied to pine stumps to prevent *H. basidion* infection (Pratt *et al*., 1999). A secondary effect of *P. gigantea* is its effect on pine weevil: pine colonized by the fungus suppressed weevil egg-laying and reduced the survival of weevil larvae (Skrzecz, 1996), while in Sitka spruce, growth of larvae was stunted (Armendáriz, unpublished data). *P. gigantea* alters both the physical and chemical composition of wood (Behrendt & Blanchette, 1997). Results of olfactometer studies suggest that the quantity or composition of behaviourally significant volatiles emitted from pine were modified by *P. gigantea* (Skrzecz & Moore, 1997).

In this research, we assess the ability of entomopathogenic nematodes to find and invade pine weevil larvae in Sitka spruce in the laboratory, using both fresh timber and timber inoculated with *P. gigantea*. Larvae of the wax moth (*Galleria mellonella* L.), which do not feed on wood, were also included in order to distinguish between direct and indirect (*Hylobius*-mediated) effects of timber condition on the nematodes. We tested two species of nematode with different behavioural characteristics: *Steinernema carpocapsae* Weiser is classed as an ambush or 'sit and wait' strategist: IJs tend to remain close to the soil surface where they nictate (IJs stand on their tail with more than 75% of the body held straight) as part of their ambush strategy that enables them to attach to passing insects, while *Heterorhabditis megidis* Poinar, Jackson and Klein displays a greater tendency to disperse in search of sedentary hosts and is classed as a cruise forager (Grewal *et al*., 1994).

MATERIALS AND METHODS

Cultures

Nematodes (*S. carpocapsae* UK strain and *H. megidis* UK211) were cultured in last instar larvae of *G. mellonella* (Woodring & Kaya, 1988). After harvest, IJs were washed three times by sedimentation in tapwater. IJs were stored in tapwater at 9ºC for a maximum of four weeks prior to use. *H. abietis* larvae were obtained from a laboratory culture fed on Sitka spruce. Larvae were selected on the basis of weight: only those <200 mg were used, corresponding to first and second instar (Henry, 1995; Armendáriz, unpublished data). A UK strain of *P. gigantea* (obtained from the American Type Culture Collection, ATTC no. 38030) was cultured on malt agar (Oxoid) under black light to encourage sporulation. *G. mellonella* were obtained from The Mealworm Company (Sheffield, UK).

Billet Assay

Stems of fresh Sitka spruce with a diameter of 5-7 cm were cut into pieces 30 cm in length. These billets were washed in detergent solution and inoculated with 6 ml of a spore suspension of *P. gigantea* (approx. 12×10^6 spores in 0.1% Tween). They were incubated at 20ºC for 4, 8, 12 or 16 weeks in plastic propagators (36×21.5 cm) containing moist sand (3.3 kg, heat sterilized sand moistened with 10% w/w tapwater). Billets were laid horizontally, partially buried to a depth of 2-3 cm in the sand. Fifteen first or second instar pine weevil larvae were placed in the sand close to each billet. After 24 h, 30.000 *S. carpocapsae* or *H. megidis* IJs were applied to the sand surface around the billet. The temperature was maintained at 20ºC throughout the assay. After 4 days, all bark was removed and larvae were recovered. Dead insects were dissected and the number of nematodes recovered was used as a measure of infectivity. All billets for an experiment were inoculated with *P. gigantea* at the same time, and hence the nematode assay for each fungal growth time was carried out on a different date and using a different batch of nematodes. Each assay also included pine weevil larvae in fresh timber treated with nematodes, and in fresh and *P. gigantea* inoculated timber without nematodes. The experiment was repeated three times.

Disk Assay

Disks 3.5 cm high and 4-5 cm diameter were prepared from the trunks of young Sitka spruce trees. Disks were washed and inoculated with 2 ml of a spore suspension of *P. gigantea* (approx. 4×10^6 spores in 0.1% Tween). Inoculated disks were placed individually in plastic drinking beakers ('Glacier', Rexam Plastic, Bristol, UK; 8 cm high, 5.2 cm diameter at base) with moist sterile paper in the bottom and incubated at 20ºC. Inoculations were performed at 2- or 4-week intervals so that disks with fungus at different stages of growth (2 to 16 weeks) could be used in a single assay. Assays were performed with both *H. abietis* and *G. mellonella* as the target insect.

H. abietis. A hole (5-mm diameter, 1-cm deep) was bored through the bark of fresh or fungus-colonized timber disks at a point equidistant from both cut surfaces. A single first or second instar *H. abietis* larva was placed into the hole which was then sealed with spruce sawdust. For the fungus treatments, the sawdust used to plug the insect chambers had been inoculated with *P. gigantea* spores 2-3 weeks previously. Larvae migrated under the bark and eventually continued to eat.

G. mellonella. These insects neither feed on nor migrate in timber. A hole (6-mm. diameter) was drilled horizontally through the disk to within 3-5 mm of the bark at the far side. The remaining wall of timber and bark was perforated with a needle. A single last instar wax moth larva was inserted into the cavity and the entrance was plugged with sawdust and parafin wax. The only access to the exterior was via the needle perforation.

Timber disks containing a pine weevil or a wax moth larva were placed individually in plastic drinking beakers and covered with moist sand (heat sterilized sand moistened with 10% w/w tapwater) to within 3 cm of the top of the beaker. A nematode suspension containing approx. 2000 *S. carpocapsae* or *H. megidis* IJs in 350 μl tap water was applied to the sand surface and the beakers were capped and incubated at 20ºC. After two days, the insects were recovered from the timber and incubated at 20ºC for a further 2 days. Dead insects were dissected and the number of nematodes recovered was used as a measure of infectivity.

For each nematode species, two experiments were conducted. In one of these (exp. 1 for *S. carpocapsae* and exp. 3 for *H. megidis*), there were five timber treatments: fresh timber, and timber inoculated with *P. gigantea* 2, 4, 6 and 8 weeks previously. In the second experiment for each species (exps 2 and 4), there were seven timber treatments: fresh timber, and timber inoculated with *P. gigantea* 2, 4, 6, 8, 12 and 16 weeks previously. In each experiment, *G. mellonella* larvae were also placed directly in the sand, to quantify nematode invasion in the absence of timber. There were 10 replicates per treatment in each experiment.

Statistical Analysis

Data were subjected to one-way or two-way analysis of variance (ANOVA) followed by Tukey's test for separation of means. Pairs of treatments were compared using Student's *t*-test In the disk assay, the number of nematodes recovered in insects was expressed as a proportion of the nematodes applied for statistical analysis. Proportion data were subject to the arcsine transformation prior to analysis. Minitab 13.1 was used for all statistical tests.

RESULTS

Parasitization of *H. abietis* Larvae in Fresh and *P. gigantea* Inoculated Sitka Spruce Billets

Fewer than 7% of the pine weevil larvae recovered from timber billets were alive in any of the nematode treatments, compared to 70-80% where no nematodes were applied (Table 1). There was no difference in larval survival between fresh and *P. gigantea*-inoculated timber either when no nematodes or when *S. carpocapsae* was applied, but the efficacy of *H. megidis* was affected by timber condition: 7% of the weevil larvae survived in fungus-inoculated billets compared to 2% in fresh billets (Table 1). Four times as many *H. megidis* as *S. carpocapsae* invaded weevil larvae (353 and 89 nematodes per insect, respectively, averaged over all assays).

On each assay date, a single fungal growth time was tested, together with fresh timber for comparison. Each assay was performed using a different batch of nematodes. To account for between-batch variation in nematode quality, infectivity was expressed as a fungus:fresh ratio, defined as the number of nematodes invading insects in *P. gigantea*-inoculated timber relative to the number invading insects in fresh timber on the same date. For *S. carpocapsae*, the fungus:fresh ratio was always greater than 1 (average 2.0), while for *H. megidis*, it was lower than 1.0 for each fungal growth time (average 0.76) (Figure 1). Two-way ANOVA showed that growth time of the fungus did not affect the fungus:fresh ratio ($P > 0.05$), but nematode species had an effect at $P = 0.062$.

Parasitizition of *G. mellonella* Larvae in Fresh Sitka Spruce Disks and Free in Sand

Fewer *H. megidis* invaded *G. mellonella* larvae in sand-embedded timber disks than invaded insects free in sand: 385.7 ± 60.2 in the timber treatment compared to 627.5 ± 52.2 in the sand treatment ($P < 0.01$). For *S. carpocapsae*, the reverse was observed: the number of nematodes invading insects in timber was three times higher than in free insects: 91.0 ± 11.9 vs. 28.5 ± 4.2 ($P < 0.001$).

TABLE 1. Percentage of *Hylobius abietis* larvae recovered alive from Sitka spruce billets four days after addition of entomopathogenic nematodes (*Steinernema carpocapsae* or *Heterorhabditis megidis*). Billets were either fresh or had been inoculated with *Phlebiosis gigantea* 4-16 weeks previously. Mean (±SEM) of three experiments. Within each nematode treatment, values followed by the same letter are not significantly different ($P < 0.05$; paired Student's *t*-test on arcsin transformed data)

Treatment		
Nematodes	Timber	% *H. abietis* larvae alive
S. carpocapsae	Fresh	2.3 (0.53) a
	+ *P. gigantea*	2.3 (1.17) a
H. megidis	Fresh	1.7 (0.05) b
	+ *P. gigantea*	6.9 (1.19) a
None	Fresh	78.4 (2.02) a
	+ *P. gigantea*	71.9 (5.11) a

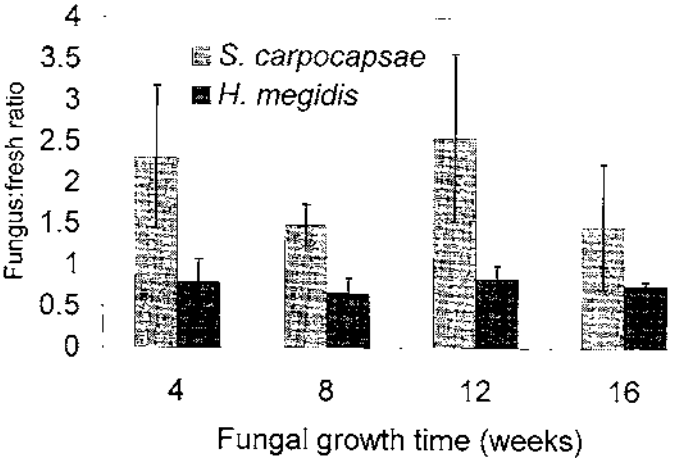

FIGURE 1. Fungus: fresh ratio: number of nematodes recovered from *Hylobius abietis* larvae in timber billets inoculated with *Phlebiopsis gigantea* 4-16 weeks previously expressed as a proportion of the number recovered from larvae in fresh timber. Timber billets exposed in moist sand to the entomopathogenic nematodes *Steinernema carpocapsae* or *Heterorhabditis megidis*.

Parasitization of *H. abietis* and *G. mellonella* Larvae in Fresh and *P. gigantea* Inoculated Sitka Spruce Disks

The main purpose of these assays was to test whether nematode invasion of wax moth larvae, which do not feed on timber, was affected by timber condition similarly to the invasion of pine weevil larvae. The results were initially analysed by two-way ANOVA, separately for each experiment, to detect effects of timber condition, insect species, and any interaction between these factors. In each of the two experiments conducted with *S. carpocapsae*, insect species had a highly significant effect on the number of nematodes recovered from the insects (Table 2), with higher numbers found in wax moth than in pine weevil larvae (Figures 2(a)-(d)). Timber condition also had a significant effect on *S. carpocapsae* invasion in experiment 1 (Table 2), where the number of nematodes in fungus-inoculated timber tended to be higher than in fresh timber for both insect species (Figures 2(a) and (c)).

TABLE 2. Effect of timber condition and insect species (*Hylobius abietis* or *Galleria mellonella* larva) on proportion of entomopathogenic nematodes infecting insects in timber disks. Disks were either of fresh timber (time = 0) or had been inoculated with the fungus *Phlebiopsis gigantea* 2-16 weeks prior to the application of nematodes. Results of 2-way ANOVA for each of four experiments

		Factor		
Nematode species	Expt no.	Timber condition	Insect species	Timber condition × insect species
Steinernema carpocapsae	1[a]	***[c]	***	NS
	2[b]	NS	***	NS
Heterorhabditis megidis	3[a]	*	***	NS
	4[b]	***	NS	**

[a]Fresh, and timber inoculated with *P. gigantea* 2, 4, 6 and 8 weeks previously.
[b]Fresh, and timber inoculated with *P. gigantea* 2, 4, 6, 8, 12 and 16 weeks previously.
[c]Asterisks indicate significant effect at $P < 0.001$ (***), $P < 0.01$ (**) or $P < 0.05$(*). NS = not significant.

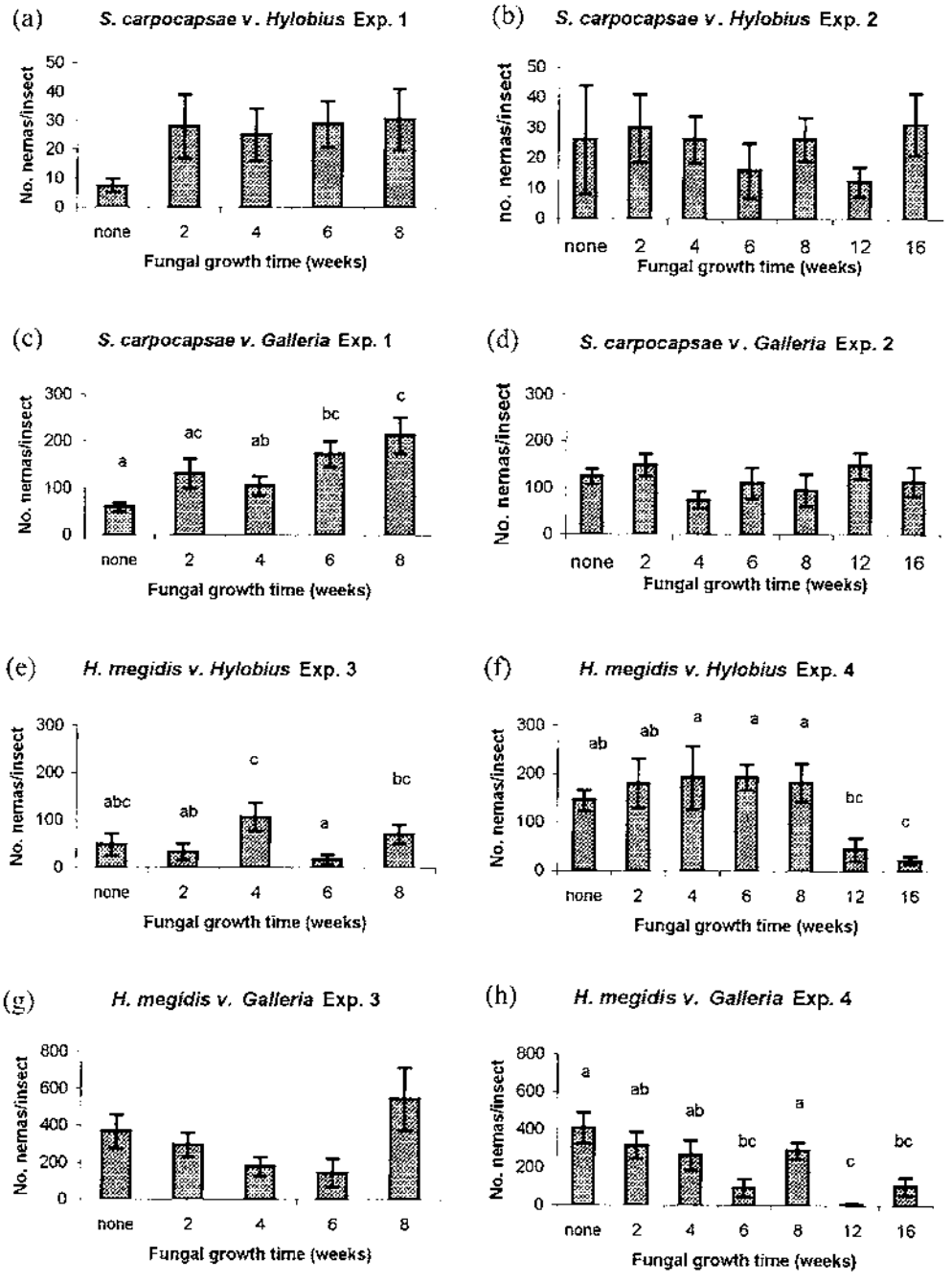

FIGURE 2. Mean (± s.e) number of *Steinernema carpocapsae* recovered from *Hylobius abietis* (a, b) or *Galleria mellonella* (c, d) larvae, or of *Heterorhabditis megidis* recovered from *Hylobius abietis* (e, f) or *Galleria mellonella* (g, h) larvae, in timber disks inoculated with the fungus *Phlebiobis gigantea* up to 8 (exp. 1, 3) or 16 (exp. 2, 4) weeks previously and exposed to the nematodes in moist sand for 48 hours. Treatments accompanied by the same or no letter are not significantly different ($P < 0.05$, Tukey's test).

In the first of two experiments conducted with *H. megidis* (exp. 3), insect species and timber condition each had a significant effect on the number of nematodes invading the insects (Table 2). In wax moths, the trend indicated a decrease in numbers invading with increasing fungal growth time for the first six weeks, followed by an increase in week 8 (Figure 2(g)). The pattern was similar in pine weevil larvae, except that the number invading after 4 weeks fungal growth time was higher than after 2 or 6 weeks (Figure 2(e)). In the second *H. megidis* experiment (exp. 4), fungal growth time had a highly significant effect on nematode invasion: in both insect species, the numbers invading were greatly reduced in timber inoculated 12 and 16 weeks previously (Figures 2(f) and (h)). There was a distinct change in the appearance of the 12 and 16 week old disks in this experiment. Small pieces were removed to malt agar plates and the resulting growth was observed. From the appearance of the timber and the results of culturing, it was apparent that other fungi (including *Penicillium* and *Aspergillus*) were replacing *P. gigantea* as the dominant species on these disks.

As was seen in the billet experiment, *H. megidis* invaded pine weevil larvae in timber disks in higher numbers than *S. carpocapsae*, averaging 102 nematodes per insect (Figures 2(e) and (f)) compared to 29 nematodes per insect for *S. carpocapsae* (Figures 2(a) and (b)). When the host was a wax-moth larva, more *H. megidis* than *S. carpocapsae* also invaded, but the difference between the species was less pronounced, averaging 249 nematodes per insect for *H. megidis* (Figures 2(g) and (h)) and 124 for *S. carpocapsae* (Figures 2(c) and (d)).

DISCUSSION

Entomopathogenic nematodes are typically found parasitising soil-dwelling stages of insects. There are no reports of pine weevil naturally infected by *Steinernema* or *Heterorhabditis*. In their natural habitat, pine weevil larvae are physically protected from natural enemies. There is also the possibility that volatiles from fresh or decaying stumps may mask the chemical indications of the insect, or deter searching parasites. In order to assess to what extent fresh coniferous timber inhibits nematode host-finding and/or invasion, a comparison was made between insect larvae free in sand and larvae within spruce disks buried in sand. Non-feeding wax moth larvae rather than pine weevils were used in this exercise in order to eliminate potential differences in attractiveness between a feeding (in timber) and non-feeding (free) insect. The timber disk, with only a small needle-made aperture providing access to the insect chamber, presented minimal obstruction to either nematode species; wax moth larvae in the timber contained 60% as many *H. megidis* as larvae free in sand, and three times as many *S. carpocapsae*. Although the distance the IJs had to travel to the surface of a free or timber-enclosed insect was approximately equal, the higher number of *S. carpocapsae* invading the latter may reflect the shorter distance the nematodes had to migrate through sand: 3.5 cm to reach the opening of the timber disks compared to 5 cm to reach the free insect; *S. carpocapsae* migrates poorly in sand compared to other species of entomopathogenic nematode (Grewal *et al*., 1994). The interior of the timber may also have provided a more suitable substrate for invasion by nictating IJs of *S. carpocapsae* than sand (Mannion & Jansson, 1993).

Invasion of insects by *S. carpocapsae* was higher in *P. gigantea* inoculated timber than in fresh timber, both in the billet assay and in one of the two disk experiments conducted with that nematode. In field experiments, entomopathogenic nematodes were reported to be more succesful at finding mature larvae and pupae than early instars of *H. abietis*, and this was attributed to the loosening of the bark by degradation and insect gallery formation in the older stumps (Brixey, 1997). Softening of bark in fungal treatments was observed in the present experiments, and this may have facilitated access by nematodes to the insects. The discovery that *S. carpocapsae* was more likely than *H. megidis* to be aided in parasitization could reflect a greater requirement of that species for physical facilitation to penetrate through bark.

In contrast to *S. carpocapsae*, there is little evidence that invasion of insects by *H. megidis* was enhanced in the fungal treatments. Indeed, invasion was lower in billets inoculated with *P. gigantea* than in fresh billets, and there was strong inhibition of *H. megidis* invasion after 12 and 16 weeks fungal growth time, associated with the dominance of other fungal species.

In addition to inducing physical changes, wood-rotting micro-organisms also alter the chemical composition of timber (Rayner & Boddy, 1988). There are two possible ways in which altered substrate chemistry might affect the infectivity of entomopathogenic nematodes: directly, through effects on IJ activity or host-finding, or indirectly, through trophic effects on the host. Trophic effects on infectivity of entomopathogenic nematodes have been reported: both the species of plant on which insects fed and the presence of endophytic fungi affected their susceptibility to entomopathogenic nematodes (Jaworska & Ropek, 1994; Barbercheck *et al*., 1995; Grewal *et al*., 1995). Pine weevil larvae were observed to feed in both billet and disk assays, and although the assays lasted no longer than four days, feeding on fungus infected timber may have altered the attractiveness or susceptibility of pine weevil to nematodes, contributing to observed effects on nematode invasion.

However, trophically mediated effects cannot have contributed to the effects of timber condition on nematode invasion of wax moth larvae. The normal diet of these insects is beeswax and honey, and moreover the last instar used in the assay had ceased feeding. The alternative explantations are physical facilitation, which may explain increased (but not decreased) nematode invasion in older timber as discussed above, or altered allelochemicals emanating from fungus-inoculated timber. There is evidence that *P. gigantea* induced changes in the quantity or composition of the volatiles emanating from infected pine, making it less attractive to pine weevil larvae (Skrzecz & Moore, 1997). A behavioural effect of allelochemicals from the decaying timber, interfering with host finding or invasion, is the most plausible explanation for reduced invasion of wax moths by *H. megidis* in older timber in disk expt. 4. In this case the reduced invasion may be attributed to the presence of *Penicillium* and *Aspergillus* which were replacing *P. gigantea*. Activation of *S. carpocapsae* by chemicals emanating from fungus-infected timber may have contributed to the enhanced invasion by this species. In the absence of exogenous stimulation, nematode IJs characteristically become inactive and remain thus (Croll, 1972). The tendency to become inactive is more pronounced for *S. carpocapsae* than for *H. megidis*, but IJs of this species could be chemically stimulated into persistent activity (Ishibashi & Takii, 1993).

Entomopathogenic nematodes are being considered as one element in an integrated approach to suppressing populations of pine weevil. In general, these nematodes are required in large doses, and an application rate of 7.5×10^9 nematodes ha^{-1} was recommended for control of pine weevil in stumps (Brixey, 1997). *H. megidis* invaded pine weevils in higher numbers than *S. carpocapsae*, but may be more sensitive to adverse effects of timber condition associated with the growth of naturally occuring or artificially inoculated micro-organisms.

Clearly, the behaviour of the two nematode species was differently affected by the condition of the timber. It is also clear that elements of the biotic environment apart from the target host can exert a significant influence on the efficiency of entomopathogenic nematodes in searching for or invading hosts, contributing to the difficulty of predicting field performance from laboratory assays.

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