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# The lethal and sub-lethal consequences of entomopathogenic nematode infestation and exposure for adult pine weevils, *Hylobius abietis* (Coleoptera: Curculionidae)

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ABSTRACT

Entomopathogenic nematodes (EPN) frequently kill their host within 1–2 days, and interest in EPN focuses mainly on their lethality. However, insects may take longer to die, or may fail to die despite being infected, but little is known about the effects of EPN infection on insects, other than death. Here we investigate both lethal and sub-lethal effects of infection by two EPN species, *Steinernema carpocapsae* and *Heterorhabditis downesi*, on adults of the large pine weevil, *Hylobius abietis*. Following 12 h nematode–weevil contact in peat, *S. carpocapsae* killed a significantly higher proportion of weevils (87–93%) than *H. downesi* (43–57%) at all concentrations tested. Less than 10% of weevils were dead within 2 days, and weevils continued to die for up to 10 days after exposure ($LT_{50}$ of 3 days or more). In a separate experiment, live weevils dissected 6 days after a 24 h exposure to nematodes on filter paper harbored encapsulated and dead nematodes, showing that weevils could defend themselves against infection. Some live weevils also harbored live nematodes 6 days after they had been removed from the nematode infested medium. Feeding by weevils was not affected by infection with, or exposure to, either species of EPN. We discuss these results in relation to the use of EPN in biological control against *H. abietis*.



## 1. Introduction

The large pine weevil, *Hylobius abietis* Linnaeus, is the major economic threat to reforestation in Europe (Leather et al., 1999; Langstrom and Day, 2004). In North America its niche is filled by two species from the same genus, *Hylobius congener* Dalla Torre (Martin, 1964) and *Hylobius pales* Herbst (Coleoptera: Curculionidae) (Lynch, 1984). It is the adult weevils that cause the financial damage, by feeding on and killing replanted seedlings (Orlander and Nilsson, 1999). This feeding not only causes seedling mortality but also reduced growth and stem deformation (Eidmann et al., 1996). It was estimated in 2004 that if pesticides were not used the cost of the resulting damage across Europe would be approximately €140 million per year (Langstrom and Day, 2004).

Female adult weevils lay their eggs on or nearby tree stumps and other recently dead or dying wood (Scott and King, 1974; Nordenhem and Nordlander, 1994; Nordlander et al., 1997). Larvae develop in the stumps, living and feeding just under the bark. They pass through four larval moults over a period of 12–36 months prior to pupation and emergence of adults (Leather et al., 1999). Clear felling a forest creates many tree stumps, which provide an excellent habitat for the development of larval *H. abietis*. Stumps remain a useable resource for *H. abietis* larvae for up to 3 years after felling (Nordenhem, 1989). Large population levels can build up in an area due to the continual provision of breeding material provided by rotational harvesting of forests within the weevils' dispersal range (Nordlander, 1987). The adults feed on the bark and cambium of live trees, commonly that of young seedlings (replants used to restock the site). Adult feeding can drastically reduce the numbers of young replants, by in some cases up to 100%, due to the high population densities of weevils on a site relative to the available feeding material (Tilles et al., 1986). Therefore, seedling death can either occur due to the sheer volume of feeding damage or when weevil damage forms a complete ring around the stem, killing the plant. Seedlings can be susceptible to weevil attack for the first 2–3 years following restocking (Petterson et al., 2005). Furthermore, adult weevils are extremely mobile, with high dispersal ability, and can therefore cause damage over a broad area (Solbreck, 1980).

Currently, the main method of control of *H. abietis* in Ireland and the UK is by the use of pesticides. These controls include the treatment of seedlings with the pyrethroids cypermethrin or α-cypermethrin. It is likely that the European Union will look to increase its current restrictions on the use of pesticides in forestry (Georgis et al., 2006). Currently, *H. abietis* is the only insect pest

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in forestry in the UK and Ireland against which pesticide is routinely applied (Willoughby et al., 2004). As a possible alternative, entomopathogenic nematodes (EPN) have been trialed for the biological control of *H. abietis* (Pye and Pye, 1985; Brixey, 2000; Brixey et al., 2006; Dillon et al., 2006, 2007; Torr et al., 2007). EPN in the families Steinernematidae and Heterorhabditidae are lethal parasites of a broad variety of insect species (Poinar, 1979). These EPN have a symbiotic association with bacteria of the genera *Xenorhabdus* and *Photorhabdus*, respectively, which are in part the cause of their pathogenicity (Forst et al., 1997). Non-feeding infective juveniles (IJs) search for insect hosts in the soil and infect the insect by entering through its natural openings, mainly the mouth, anus, spiracles and also through the cuticle (Mràcek et al., 1988; Peters and Ehlers, 1994). Once in the hemolymph the IJs release their symbiotic bacteria from their gut, which proliferate, killing the insect. The nematodes develop and reproduce inside the insect cadaver and produce more IJs that emerge from the cadaver within 1–3 weeks and search out new hosts for infection. The use of EPN against *H. abietis* has been directed at both their larval and pupal stages (Dillon et al., 2006, 2007), and their pupal and callow adult stages (Brixey et al., 2006) whilst still in the stumps, but all stages, including adults, are susceptible to EPN infection (Pye and Burman, 1978; Pye and Pye, 1985; Brixey, 2000). The population of *H. abietis* on a clearfell site 2–3 years after felling consists of the indigenous weevil population (those which developed in the stumps and survived control measures on that site) and the migrant population (adult weevils attracted into the area by nearby felling). Under sustainable forest management, as per Forest Stewardship Council guidelines, there has been a move away from large felling coupes, with smaller felling coupes preferred. While smaller felling coupes may be environmentally advantageous in terms of soil and water protection, continuous felling in a forest property may increase the size of the migrant *H. abietis* population, as volatiles are continuously emitted during the prolonged felling period. As it is not always possible to treat all felling coupes within a forest property (e.g. steep sites preclude the use of the machinery used to apply the nematodes), EPN applied in a biocontrol program should ideally kill the immature weevils developing in the stumps and also kill or reduce feeding by the migrant/emerging adult weevils. Furthermore, field trials have been conducted where seedlings and their surrounding soil were dipped in a suspension of *Steinernema carpocapsae*, which significantly reduced seedling mortality (Pye and Pye, 1985). Little consideration has been given to the effects, both lethal and sub-lethal, that EPN may have on adult *H. abietis*.

While the main aim of using EPN in biological control is to kill the target insect, infection with EPN of both adult and larval stages of insects has also been shown to have sub-lethal effects. For example, mature *Spodoptera littoralis* larvae fed with *Steinernema riobrave* had decreased rates of leaf consumption and ate fewer meals in comparison to control treatments (Alchanatis et al., 2000). Additionally, Simões et al. (2000) showed that *Galleria melonella* infected with *S. carpocapsae* had reduced silk production. Chemical insecticides also have sub-lethal effects on insects, including on *H. abietis* (Rose et al., 2006). However, it has not previously been investigated whether exposure of *H. abietis* to sub-lethal concentrations of EPN has behavioral effects on the insect. If infection/exposure with EPN were to affect the behavior of *H. abietis*, such as reducing their feeding rate, then it would make EPN an even more effective biological control agent.

Therefore, in the current study we compared the lethal and sub-lethal effects of two EPN species, *S. carpocapsae* and *Heterorhabditis downesi*, which have different foraging strategies, on adult *H. abietis*. EPN can be classified in terms of their foraging behavior, being ambush, cruise or intermediate foragers (Campbell and Gaugler, 1993). *S. carpocapsae* is classed as an ambush forager and is reported to remain near the soil surface where it attaches onto passing mobile hosts. *Heterorhabditis* spp. are classified as cruise foragers, ranging widely and responding to volatiles from sedentary hosts (Campbell and Gaugler, 1993). However, this classification should not be given undue weight, as shown by fact that *S. carpocapsae* were able to infect pine weevils within tree stumps at depths of more than 30 cm below soil surface (Dillon et al., 2006). To investigate lethal effects of EPN on *H. abietis*, we exposed adult weevils to a range of concentrations of IJs and determined the percentage mortality and speed of kill ($LT_{50}$ and $LT_{90}$ values). To investigate sub-lethal effects we tested whether exposure to and infestation by EPN affected *H. abietis* feeding rate on bark disks. In addition, to examine whether EPN could be used to protect tree seedlings against adult weevil feeding, we exposed weevils to EPN and measured the weevils feeding rate on live seedlings.

## 2. Materials and methods

### 2.1. Insects

For all experiments, adult *H. abietis* weevils were collected from pine, Sitka spruce and larch tree stumps, from sites across Ireland. Weevils were collected using emergence traps set over stumps (Dillon et al., 2006), which were emptied every 2 weeks between ca. June–November 2007. After collection, weevils were maintained in the laboratory in plastic Tupperware boxes containing a freshly cut piece of Sitka spruce branch (for food) and moist tissue paper, which were both replaced on a weekly basis, or when all bark had been consumed. Boxes were kept in a constant temperature room at 20 °C.

### 2.2. Nematodes

*S.* carpocapsae All strain and *H. downesi* K122 were produced in late instar larvae of the greater wax moth *Galleria mellonella* (L.), at 20 °C (Woodring and Kaya, 1988). Nematode-killed insects were placed on White traps. IJs were harvested daily for 3 days from the time of first emergence and harvests were pooled. Harvested IJs were washed by sedimentation in three changes of tap water and stored for up to 3 months at 9 °C. Storage was in 50 ml aliquots (2000 IJs/ml) in 9 cm diameter food containers with snap-on lids.

### 2.3. Pathogenicity of EPN for adult H. abietis

An attempt was made to simulate how weevils may be exposed to EPN in forestry biocontrol programmes, where nematodes are sprayed on and around tree stumps and adult weevils emerging from stumps would need to move through the soil, which is frequently peat-based. Weevils were buried in peat-moss compost within 50 mm × 17 mm bijou screw-cap tubes, one weevil per tube. The compost was packed so that the weevil was surrounded by soil at all times. IJs of either *H. downesi* or *S. carpocapsae* were applied to the top of the compost before the lid was closed. For each nematode species the following concentrations of IJs were applied: 500, 1000, 2000, and 4000 in 500 µl of water. In addition, a water control was included. For each nematode and concentration combination ten weevils were tested and the whole experiment was repeated three times. Tubes were placed in a constant temperature room at 20 °C for 12 h, after which the weevils were removed and placed individually in wells of 24-well multiwell tissue-culture plates, containing moist filter paper. Weevils were then checked daily for mortality, for 10 days. Data from the three replicates were pooled to calculate $LT_{50}$ and $LT_{90}$ values by probit analysis.

### 2.4. Effects of EPN on feeding by adult H. abietis: Bark disk experiment

Weevils were initially exposed to nematodes: they were placed in 24-well multiwells, one weevil per well, containing a 1 cm circle of filter paper, treated with one of five treatments: (1) 50 μl of water (no nematodes); (2) 400 IJs of *S. carpocapsae* (low concentration); (3) 2000 IJs of *S. carpocapsae* (high concentration); (4) 2000 IJs of *H. downesi* (low concentration); or (5) 10,000 IJs of *H. downesi* (high concentration). Nematodes were applied in 50 μl of water. Weevils were left for 24 h in the wells with no access to food. Trial experiments, using this experimental procedure, demonstrated that these concentrations resulted in approximately equivalent killing rates for the two EPN species, at both the high and low concentrations.

For the feeding assay, food containers (9 cm diameter by 5 cm high) with snap-on lids were filled with ca. 50 g of dry sterile sand. Disks of bark, with a diameter of 10 mm, were punched from a freshly cut Sitka spruce trunk, using a metal corer, and a single disk was placed in the center of a food container, as a food source for the weevils. One weevil was then added to each food container. Bark disks were removed and replaced by fresh ones every day and were weighed both before being put into the food containers, and on their removal. On removal the disks were also checked for visible evidence of feeding. Two experiments were conducted. In the first 40 blocks were tested, and in the second 20 blocks were tested. Each block contained one replicate of each of the five treatments plus a control treatment, which consisted of only a bark disk with no weevil in the food container. The first experiment was conducted for 5 days, with bark disks weighed every day. The second experiment was conducted over six days with bark disks being weighed only on days 1, 2, and 6. All weevils were dissected at the end of each experiment to check for live, dead or encapsulated nematodes. Experiments were conducted in a constant temperature room at ca. 20 °C under a L16:D8 light regime.

### 2.5. Effects of EPN on feeding by adult H. abietis: seedling experiment

An experiment was conducted to investigate whether exposing *H. abietis* to EPN would protect tree seedlings against adult *H. abietis* feeding, by reducing adult feeding rates. Containerised Sitka spruce seedlings, standardized to be of approximately equal size (mean height 23 cm), were transplanted into 9 cm plant pots containing peat-moss compost and acclimated for 1 week at 17 °C. Individual seedlings were treated with a suspension of 3000, 30,000, or 300,000 IJs per plant of either *S. carpocapsae* or *H. downesi*, which was applied to the surface of the peat-moss around the base of the seedling in 5 ml of water. Eidt et al. (1995) recommended an application rate of 300,000 IJs per seedling for control of *H. congener*. Control seedlings were treated with 5 ml of water. Twenty-four hours after nematode application, either 1 or 3 adult weevils were introduced into the arena. In order to ensure weevils remained at the seedling, individual seedlings were placed in an enclosure (45 cm height, 10 cm diam.), which slotted into the top of the pot. The enclosure was constructed using a 2 l plastic bottle, including a 7 cm strip of wire mesh encircling the bottle, to provide ventilation to prevent excessive moisture due to high humidity. Weevils were allowed to feed for 6 days, after which time they were removed and the area of bark consumed was measured using image analysis software (Image Pro, Media Cybernetics, MD, USA). Nine blocks were tested, and each block contained 1 replicate of each of the 14 treatments (two nematode species at three concentrations and a water control at both weevil densities).

### 2.6. Statistics

$LT_{50}$ and $LT_{90}$ values for the bioassay were obtained by Probit analysis, assuming a Weibull distribution of data, using MINITAB v. 14.0 (Minitab Inc., State College, Pa., USA). These values were calculated using only the data for insects that died during the course of the 10 day experiment, rather than for all of the insects that were exposed. Therefore, the $LT_{50}$ and $LT_{90}$ values are the times taken for 50% and 90% of individuals to die of those individuals that died within 10 days. In comparisons between nematode species, of $LT_{50}$ and $LT_{90}$ values for all concentrations, two values were considered different if their 95% confidence intervals did not overlap.

All comparisons of probability data were made using $\chi^2$ tests, with Yate's correction for continuity and Bonferroni's correction for multiple tests made where necessary. A series of GLMs was performed to compare weevil feeding, for both disk feeding and seedling feeding data, using MINITAB v.14.0. Data for the disk feeding and seedling feeding experiments were tested for normality and data found to be non-normal were transformed before analysis.

## 3. Results

### 3.1. Pathogenicity of EPN for adult H. abietis

By day 10, *S. carpocapsae* killed significantly more *H. abietis* than *H. downesi* at all nematode concentrations (Table 1). Within *S. carpocapsae* treatments highest mortality (93.3%) occurred at the 2000 IJ concentration and lowest (86.7%) at 500 IJs, although there were no significant differences between concentrations. Within *H. downesi* treatments highest mortality (56.7%) occurred at 4000 IJs and lowest (43.3%) at 500 IJs, but again within-species differences were not significant. Furthermore, in comparisons of all the EPN concentrations tested, the cumulative mortality of adult *H. abietis* was higher at each day for *H. abietis* exposed to *S. carpocapsae* rather than to *H. downesi* (shown for 500 and 4000 IJ concentrations in Fig. 1).

Probit analyses were conducted to calculate $LT_{50}$ and $LT_{90}$ values, in days, for each nematode at each concentration. Goodness of fit tests for each concentration showed that the Weibull distribution was the best fit to the data of all available distributions. Slopes of the lines did not differ significantly between EPN species for the 500 IJ ($\chi^2 = 3.8$, df = 1, $P = 0.052$), 2000 IJ ($\chi^2 = 2.3$, df = 1, $P = 0.13$) and 4000 IJ ($\chi^2 = 1.3$, df = 1, $P = 0.26$) concentrations and therefore the comparisons of different EPN were similar, regardless of the day. However, for the 1000 IJ concentration the test for equal slopes was significantly different ($\chi^2 = 4.3$, df = 1, $P = 0.04$) and therefore the comparison of different nematodes against *H. abietis* will not be similar regardless of the time.

Overall, $LT_{50}$ and $LT_{90}$ values for all concentrations ranged between 2.99–4.07 days and 4.92–6.64 days, respectively, for *H. downesi*, and 3.49–4.52 days and 6.02–7.44 days, respectively, for *S. carpocapsae* (Table 1). At a concentration of 500 EPN the 95% confidence intervals of $LT_{50}$ and $LT_{90}$ values did not overlap between *S. carpocapsae* and *H. downesi*, with the latter having significantly lower values. However, at higher concentrations all CI overlapped, indicating no significant differences in either $LT_{50}$ or $LT_{90}$ values between EPN species. Comparing $LT_{50}$ and $LT_{90}$ values for each concentration, within each nematode species, there were significant differences for *S. carpocapsae* between the 500 and 2000 IJ concentrations, for both $LT_{50}$ and $LT_{90}$ values, and between 1000 and 2000 IJ concentrations for $LT_{50}$ values, in all cases with the 2000 IJ concentration having significantly lower values. For *H. downesi* there were significant differences between the 500 and 1000 IJ concentrations for both $LT_{50}$ and $LT_{90}$ values, with the 500 IJ concentration having significantly lower values.

### 3.2. Effects of EPN on feeding by adult H. abietis: bark disk experiment

In both experiments there were no significant differences between treatments, both control and experimental, in the numbers

**Table 1**
Percentage of insects dead after 10 days, $LT_{50}$ and $LT_{90}$ values (in days) for two entomopathogenic nematode species, at four concentrations, against adult pine weevil, *Hylobius abietis*. Percent mortality was compared between nematode species at each concentration by $\chi^2$ tests.

| Nematode species | EPN conc. (IJs insect$^{-1}$) | Percent mortality at day 10 | | $LT_{50}$ ± SE in days (95% CI) | $LT_{90}$ ± SE in days (95% CI) |
|---|---|---|---|---|---|
| *H. downesi* | 500 | 43.3 | *** | 2.99 ± 0.24 (2.52 – 3.47) | 4.92 ± 0.40 (4.23 – 5.83) |
| *S. carpocapsae* | 500 | 86.7 | | 4.52 ± 0.23 (4.04 – 4.95) | 7.44 ± 0.34 (6.83 – 8.22) |
| *H. downesi* | 1000 | 53.3 | * | 4.07 ± 0.25 (3.57 – 4.56) | 6.64 ± 0.38 (5.96 – 7.48) |
| *S. carpocapsae* | 1000 | 83.3 | | 4.37 ± 0.22 (3.92 – 4.79) | 7.12 ± 0.34 (6.51 – 7.90) |
| *H. downesi* | 2000 | 53.3 | *** | 3.56 ± 0.24 (3.07 – 4.04) | 6.14 ± 0.40 (5.42 – 7.04) |
| *S. carpocapsae* | 2000 | 93.3 | | 3.49 ± 0.20 (3.07 – 3.87) | 6.02 ± 0.29 (5.49 – 6.67) |
| *H. downesi* | 4000 | 56.7 | ** | 3.71 ± 0.23 (3.25 – 4.18) | 6.16 ± 0.38 (5.49 – 7.02) |
| *S. carpocapsae* | 4000 | 90.0 | | 3.94 ± 0.21 (3.51 – 4.33) | 6.54 ± 0.30 (5.99 – 7.21) |

* $P < 0.05$.
** $P < 0.01$.
*** $P = 0.001$.

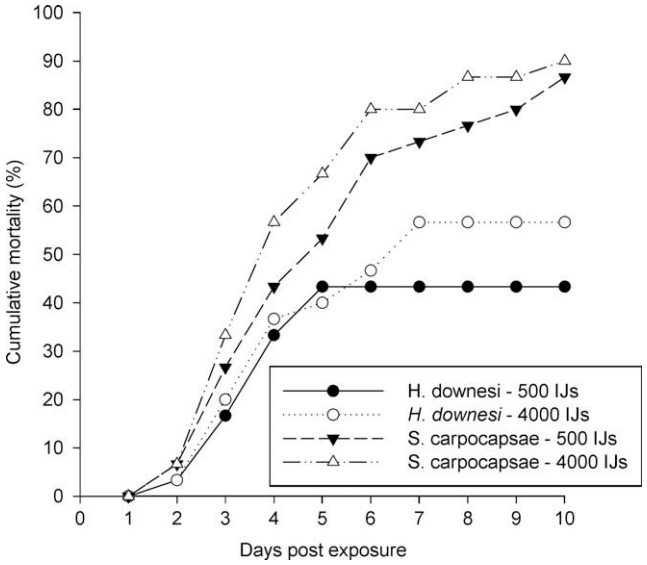


**Fig. 1.** Cumulative mortality of adult pine weevil, *Hylobius abietis*, exposed for 12 h to one of two concentrations (500 and 4000 IJs insect$^{-1}$) of *Heterorhabditis downesi* or *Steinernema carpocapsae*.

of weevils killed by nematodes (Table 2; Fig. 2A). Dissection of live and dead weevils at the end of each experiment showed which weevils had been invaded by nematodes. This allowed analysis of weevil feeding data to be conducted on either: (1) only infected weevils i.e. those weevils that at the end of the experiment contained live, dead or encapsulated nematodes on dissection (weevils could be both alive or dead at point of dissection) or (2) exposed weevils i.e. all weevils that had been exposed to nematodes. Dissection demonstrated that the pine weevils were capable of encapsulating both species of nematode (Fig. 2).

There were no significant differences between treatments in the percentage of surviving weevils, either infected (Table 3), or exposed (data not shown), which fed each day, for either of the two experiments. This indicated that neither EPN invasion nor exposure had an effect on a weevil's tendency to feed over a 24 h period, at any point, for up to a week after exposure. In general the percentage of weevils that fed increased from the first day onwards.

In the first experiment, when analyzing only weevils (live and dead) which had been infected by nematodes, the mean percentage weight loss from bark disks was significantly different between the six treatments ($F_{5,635} = 43.17$, $P < 0.001$) (Fig. 3B), and also between the 5 days ($F_{4,635} = 4.69$, $P = 0.001$) (Fig. 3A). However, there was no significant interaction between treatment and day ($F_{20,635} = 0.77$, $P = 0.75$), therefore the values collected for each day and each treatment were pooled (Fig. 3). Mean percentage weight loss of bark decreased over the 5 day period, with weight loss significantly lower on the fourth and fifth days than on the first (Fig. 3 A). In all treatments where weevils were present, weight loss was significantly higher than for the control treatment, without weevils (Fig. 3 B). The only significant difference between

**Table 2**
Cumulative mortality of adult pine weevils, *Hylobius abietis*, either not exposed to nematodes, or exposed to either *Heterorhabditis downesi* at a low (2000 IJs insect$^{-1}$) or high (10,000 IJs insect$^{-1}$) concentration, or *Steinernema carpocapsae* at a low (400 IJs insect$^{-1}$) or high (2000 IJs insect$^{-1}$) concentration. Experiments were performed over 1 week (Experiment 1: $n = 40$ weevils per treatment; Experiment 2: $n = 20$ weevils per treatment). Actual cumulative number of dead weevils, for each treatment on each day, is shown in brackets.

| Experiment | Days after exposure | Cumulative% (and no.) of *H. abietis* dead for each treatment | | | | |
|---|---|---|---|---|---|---|
| | | No nematodes | *H. downesi* low | *H. downesi* high | *S. carpocapsae* low | *S. carpocapsae* high |
| 1 | 1 | 0 (0) | 2.5 (1) | 10 (4) | 5 (2) | 15 (6) |
| | 2 | 2.5 (1) | 2.5 (1) | 12.5 (5) | 10 (4) | 22.5 (9) |
| | 5 | 2.5 (1) | 5 (2) | 12.5 (5) | 12.5 (5) | 22.5 (9) |
| 2 | 1 | 0 (0) | 0 (0) | 0 (0) | 5 (1) | 0 (0) |
| | 2 | 0 (0) | 10 (2) | 25 (5) | 15 (3) | 10 (2) |
| | 6 | 5 (1) | 25 (5) | 45 (9) | 25 (5) | 15 (3) |

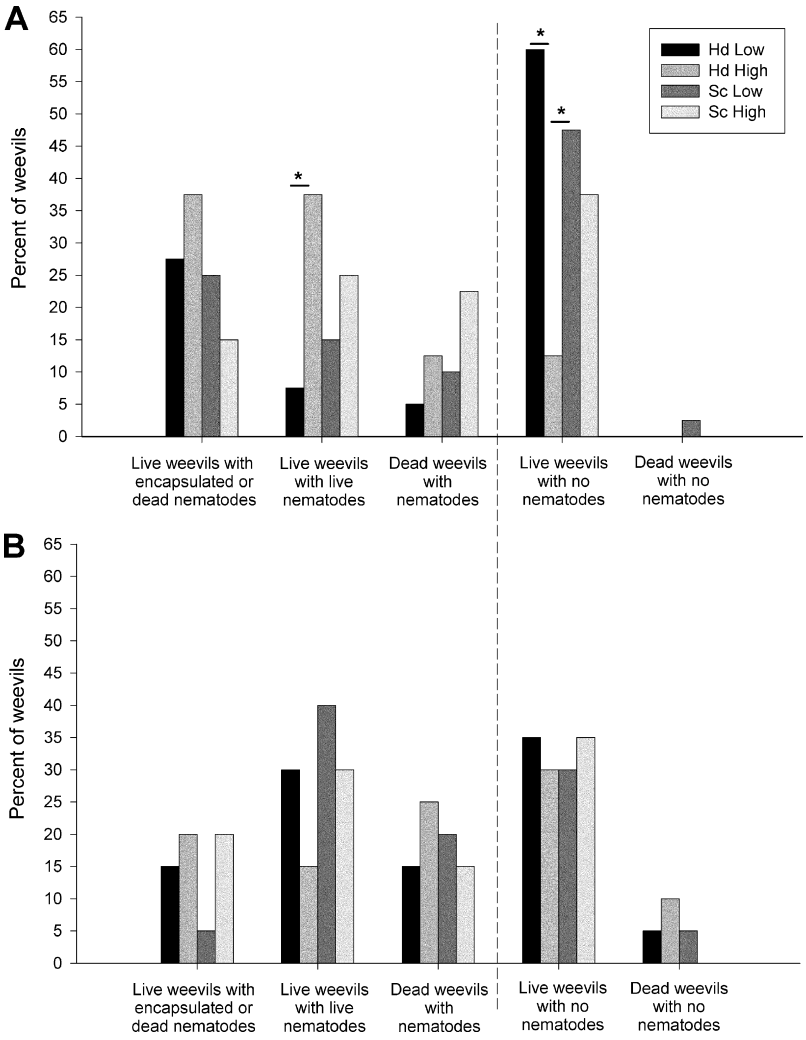


**Fig. 2.** Invasion of two entomopathogenic nematode species into adult pine weevils, *Hylobius abietis*. Weevils were exposed either to *Heterorhabditis downesi* at a low (2000 IJs insect$^{-1}$) or high (10,000 IJs insect$^{-1}$) concentration, or *Steinernema carpocapsae* at a low (400 IJs insect$^{-1}$) or high (2000 IJs insect$^{-1}$) concentration. One week after exposure to nematodes, live and dead weevils were dissected and checked for the presence of dead, encapsulated or live nematodes. Two independent experiments were conducted: (A) $n = 40$; and (B) $n = 20$. Asterisks represent a significant difference at $P < 0.05$ using $\chi^2$ tests with Bonferroni correction.

**Table 3**
The percentage of surviving adult pine weevils, *Hylobius abietis*, which fed on bark disks during any part of a day. In a control treatment weevils were not exposed to nematodes, and in experimental treatments weevils were exposed to either *Heterorhabditis downesi* at a low (2000 IJs insect$^{-1}$) or high (10,000 IJs insect$^{-1}$) concentration, or *Steinernema carpocapsae* at a low (400 IJs insect$^{-1}$) or high (2000 IJs insect$^{-1}$) concentration (for experimental treatments only data from weevils that were confirmed by dissection to have been infected by nematodes are shown in this table). Total numbers ($n$) of surviving nematode–infected weevils, for each treatment on each day, are shown in brackets.

| Experiment | Days after exposure | % of *H. abietis* feeding in each treatment (number of surviving weevils in brackets) | | | | |
|---|---|---|---|---|---|---|
| | | Control | Weevils infected by nematodes | | | |
| | | No nematodes | *H. downesi* low | *H. downesi* high | *S. carpocapsae* low | *S. carpocapsae* high |
| 1 | 1 | 60.0 (40) | 53.3 (15) | 57.1 (35) | 35.0 (20) | 76.0 (25) |
| | 2 | 60.0 (40) | 60.0 (15) | 54.8 (31) | 77.8 (18) | 84.2 (19) |
| | 5 | 89.7 (39) | 100.0 (14) | 90.0 (30) | 87.5 (16) | 100.0 (16) |
| 2 | 1 | 15.8 (19) | 9.1 (11) | 16.7 (12) | 38.4 (13) | 23.1 (13) |
| | 2 | 57.9 (19) | 45.5 (11) | 41.7 (12) | 50.0 (12) | 61.5 (13) |
| | 6 | 89.5 (19) | 77.8 (9) | 85.7 (7) | 85.7 (7) | 77.8 (9) |

weevil treatments was that the weight loss of bark was significantly lower for the *S. carpocapsae* low concentration than the *H. downesi* high concentration ($P < 0.05$).

Analysis of all nematode exposed weevils, for the first experiment, mirrored the findings for infected weevils, with significant differences between all treatments ($F_{5,905} = 52.47$, $P < 0.001$), and all days ($F_{4,905} = 9.03$, $P < 0.001$) (data not shown), but no interaction between treatment and day ($F_{20,905} = 1.04$, $P = 0.41$). Again, mean percentage weight loss of bark decreased over the 5 day period, with weight loss significantly lower on the fourth and fifth days than on the first. Weight loss was significantly higher for all weevil treatments than for the control treatment, but there were no differences between any of the weevil present treatments. The results for the two data sets (infected and exposed weevils),

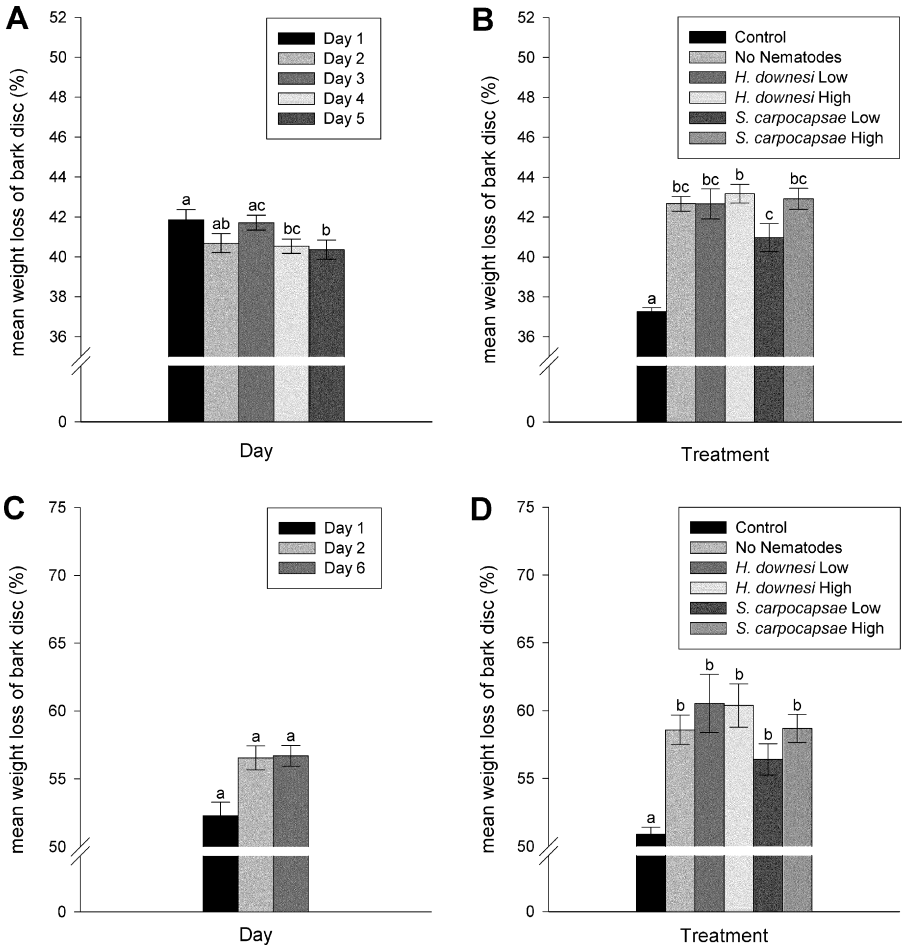


**Fig. 3.** Mean percentage weight loss of bark disks fed upon by adult pine weevils, *Hylobius abietis*, which were either not exposed to nematodes or exposed to *Heterorhabditis downesi* at a low (2000 IJs insect$^{-1}$) or high (10,000 IJs insect$^{-1}$) concentration, or *Steinernema carpocapsae* at a low (400 IJs insect$^{-1}$) or high (2000 IJs insect$^{-1}$) concentration (only data from weevils that were confirmed by dissection to have been invaded by nematodes are shown in this graph) and an unfed upon control. Experiment 1 (5 days): (A) Pooled percentage weight loss per day of all six treatments ($n$ = 117–146 per day); and (B) Pooled percentage weight loss per treatment of all 5 days ($n$ = 55 to 199 per treatment); and the second over 6 days, with bark disks weighed at the end of days 1, 2, and 6. Experiment 2 (6 days): (C) Pooled feeding rate per day of all six treatments ($n$ = 34–63 per day); and (D) Pooled feeding rate per treatment of all 3 days ($n$ = 13–60 per treatment). Within each graph, bars with different letters were significantly different by Tukey's tests to at least $P < 0.05$.

suggest that neither infection with nor exposure to nematodes had an effect on weevil feeding rate, on any day, for the first week after infestation.

In the second experiment, for infected weevils only, there were significant differences between the six treatments ($F_{5,135} = 13.03$, $P < 0.001$) (Fig. 3D), but not between the 3 days ($F_{2,135} = 2.17$, $P = 0.12$) (Fig. 3C) and there was no interaction between treatment and day ($F_{10,135} = 0.72$, $P = 0.71$). As in the previous experiment, in all treatments where weevils were present weight loss was significantly higher than for the control treatment, but there was no difference between any of the weevil present treatments. However, as in the first experiment, *S. carpocapsae* low concentration again showed the lowest bark weight loss. Comparisons of data for all weevils exposed to nematodes in the second experiment displayed similar results to those for nematode-infected: there were significant differences between all treatments ($F_{5,177} = 15.66$, $P < 0.001$), but not between all days ($F_{2,177} = 1.48$, $P = 0.23$), with no significant interaction between treatment and day ($F_{10,177} = 0.72$, $P = 0.71$).

The majority of weevils that died in these experiments contained nematodes and, in addition, 35–75% of live weevils also contained nematodes (Fig. 2). Live weevils either had dead and encapsulated nematodes or live ones, but not both. In the first experiment, there were significantly more live weevils containing live nematodes at the high, rather than the low concentration of *H. downesi* (Fig. 2A). In the second experiment it was noted that 12 weevils from all nematode treatments had large numbers of IJs clumped under their elytra and that 11 of these weevils had also been invaded by nematodes.

### 3.3. Effects of EPN on feeding by adult H. abietis: seedling experiment

At the end of the experiment mortality of weevils was recorded at 8% in the control treatment, 14% in the *S. carpocapsae* treatments and 32% in the *H. downesi* treatments. The remaining live weevils were then incubated for 2 weeks to monitor subsequent death, in total 8% of control weevils died, 24% in the *S. carpocapsae* treatment and 44% in the *H. downesi* treatment, suggesting that a fair proportion of the weevils in the treatment had become infected.

Over 6 days, a single weevil consumed a mean of 1072 ± 264 mm$^2$ of bark, while three weevils consumed a mean of 2125 ± 217 mm$^2$. In total 23 seedlings had their entire bark stripped and the mean feeding area for these seedlings was 2155 ± 130 mm$^2$. Therefore, the mean feeding recorded for three weevils during the course of the experiment was on average equivalent to almost the entire seedling (given that seedling size had been standardized). The duration (6 days) of the experiment was too short for seedling death to be evident, but in all likelihood, given that stem girdling results in death, most if not all of the seedlings fed on in this

experiment would eventually die. The area of bark consumed in the nematode treatments did not differ from that in the control treatments ($F = 0.25$; $df = 2, 120$; $P > 0.05$), but was affected by weevil density ($F = 8.56$; $df = 1, 120$; $P < 0.01$; Fig. 4). Within the nematode treatments, the area of bark consumed was affected by weevil density ($F = 14.86$; $df = 1, 96$; $P < 0.001$), but not by nematode species or concentration ($F = 2.89$; $df = 1, 96$; $P > 0.05$ and $F = 0.51$; $df = 1, 96$; $P > 0.05$). There was an interaction between nematode and concentration ($F = 3.69$; $df = 2, 96$; $P < 0.05$), but not between any of the other factors ($P > 0.05$). Therefore, these data also suggest that exposure to EPN had no effect on overall bark consumption by weevils.

## 4. Discussion

Currently, control of the large pine weevil by EPN involves applying large numbers of IJs to the soil around tree stumps, targeting all stages – larvae, pupae, and callow adults developing within the stump (Pye and Pye, 1985; Brixey, 2000; Brixey et al., 2006; Dillon et al., 2006, 2007; Torr et al., 2007). *S. carpocapsae* has been most widely used for this to date (Brixey, 2000; Brixey et al., 2006), but *H. downesi* consistently reduces number of adults to a greater extent (Dillon et al., 2006, 2007). Since nematodes kill at most 77% of the immature weevils (Dillon et al., 2007), inevitably a proportion will emerge from the stump as adults, posing a threat to newly planted seedlings. Furthermore, adults are less susceptible to EPN infestation than all other life stages (Pye and Burman, 1978; Dillon et al. 2007). Damage to seedlings could be reduced if adult weevils became infected as they emerged through nematode-infested soil and if such infection were to result in death and/or reduced feeding rate. Previously, *S. carpocapsae* has been trialed for direct protection of seedlings against *H. abietis* (Pye and Pye, 1985) and *H. congener* (Eidt et al., 1995); with this approach, nematodes are concentrated around the base of the seedling and so the weevil will only encounter them once it has reached its food supply; rapid kill and/or rapid cessation of feeding would in this case be even more critical in protecting seedlings from damage.

In our bioassay, immersion in nematode-infected soil for 12 h resulted in subsequent death of about half (43–57%) of the weevils when the nematode was *H. downesi*, and nearly all of them (83–93%) when it was *S. carpocapsae* at comparable concentrations. Weevils continued to die for up to 10 days, despite the fact that they were no longer in contact with nematodes after the first day. As shown in the seedling experiment, one weevil feeding for 6 days can consume over 1000 $mm^2$ of bark; more than enough to kill a young plant. The reason for the relatively long survival time after exposure to nematodes is not clear, but one possible explanation relates to the immune response mounted by the weevils against the nematodes. Encapsulation of IJs of both nematode species by adult *H. abietis* in the feeding assay demonstrated that the insects were capable of mounting an immune response against EPN. Encapsulation and melanisation of EPN is a common immune response amongst insects (Li et al., 2007), and melanisation of *S. carpocapsae* by the larvae of *H. abietis* has previously been noted (Pye and Burman, 1977). A proportion of the insects dissected after 5–6 days in the feeding assay were alive but harbored encapsulated or dead nematodes: these weevils may have successfully defended themselves against the nematodes and might have survived had they not been sacrificed. However, a substantial proportion of live weevils sacrificed at this time harbored live nematodes instead. Li et al. (2007) noted that nematodes may escape from encapsulation, and this could explain both the finding of live nematodes within the weevils so long after exposure to nematodes, and also the delayed death of weevils following exposure. An alternative explanation for both of these phenomena is delayed entry by IJs into the host's body. In the feeding experiment, live IJs were found under the elytra of some weevils, providing a private source of infection through the spiracles several days after weevils were removed from the nematode-contaminated environment. Adult *H. congener* with both mouth and anus sealed still became infected with *Steinernema* spp. (Eidt et al., 1995), pointing to the spiracles as a route of entry into adult *Hylobius*.

In the bark feeding experiments, neither infection with, nor exposure to, either species of nematode had an effect on whether an adult *H. abietis* fed or not on a given day, nor on the average amount consumed; nor did weevil feeding rates decline over the 5–6 day period after exposure. Furthermore, the results of the seedling experiment showed that by the end of the experimental period weevil mortality was significantly higher in the *H. downesi* treatments than in the *S. carpocapsae* or control treatments, suggesting that weevil feeding pressure was higher in the control. Despite this fact, the area of bark consumed by weevils in the nematode treatments was not significantly lower than in the untreated control. These results indicate that both exposure to and infection with either species of nematode did not have an effect on their feeding rate or total bark consumption. Insects that are infected with a lethal concentration of EPN become less active as they approach death, and Alchanatis et al. (2000) demonstrated "pre-mortal" reduction in feeding rate of *Spodoptera littoralis* larvae; leaf consumption was reduced within hours of infection by *Steinernema riobrave*, although death did not occur until 48 h afterwards. We assume that pine weevil do, indeed, cease feeding at some point before they die, but acute "pre-mortal" feeding reduction was not detected in our study. Mortality during the experiment was relatively low (rarely exceeding 25% in any treatment), and only the consumption of weevils that had survived the previous 24-h feeding period was recorded, which may have been too long an interval to detect cessation of feeding shortly before death. However, the experiment was not designed to detect this immediate pre-death cessation of feeding. What our results show is that weevils that are fighting and/or succumbing to nematode infection do not reduce their food intake. However, these data do not rule out the possibility that sub-lethal infection by EPN may affect other behaviors in *H. abietis*, for example their foraging behavior, i.e. whether they are able to locate seedlings or even their preferences for certain seedlings. Preference to plant host volatiles varies

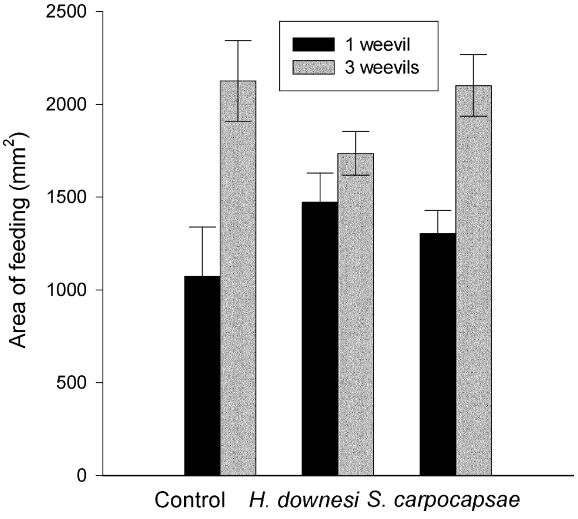


**Fig. 4.** The mean area of bark consumed by either one or three adult pine weevils, *Hylobius abietis*, on Sitka spruce seedlings that had been treated with entomopathogenic nematodes. Seedlings were treated with either *Heterorhabditis downesi* or *Steinernema carpocapsae*, and a control group of seedlings were not treated with nematodes. Weevils were allowed feed on the seedlings and the area of bark consumed was recorded after 6 days. (Control, $n = 18$; *H. downesi*, $n = 54$; *S. carpocapsae*, $n = 54$).

at different phases of the adult pine weevil life cycle (Nordenhem and Eidmann, 1991) and therefore may also be influenced by other factors. An insect's food choice may be influenced by the energetic and nutrient demands of mounting an immune response (Povey et al., 2009), possibly influencing choice of food plant.

The results of this study suggest that *S. carpocapsae* is a more effective nematode than *H. downesi* for killing adult *H. abietis*. This was confirmed in other experiments using different assay types, showing that it is not just a feature of the assay conditions. For example, in continuous exposure on filter paper, the $LC_{50}$ for *H. downesi* was approximately 200 IJs/weevil, while for *S. carpocapsae* it was less than 50 (Ennis, unpublished data). The reasons for the superiority of *S. carpocapsae* over *H. downesi* in killing adult *H. abietis* (such as behavior of IJs, differential immune response of insect, suitability of nematode/bacterial pathogenicity factors for this host) are outside the scope of this study. Although *S. carpocapsae* kills more weevils than *H. downesi*, it does not kill them substantially faster. If considering nematodes for seedling protection, a species or strain with faster speed of kill would be desirable. Three weevils feeding for 6 days can completely strip the bark form a seedling; however, a seedling can be killed by much less weevil feeding pressure if it is girdled.

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