Behavioural interactions between the large pine weevil, *Hylobius abietis* L. (Coleoptera: Curculionidae), and entomopathogenic nematodes

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Declaration of Authorship

I certify that the work presented here is, to the best of my knowledge, original and the result of my own investigations, except where explicitly acknowledged otherwise.

This work has not been submitted, either in whole or in part, for a degree at this or any other University.

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Abstract

Entomopathogenic nematodes (EPN) of the families Steinernematidae and Heterorhabditidae are a promising prospective control agent of the large pine weevil, *Hylobius abietis* L. (Coleoptera: Curculionidae), a major pest in Irish forestry. Applications of EPN to tree stumps in Ireland and the UK have been shown to

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significantly reduce adult *H. abietis* populations emerging from the stumps. To date however, little work had been done on the host-finding abilities of applied EPN in the field and how those EPN will interact with adult *H. abietis* either already on site or those that emerge from the stumps. The main objectives of this study were to investigate the behavioural interactions between two EPN species and adult *H. abietis* and to assess one of the methods used to investigate the impact of applied EPN strains on the indigenous EPN population.

To investigate if adult *H. abietis* react to the presence of EPN, one thousand nematodes were applied to the head region of adult weevils and grooming responses were recorded (Chapter 3). Insects that had *Steinernema carpocapsae* applied on average groomed for significantly longer (44.16 seconds), than those that had *Heterorhabditis downesi* applied (11.33 seconds). In order to gain an insight into the possible cause of these different insect reactions to these two EPN species observations of nematode behaviour on adult *H. abietis* were carried out (Chapter 3). Infective juveniles (IJs) of *S. carpocapsae* were more likely to be observed standing or moving across the body of the insect while *H. downesi* IJs were more likely to be observed not moving. It is thought that this difference in behaviour has caused the differences observed in *H. abietis* grooming reactions.

Adult *H. abietis* were tested to see if the presence of EPN in the substrate would alter the amount of time the insects would take to leave the area (Chapter 3). There was no difference found in the time taken to leave *S. carpocapsae* or *H. downesi* treated substrates when compared to controls. The impact of EPN presence in the substrate on *H. abietis* feeding was also investigated (Chapter 3). *Steinernema carpocapsae* was found to reduce the level of feeding on bark discs after two days when compared to controls in a choice test. This difference was not found with *H. downesi* or after two days post-application with either nematode species.

Adult *H. abietis* that had been exposed to EPN did not significantly change the amount of feeding on bark discs compared to non-exposed controls (Chapter 3). In all nematode treatments, infected weevils fed more than non-infected weevils in that treatment. Weevils that died during the assay fed more than those that did not die when exposed to a low dose of *H. downesi* but not in the other EPN treatments. As

weevils that died that were not exposed to EPN also fed more it is thought that this is not due to nematode exposure. Weevils were able to mount an immune response to both *S. carpocapsae* and *H. downesi* as encapsulated IJs of each species were found upon dissection of the exposed insects.

Foraging strategies of entomopathogenic nematodes have been the subject of several studies but most have been based on simple substrates such as agar and have not reflected the ability of certain EPN species to infect hosts in cryptic field habitats. The presence of twigs in the substrate increased *H. abietis* larval mortality by *S. carpocapsae.* Insects that were able to feed differed in their mortality from those that could not in a sand/peat mixed substrate but this difference was not found in a sand only substrate (Chapter 4). The presence of twigs or insect feeding did not alter the mortality of *H. abietis* larvae to *H. downesi* in a sand substrate.

The various life stages of *H. abietis* were tested for susceptibility to EPN infection (Chapter 4). The adult proved to be relatively insusceptible to EPN infection at low doses, with a two day exposure of 200 IJs killing none of the adult weevils exposed. Continuous exposure on filter paper at doses up to 4000 IJs resulted in a higher mortality rate. In a peat medium with an eight hour exposure mortality was different for each EPN species tested with *S. carpocapsae* killing more adult insects than both *S. feltiae* and *H. downesi* at all doses. When larvae and pupae were tested for EPN susceptibility it was found that larvae were highly susceptible to even low concentrations for both continuous and limited exposure. Pupae were found to be less susceptible than callow adults as insects that fully pupated during assays became readily infected by EPN (Chapter 4).

The amplified fragment length polymorphism (AFLP) method for assessing levels of hybridisation between EPN strains was assessed to examine the level of accuracy possible with this method (Chapter 5). A certain level of inheritance patterns of strains of known parentage was possible but this level proved too low for statistically valid examination. The AFLP method proved to be difficult to reproduce when assessing EPN gene flow.

Chapter 1

Introduction

1.1 <u>The Large Pine Weevil</u>

The large pine weevil, *H. abietis* (Coleoptera: Curculionidae), is a major pest of forests in Northern and central Europe. Under natural conditions weevils feed in the actively growing sections of mature trees, e.g. crowns of conifers (Orlander *et al.* 2000). Adults use stumps of fallen trees as a substrate for oviposition and populations were limited by the availability of such sites (Eidmann et al., 1979). Modern forestry has however provided the insect with much more suitable conditions. Large sites are clear-felled, providing the weevil population with a large number of suitable ovipositing sites. Eggs are laid on or near stumps and they then develop under the bark of stumps into adults that emerge between 12 and 36 months later (Leather *et al.*, 1999). The newly emerged adults feed on seedlings that are routinely planted on clear fell sites. It is the damage to these seedlings that causes financial losses and requires the control measures currently in use. Without control, damage of seedlings can be extensive, up to 100% mortality in some cases, with an average of 50% within 5 years in the UK (Heritage and Moore, 2001). Low levels of feeding on seedlings can be withstood and healed. Previously attacked seedlings are more likely to be attacked than undamaged seedlings although previously wounded sections of stem are less likely to be attacked, possibly due to chemical changes in resin acids brought on by feeding damage (Ericsson *et al.*, 1988). Complete girdling, i.e. removal of bark in a ring around the stem, causes seedling death but even non-mortal damage can affect the growth of the plant and quality of wood produced. Girdling is less common in larger seedlings, possibly due to a larger stem diameter increasing the amount of bark to be removed in order to girdle the seedling (Orlander *et al.*, 1999). The bark of coniferous plants is the preferred food of *H. abietis* though they will feed on other woody plants such as *Fraxinus excelsior* (Leather *et al.,* 1994) and bramble (Personal observation). Modern forestry practices in Ireland with an emphasis on large monocultures of coniferous trees, felled in blocks up to several hectares in size, have

created ideal breeding conditions for *H. abietis*. This has allowed populations to reach extremely high densities on some sites. Replanted sites in the Republic of Ireland have been estimated to have up to 65,000 adults emerging per hectare (Ward, 1991). The numbers of adults on newly planted sites is estimated to be lower than replanted sites as the lack of stumps as breeding sites means only adults travelling from off-site would be present (Wilson and Day, 1996).

1.1.1 <u>Life Cycle</u>

Adult female *H. abietis* locate suitable stumps for egg depositing by detection of host volatiles (Norlander *et al.*, 1986). Resin from newly felled conifer stumps emits terpenes and ethanol which are detected by specialised olfactory organs on the insects' antennae (Mustaparta, 1973). The females then oviposit between 60 and 120 eggs per year in niches chewed in the bark of roots of the newly felled coniferous tree (Eidmann, 1974; Novak, 1976). Eggs can also be laid in soil, usually in the upper layers (Pye and Claesson, 1981; Nordenhem and Norlander, 1994). Larvae that hatch from eggs laid in the soil can migrate at least 5cm towards roots (Pye and Claesson, 1981; Nordenhem and Norlander, 1994; Henry, 1995). Larval mortality is high with less than 10% of the population found to reach the adult stage in a study by Elton *et al.* (1965). It has also been reported that larval mortality is higher in Sitka spruce than in Corsican pine (Thorpe and Day, 2002).

Larvae are white with a highly sclerotised brown head. They develop through 4 to 5 instars before pupation (Christiansen, 1971). Larvae feed on the phloem layer under the bark in single galleries. The larvae, when developed fully, form a pupal chamber either in the bark, between the bark and the timber or in the timber itself. The type of chamber is dependent on bark thickness. The pupal stage lasts for several weeks until a callow adult is formed. This stage remains in the pupal chamber for a further 2-3 weeks before emerging from the stump as a fully hardened adult (Novak, 1976). The length of development in the stump varies depending on host species, temperature and time of oviposition. In Britain development from egg to adult has been recorded as 1-3 years (Leather *et al.*, 1999) with up to 4 years recorded in Scandinavia (Bakke and Lekander, 1965). The longer development time in Scandinavia is due to the colder

climate though the majority of adults emerge within 2 years in Sweden (von Sydow and Birgersson, 1997). While the majority of studies on *H. abietis* development have been carried out in Scandinavia, those conducted in Britain are more relevant to Ireland due to similar climate conditions. Under those conditions the time of year of oviposition influences the length of time taken for larvae to develop. Adults become active in spring (Munro, 1928) and eggs laid early in the year will hatch and develop through all their larval instars by late autumn and generally will begin to form pupal chambers before winter. The following spring these larvae will pupate and emerge as adults in the middle of summer. Eggs laid later in the summer will develop into pupae later in the following year and will emerge as adults in the autumn or the spring of the third year.

Upon emergence from stumps adults require a feeding period before they sexually mature (Orlander *et al.*, 2000, Wainhouse *et al.,* 2001; Wainhouse *et al.*, 2004). The amount consumed during maturation feeding is dependent on weevil size with larger weevils feeding more (Thorpe and Day, 2007). It is also dependent on the quality of the bark on the host plant. Weevils need to feed less on bark with high nitrogen content in order to sexually mature. As seedlings are high in nitrogen content and have fewer resin ducts than adult trees the preoviposition period for *H. abietis* that feed on seedlings is shorter (Wainhouse *et al.,* 2004). The species of tree fed upon prior to oviposition can affect sexual maturation and oviposition behaviour. Wainhouse *et al.* (2001) found that weevils fed on Corsican and Scots pine matured to the reproductive stage quicker and laid more eggs than those fed on Sitka spruce.

1.1.2 <u>Dispersal</u>

In Scandinavia and continental Europe emerging females undergo a migratory flight before they reproduce. This occurs primarily in May and June and the migration may be up to 80 km, though distances up to 10km are believed to be more common (Solbreck, 1980). *H. abietis* will only fly when wind speeds are less than 4 m/s and temperatures are above 18°C (Solbreck and Gyldberg, 1979). Males are also more likely to fly early in the flight period (Solbreck and Gyldberg, 1979). In Britain and Ireland flight is rare, as the climatic conditions are relatively uncommon (Met Eireann, Annual Report, 2007), and the main method of migration is assumed to be by walking and along with timber being transported. A study by Conord *et al.* (2006)

found that Irish weevil populations less than 200 km apart could be distinguished using genomic analysis. In other populations from mainland Europe where migration by flight is more common this distinction was not possible giving further weight to the theory that dispersal by flight in Ireland is rare. Any increase in temperatures associated with a change in climate would be likely to increase the frequency of flight in *H. abietis* and this would cause an increase in the dispersal range of this pest.

1.2 <u>Reducing damage to seedlings</u>

1.2.1 <u>Forestry practices</u>

As the amount of bark eaten by a single adult *H. abietis* in one day is large relative to the size of a young coniferous seedling it is highly unlikely that seedlings are an emerging adult's sole source of food. Other bark sources such as branches felled during thinning and other wood on site form part of their diet. Orlander *et al.* (2001) showed that the presence of fresh pine branches near re-planted seedlings significantly reduced feeding damage by *H. abietis.* These branches will eventually dry out and become less attractive as a food source for weevils so the effect is only temporary (Axelsson, 1987) and this method would not rule out damage to seedlings in the medium to long term.

Other forestry practices can also affect feeding levels on seedlings (Heritage and Moore, 2001). Stump removal was practised in many countries in order to reduce the number of potential breeding sites. This practice has largely been discontinued due to the costs involved and problems incurred due to soil compaction. The forestry industry uses a process called scarification to remove the top layer of humus around freshly planted seedlings, exposing the mineral soil underneath. This has been shown to reduce damage caused by *H. abietis* attack (Eidmann *et al.*, 1996; Orlander and Nilsson 1999; Hannerz *et al.*, 2002; Petersson and Orlander, 2003).

 The timing of replanting is also used to reduce the level of *H. abietis* damage. As the adults emerging from the stumps are thought to cause the most damage, delaying of replanting was tried in an attempt to allow *H. abietis* numbers to gradually fall back. Damage could not be eliminated as adult weevils could migrate into the area from other nearby clear-fell sites (Doom and Frenken, 1978). As the delay required could

be up to six years because seedlings are susceptible for up to 5 years this is not commercially viable for many growers (Selander, 1993). Current best-practice is to replant felled sites as soon as possible in order to allow seedlings time to establish before weevils emerge from the stumps (Schaible, 1999; Heritage and Moore, 2001).

The size of the felled compartments in a forest can influence the amount of damage suffered by the seedlings replanted in that compartment, with larger compartments having a proportionately lower level of damaged seedlings (Wilson *et al.*, 1996). This is probably due to the fact that seedlings nearer the edge are more likely to be attacked by adults migrating onto the site and in a larger compartment seedlings in the centre would have a longer distance between them and the edge. At present the emphasis on reducing compartment size would make it difficult to adopt a system of larger felling areas.

1.2.2 **Physical Barriers**

There have been other attempts to introduce forestry methods in order to reduce damage by pine weevils. One of the methods used was to introduce a physical barrier to prevent attack. Hagner and Jonsson (1995) tested two different physical barriers versus a chemical pesticide (Permethrin) for effectivity in reducing damage by *H. abietis*. A plastic felt-like mesh and a bag-like barrier were found to be reasonably effective but not as effective as the chemical barrier (Hagner and Jonsson 1995). The most common type of physical barrier is a plastic collar around the stem of the plant. A plastic sheath covered in polytetraflouroethylene, a chemical that discourages climbing by insects, was shown to reduce seedling mortality by up to 83% (Eidmann *et al.*, 1996). Other plastic shields with protective collars were not significantly different from permethrin protection in a study undertaken by Petersson *et al.* (2004). These physical barriers usually only cover part of the seedling yet they significantly reduce weevil damage on the whole seedling. This suggests that weevils simply partake of another food source, such as brash or nearby mature trees. Barriers such as these have several drawbacks however. The barriers may impede seedling development, are relatively expensive to produce and applying them to the seedlings is time consuming and subject to error that could render them ineffective (Zumr and Stary, 1995; Hagner and Jonsson, 1995; Eidmann *et al.*, 1996).

1.2.3 <u>Soil type</u>

The soil type can vary the level of attack by *H. abietis*. Adult weevils react differently to various soil types they walk across. It affects not only their locomotory behaviour, but how and when they feed. Bjorklund (2004) found that half as many seedlings were damaged when planted in mineral soil as those planted in humus. This was despite a higher number of weevils approaching those on mineral soil. He suggests that the "decision" not to attack was made close to the seedling and that lack of shelter on mineral soil could be an influencing factor.

1.3 <u>Chemical Control</u>

Hylobius abietis is the only insect pest in Irish forestry that routinely has chemical insecticides used to control it (Ward *et al.,* 1993). Early control measures included dipping and spraying of seedlings with DDT and following the ban on use of DDT lindane was used. Only 2% treated seedlings were killed by *H. abietis* attack following pre-planting dipping in lindane (Heritage *et al.,* 1988). Due to environmental concerns over its long-term persistence the use of lindane was banned in many countries and its licence for use in Ireland was withdrawn in 1999. Lindane was replaced in Irish forestry by permethrin which is more readily broken down. Concerns over permethrin's toxicity to freshwater organisms have seen it replaced by alpha-cypermethrin in recent years. Plants are treated prior to planting by dipping seedlings in the pesticide solution or by spraying post-planting.

1.4 <u>Biological Control</u>

1.4.1 <u>Natural Enemies</u>

Eight to ten parasitoids are known to parasitise *H. abietis* (Kenis *et al.,* 2004). Of these only three have been recorded on more than one occasion. Adult *H. abietis* are known to be attacked by the braconid *Perilitus areolaris* (Gerdin and Hedqvist, 1985; Stary *et al.,* 1988). Parasitism levels are difficult to estimate and range from 1-4% in

samples collected from Germany (Schindler, 1964) to 15-16% in Czechoslovakia (Novak, 1965). *Dolichomitus tuberculatus* has been reared on *H. abietis* larvae but reproduction levels were found to be low (Munro, 1929).

The ectoparasitoid *Bracon hylobii* is a natural parasitoid of *H. abietis* larvae in Ireland and other European countries (Gerdin, 1977). The level of parasitism is variable in different host plant species with up to 50% parasitism reported in Sitka spruce in Scotland but was less than 1% in lodgepole pine (Henry, 1995; Brixey, 2000). As *B. hylobii* is a gregarious parasitoid each infected insect yields several parasitoids. This coupled with the wide age range of susceptible larval stages make it a good candidate for use in a biological control programmeme for *H. abietis* (Henry and Day, 2001). When laboratory reared *B. hylobii* were released in large numbers parasitism was only increased on some of the sites tested (Henry, 1999). As *B. hylobii* is host specific to *H. abietis* larvae, that are not commercially produced, production costs are relatively high. This coupled with the low increases in parasitism found by Henry (1999) make *B. hylobii* a less promising candidate for controlling *H. abietis* populations.

Predatory beetles such as carabids are common on Irish clearfell sites (Foster, NUIM, pers. comm.). In tests carabid adults have reduced migrating *H. abietis* larval populations by up to 30% (Salisbury and Leather, 1998). As these beetles are already common and many pine weevil eggs are laid in notches in the bark it is unlikely that an increase in carabid numbers would significantly reduce weevil populations.

Entomopathogenic fungi have been shown to infect *H. abietis* larvae in the field (Gerdin, 1977). Fungi of the genus *Beauvaria* have been shown to infect *H. abietis* larvae in the laboratory (Wegensteiner, 1988) though success was lower in field trials (Wegensteiner and Fuhrer, 1988).

Wood colonising fungi can also reduce populations of *H. abietis* by reducing the amount of suitable ovipositing sites. *Plebiopsis gigantea* can render stumps unsuitable as breeding sites and can inhibit the development of larvae through degradation of the stump (Skrzecz, 1996). Fungi isolated from Sitka spruce in Ireland were shown to reduce the number of eggs laid on inoculated wood (Creevey, 1999).

1.4.2 Control of *H. abietis* using Entomopathogenic Nematodes (EPN)

Several orders of nematodes have been found to parasitise *H. abietis* larvae. Two orders, the Diplogasterida and the Allentonematida, are not thought to be pathogenic to live *H. abietis* larvae (Bovien, 1937; Pye and Burman, 1977). Members of the families Steinernematidae and Heterorhabditidae have been found to infect *H. abietis* larvae in laboratory and field trials as well as once under natural conditions (Mracek *et al.*, 1993). Several studies have been carried out on control of *H. abietis* populations in stumps using EPN (Burman *et al.*, 1979; Brixey, 1997; Brixey *et al.*, 2006).

Field trials in Ireland have reduced adult emergence from stumps by up to 87% (Dillon *et al.*, 2006). Trials in Scandinavia showed a significant reduction in the number of 1st and 2nd instar larvae in *S. carpocapsae* treated stumps compared to controls. When applied to stumps containing later instar larvae however, no significant reduction was found (Burman *et al.*, 1979). In Britain trials of EPN applied directly after felling had no effect on *H. abietis* larvae numbers in stumps but EPN applied 18-24 months after felling reduced larvae numbers by up to 96% (Brixey, 1997). Laboratory tests on *H. abietis* susceptibility have shown an LD_{50} of 9 IJs when *S. carpocapsae* was assayed against 3rd to 5th instar larvae (Pye and Burman, 1978).

Programmes using EPN to reduce damage by *H. abietis* have focussed on targeting immature stages to suppress populations rather than on plant protection from adult damage. As adult *H. abietis* can travel long distances to visit oviposition sites (Heritage and Moore, 2001) it would be desirable for any control method to affect adults migrating on to a site as well as reduce numbers emerging from stumps. Adult *H. abietis* are relatively insusceptible to EPN attack, even at high doses (Pye and Burman, 1978). In trials in Scandinavia, seedlings dipped into concentrated EPN suspensions before planting had a reduced mortality rate and adult *H. abietis* populations on site were reduced by 89% while seedling mortality was reduced from 64 to 21% (Pye and Pye, 1985). In Britain however trials by Collins (1993) showed no reduction in damage by adults using EPN treated seedlings. In trials against *H. abietis congener*, the seedling debarking weevil, in Canada it was found that an

application of 400,000 IJs of *S. carpocapsae* per seedling prior to planting reduced seedling mortality from 28% to 3% (Eidt *et al.*, 1995).

Large scale applications of EPN to suppress *H. abietis* populations have been carried out in Britain and are currently underway in various locations in Ireland (; Dillon *et al.*, 2006; Torr *et al.,* 2007). In Scotland large scale applications by UK Forestry Commission have reduced damage to seedlings by up to 90% with a 70% mortality rate in stumps. It was also observed in the areas of application that emerging adults did not feed on seedlings (S. Heritage, UK Forestry Commission, pers. comm. 2005).

1.5 Entomopathogenic Nematodes (EPN)

1.5.1 Introduction

Entomopathogenic nematodes of the families Steinernematidae and Heterorhabditidae (Nematoda: Rhabditida) are lethal insect parasites that have a history of successful use as biological control agents of several insect pests (Grewal *et al*., 2005). *Steinernema kraussei* was the first EPN to be described in 1923 with *Heterorhabditis bacteriophora* being the first heterorhadbditid described in 1975. Both genera parasitise insects using a mutualistic bacterium to aid in killing their hosts. The two genera are not thought to be closely related as shown by comparisons of ribosomal DNA (Blaxter *et al.*, 1998) and the similarities in life cycle and the method of killing their insect hosts are thought to have developed through convergent evolution (Poinar, 1993; Blaxter, 1998). *Heterorhabditis* species are believed to have arisen from marine nematodes (Poinar, 1993) a theory supported by the coastal habitats in which many *Heterorhabditis* species have been isolated (Griffin *et al.*, 1994; Liu and Berry, 1995; Griffin *et al.*, 1999).

1.5.2 Life Cycle

The free-living infective juvenile (IJ) stage is the only stage of the life cycle that is found outside the host. Infective juveniles are morphologically adapted to a non-feeding life outside the insect host. Among these adaptations are a closed mouth and anus (Poinar, 1990). The IJ stage of EPN is able to survive stress factors that are fatal to other developmental stages (Fodor *et al.*, 1990). They seek out insect hosts in soil and other cryptic substrates. The IJs invade the insect through natural openings such as the mouth or anus (Poinar, 1979), or through the insect cuticle; the heterorhabditids using an anterior dorsal hook to make an opening in the cuticle (Bedding and Molyneux, 1982) or through the use of enzymes in some *Steinernema spp.* (Peters and Ehlers, 1994; Abu Hatab *et al.*, 1995). In the insect haemocoel the IJs release their symbiotic bacteria (which are carried in the gut), *Xenorhabdus spp.* in the Steinernematidae and *Photorhabdus spp.* in the Heterorhabditidae. The bacteria then proliferate and kill the insect by septicaemia, usually within 48 hours. Antibiotics produced by the bacteria prevent secondary colonisation by other species of bacteria (Akhurst and Boemare, 1990).

The IJs then develop into adults that feed on the bacteria and degraded insect remains. *Steinernema* spp. in the first generation develop into males and females that mate and the females lay eggs. There is a clear sexual dimorphism with females being substantially larger than males (Fig 1.2). One exception to this is *Steinernema hermaphroditum* that develops self fertile hermaphrodites in the first generation (Griffin *et al.*, 2001). In *Heterorhabditis* spp. the IJs develop into self-fertile hermaphroditic females. Subsequent generations consist of distinct males and females along with hermaphrodites. These develop into adults that mate and reproduce. There may be several generations until resources are depleted. Then IJs are produced and they leave the depleted cadaver in search of a new host. The number of IJs produced varies from tens to hundreds of thousands, depending on the size and species of the insect host (Strong *et al.,* 1996).
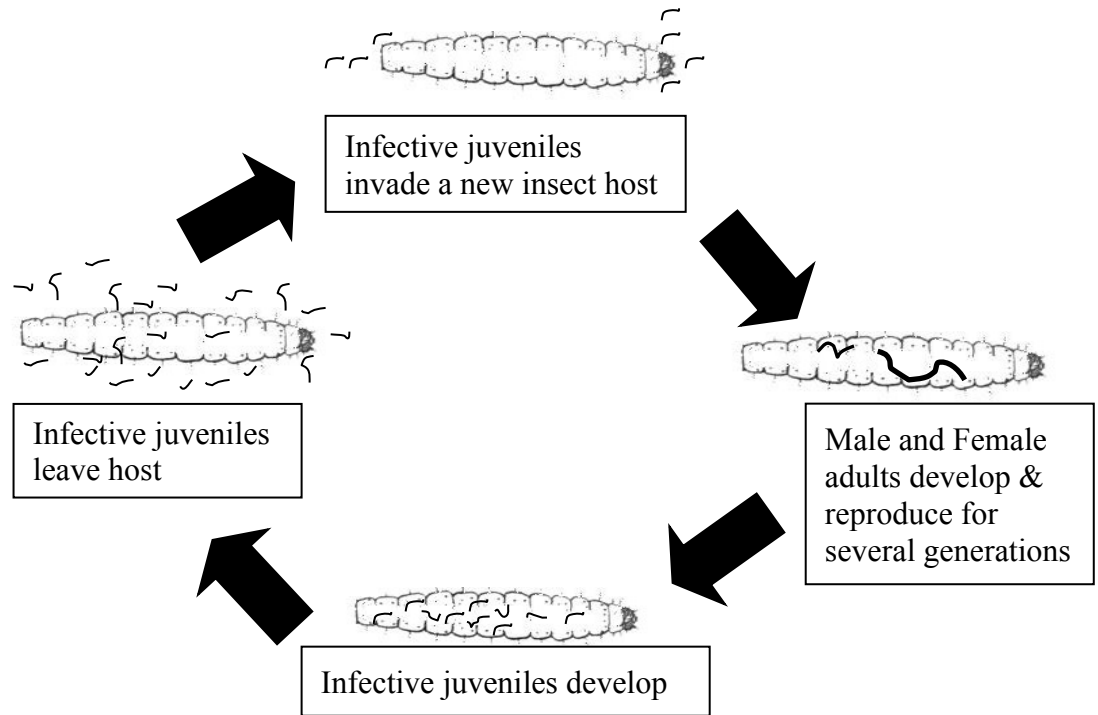
Fig 1.1a Life Cycle of *Steinernema spp.*
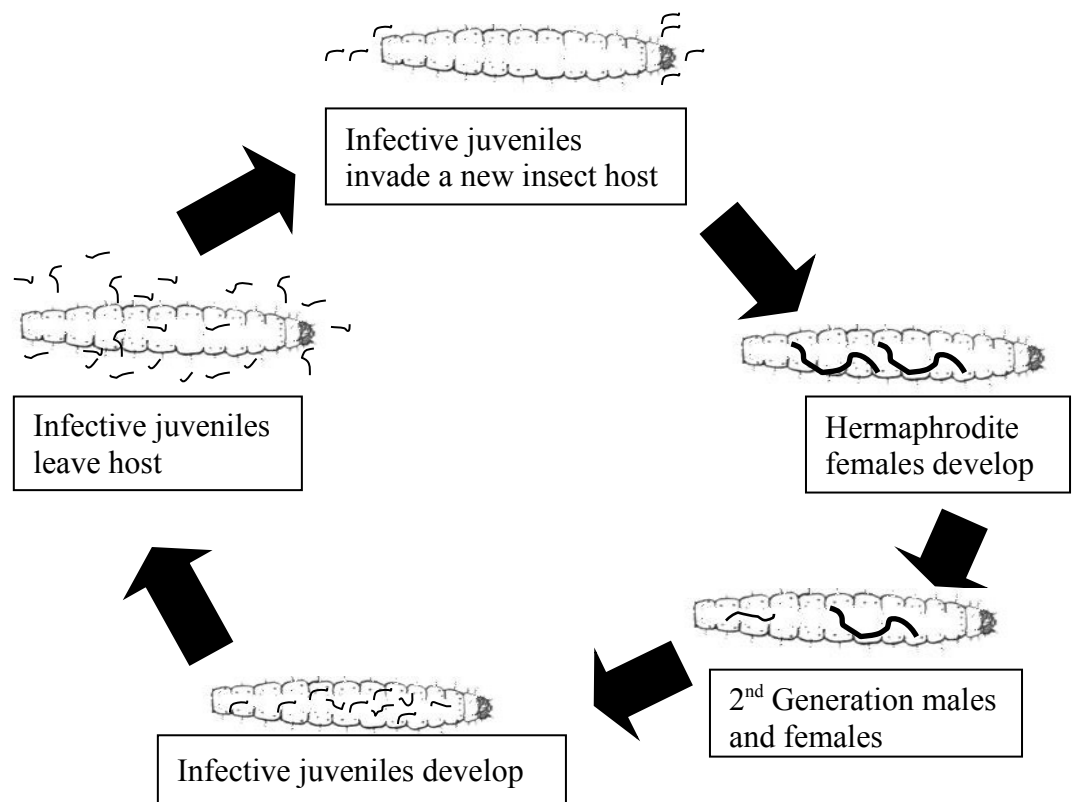
Fig 1.1b : Life cycle of *Heterorhabditis spp.*
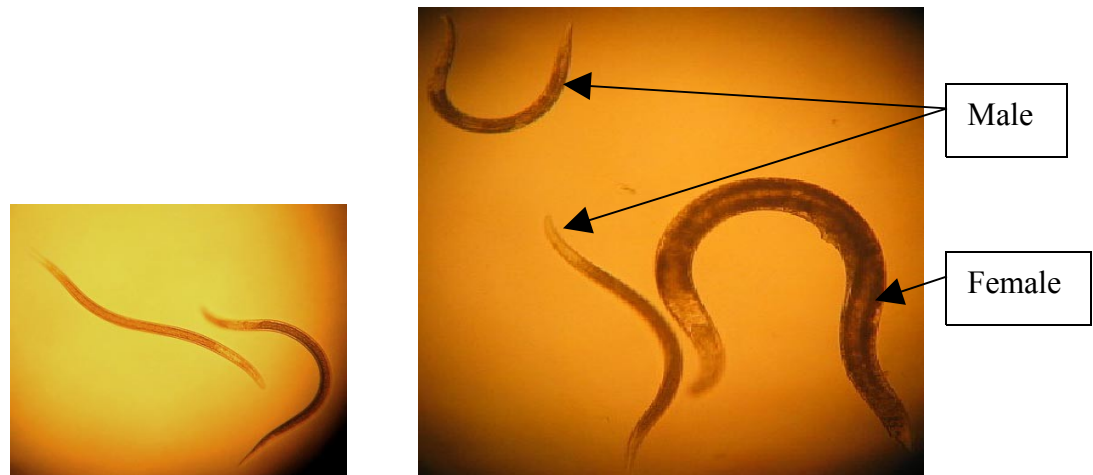
Fig 1.2 Infective Juveniles (left) and adult (right) *Steinernema feltiae.*

1.5.3 <u>Symbiotic Bacteria</u>

Both genera of EPN have mutualistic associations with bacteria. *Steinernema* spp. have associations with *Xenorhabdus* spp. and *Heterorhabditis* spp. have associations with *Photorhabdus* spp.. The nematodes provide a safe means of transport from one insect host to the other while the bacteria provide assistance in killing the insect host and supply a source of nutrition once inside the host (Akhurst and Boemare, 1990). These bacteria can be cultured in the lab and a small number of cells of *Photorhabdus luminescens* is capable of killing an insect (Gotz *et al.*, 1981). It is thought that toxins produced by the bacteria cause insect death. An example of this is a complex of toxins produced by *Photorhabdus luminescens*, known as Toxin Complex A, is thought to cause insect death after infection by *Heterorhabditis* spp. (Blackburn *et al.*, 2005). While it is possible for some EPN species to kill an insect host without their symbiotic bacteria it takes much longer (Dowds and Peters, 2002). The symbiotic bacteria can also assist the nematodes by producing antibiotic compounds that reduce the chance of re-infection by another bacterial or fungal species that would be detrimental to the development of the nematodes (Akhurst, 1982; Kaya and Koppenhofer, 1996; Boemare, 2002).

1.5.4 <u>EPN as biological control agents</u>

The first recorded use of EPN to control insect pests was in the 1930s when *S. glaseri* was applied to control *Popillia japonica*, a major pest of crops and ornamental plants, in New Jersey. As the nematodes were reared in the absence of their symbiotic bacterium the trial was not successful (Smart, 1995). Several species of EPN have been cultured commercially in different media and are sold for the purposes of insect pest control (Ehlers, 2001).

Entomopathogenic nematodes have been used to control many insect pests in a wide variety of habitats many of which cause conventional chemical pesticides to be ineffective or unavailable. In Ireland for example EPN are used to control sciarid flies in the mushroom industry where the cryptic environment of mushroom compost renders chemical control ineffective (Richardson, 1997). Their ease of use and application, low production costs and low impact on non-target organisms has made EPN a valuable means of insect pest control. The concerns of toxicity to people associated with traditional chemical pesticides during and post-application are also not a concern with EPN treatments. Health problems previously encountered with the use of chemical pesticides, such as skin hyper-sensitivity, have not been recorded after contact with EPN. Most large-scale applications can be done via traditional spraying equipment (Georgis, 1990) or with small alterations to spraying systems (Mason *et al.*, 1998) and on a small scale EPN are sold to control pests of household plants such as *Otiorhynchus sulcatus*, the black vine weevil (Personal observation).

1.5.5 <u>Distribution</u>

Entomopathogenic nematodes have a global distribution and have been isolated from every continent except Antarctica (Griffin *et al.*, 1990) and are common in soil in many habitats (Akhurst and Bedding, 1986; Hominick and Briscoe, 1990). Many individual species have localised or regional distribution with a few, such as *S. feltiae*, being found almost across the globe (Hominick, 2002) having been isolated in Britain (Boag *et al.*, 1992), mainland Europe (Steiner, 1994; Mracek *et al.*, 1999; Campos-Herrera *et al.,* 2007), the Middle East (Hazir *et al.*, 2003; Glazer, 2002), North America (Akhurst and Brooks, 1984; Stock *et al.*, 1999), South America (Stock,

1995) and Asia (Yang *et al.*, 1990). *Steinernema* spp. tend to be more prevalent in soil surveys that do not target specific soil or habitat types. *Steinernema* spp. are more numerous than *Heterorhabditis* spp. (Hominick, 2002). This increased speciation means that *Steinernema* spp. are more likely to occupy a larger number of environmental niches (Gwynn and Richardson, 1996) and thus are more likely to be found in a non-targeted survey.

Entomopathogenic nematode occurrence in Ireland has been the subject of several surveys (Blackshaw, 1988; Griffin *et al.*, 1991; Griffin *et al.*, 1994; Dillon *et al.*, 1999). Three species have been found to occur naturally in Irish soils; *S. feltiae, S. affine* and *H. downesi*. Of these *S. feltiae* is found across many soil types including forest and field soils with *S. affine* found less frequently and only found in pasture soils (Griffin *et al.*, 1991; Dillon *et al.*, 1999). *Heterorhabditis downesi* has only been found in sandy soils (Griffin *et al.*, 1991; Griffin *et al.*, 1994; Rolston *et al.*, 2005).

Despite their globally widespread distribution, EPN are recovered from a low proportion of samples in many surveys (Griffin *et al.*, 1991; Griffin *et al.*, 1994; Stuart and Gaugler, 1994; Hominick *et al.*, 1996; Dillon *et al.*, 1999; Hazir *et al.*, 2003). This irregular distribution is thought to be associated with the prevalence of insect hosts. As a suitable insect host is required for completion of the EPN life cycle any aggregated distribution in hosts will lead to an aggregated distribution of EPN. Mracek and Becvar (2000) found that targeting soil surveys to areas of known insect aggregation resulted in over 70% of sampled localities yielding samples with EPN.

1.5.6 Host Finding

Different species of EPN employ different strategies in order to find a new host. These behaviours have been classified by Lewis *et al.* (1992) as being along a continuum between an active "cruiser" and the more sedentary "ambusher" strategy. Nematodes adopting the cruiser method are thought to actively move through the soil in search of a host. These nematodes are more likely to respond to distant host cues such as carbon dioxide and other released volatiles (Burman and Pye, 1980; Lewis *et al,* 1992). Juveniles actively cruising through the soil would also be more likely to encounter non-moving subterranean hosts than an immobile ambusher juvenile.

Nematodes adopting the ambush strategy remain in the same area and adopt a "sit and wait" approach to encountering a new host. Juveniles using this approach to host-finding are most likely to encounter an actively moving host near or on the surface of the soil. One of the most common behaviours ambushers display is known as nictation. This involves the juvenile staying on the surface of the substrate and standing on its tail. The nematode then waves its body back and forth in order to attach to a passing host (Ishibashi and Kondo, 1990; Campbell and Gaugler, 1993). Ambushers are less likely to respond to distant host cues such as carbon dioxide (Lewis *et al.,* 1993). This classification of behaviours is not totally discrete with some species using a mixture of both strategies to varying degrees within their populations.

 Steinernema spp. such as *Steinernema carpocapsae* are classified as ambushers while *S. glaseri* and most *Heterorhabditis* spp. are classed as cruiser foragers. Others such as *Steinernema feltiae* are considered to be intermediate between the two types. Gaugler *et al.* (1989) suggested that a proportion of individuals in *S. carpocapsae* populations actively search for hosts with the rest of the population adopting a more passive host finding strategy. There is also some evidence that foraging strategy is dependent on conditions such as soil type, and that the laboratory results obtained on simple substrates such as agar or sand columns may not reflect what happens in the more complex soils in the field (Glazer and Lewis, 2000). The behaviour of IJs can also change over time (Dempsey and Griffin, 2002) and with decreases in lipid reserves (Patel *et al.*, 1997; Hass *et al.*, 2002). Much of the evidence supporting the classification of EPN species into certain behavioural classes is based on lab experiments conducted on surfaces such as agar and filter paper or in simple three dimensional substrates such as sand (Grewal *et al.,* 1994). It is likely that the more complex substrate of soil with differing pore space, organic content and plant roots will alter the behavioural patterns of infective juveniles. Several field trials of EPN against soil dwelling insect pests that inhabit cryptic habitats have shown that nematodes classified as ambushers are capable of infecting sedentary hosts. Trials against *H. abietis* in Ireland have shown that *S. carpocapsae*, commonly classified as an ambusher, are capable of infecting hosts up to 50 cm below the surface of the soil under the bark of tree stumps (Dillon *et al.*, 2006). It is possible that the water used to apply the EPN washed the nematodes some distance down into the soil but as insects were found parasitized across many different sites over several years it is likely that

active dispersal played a part. It was also found that a five-fold reduction in the volume of water used to apply the nematodes did not significantly affect parasitism (Dillon *et al.*, 2007).

1.5.7 Influence of Host Cues

Entomopathogenic nematode species respond differently to various cues associated with potential hosts. Lewis *et al.* (1993) showed that on an agar substrate *S. carpocapsae* responded weakly to volatile cues from *Galleria mellonella* and *S. glaseri* were strongly attracted. On a sand substrate several ambusher species, *S. carpocapsae*, *S. scapterisci* and *S. siamkayai*, showed increased jumping behaviour in response to host cues and slight air movements whereas species classified as intermediate cruise foragers, *S. ceratophorum* and *S. monticolum*, had no discernable response (Campbell *et al.*, 2000).

Carbon dioxide is believed to be one of the primary host volatiles that attract EPN. It is also thought to be a short-range attractant that assists IJs in finding the spiracles of host insects (Ishibashi and Kondo, 1990). Cruiser foragers are considered more likely to be attracted to carbon dioxide gradients as a means to locate hosts but ambush foragers are less likely to be affected by these gradients (Lewis *et al.*, 1993). Later work by Lewis *et al.* (1995) showed that *S. carpocapsae* IJs responded strongly to carbon dioxide cues only after they had come into contact with insect cuticle. The authors suggested that *S. carpocapsae* would use CO_2 emissions to locate the spiracles only after the IJs were on the body of the host and that cues in a certain order or hierarchy would elicit a response from ambusher nematodes. Other contact host cues such as faeces can cause different responses in various EPN species. *Steinernema glaseri* and *Heterorhabditis bacteriophora* responded to faeces from *Spodoptera exigua* and *Popillia japonica*, *Acheata domestica* and *Blatella germanica*. *Steinernema carpocapsae* only responded to *B. germanica* while *S. scapterisci* didn't respond to faeces from any of these insects (Grewal *et al.*, 1993).

1.5.8 Influence of Roots

Another factor often not considered in laboratory assays is the influence of roots on nematode behaviour. It has been shown presence of roots can influence the movement of entomopathogenic nematodes through soil. Some plants release chemical cues from their leaves when they are under attack by insect herbivores (Rasmann *et al.*, 2005). These 'SOS' volatile compounds attract natural enemies that then prey on or parasitise the insect. Studies such as Rasmann *et al.* (2005), Boff *et al.* (2001) and van Tol *et al.* (2001) have shown that plants release chemicals into the soil from their roots that attract entompathogenic nematodes when the plants roots are under attack. As *H. abietis* larvae develop in the stumps of felled trees it is unlikely that the plant would release specific 'SOS' compounds to attract EPN. It is likely that feeding larvae would cause the direct release of secondary compounds and other plant volatiles from the feeding site that could also attract EPN. Feeding larvae would also cause vibrations that could be carried through the wood of the stump into the soil and vibrations are known to attract IJs (Torr *et al.*, 2004). The physical presence of the surface of the root could also offer a route along which the IJs could travel. The presence of roots in soil has been shown to both positively and negatively affect the rate of host infection by EPN. Choo and Kaya (1991) showed that low density roots in a humus soil can significantly increase the infection of *Galleria mellonella* larvae by *H. bacteriophora.* The species of plant root can also influence the host finding ability of EPN. Tomato plant roots increased the infection rate of *G. mellonella* where broad bean roots in a similar system had no influence on the infectivity of EPN (Choo and Kaya, 1991). It is possible that plant roots up to a certain density increase infection rate by changing of pore size and influencing chemical makeup of soils, especially CO_2 levels, that may make roots more attractive to EPN (Cutler and Webster, 2003). Experiments that have been carried out to investigate the influence of roots on EPN host-finding have tended to use small plants such as tomato, corn and broad bean. These roots are usually small, sometimes less than 1.5g per pot (Choo and Kaya, 1991). In a felled forest system the roots are much larger and the stumps have begun to degrade. It is therefore unlikely that the roots in this system would have the same effect on soil chemistry as living roots.

1.5.9 Influence of abiotic factors on EPN

Factors such as temperature, humidity, soil pore size and aeration all influence the movement, infection and survival of EPN in soil (Shamseldean and Ab-Elgawad, 1995; Glazer, 1996; Grant and Villani, 2003). Soil texture and type play an important role in the movement and dispersal of EPN. Movement is impaired in heavy clay soils and movement impairment decreases as the percentage of silt and clay in the soil decreases (Kaya, 1990). The thickness of the organic matter on the surface of the soil can also negatively affect EPN movement into the soil after application (Georgis and Gaugler, 1991). The impact of soil moisture on EPN movement and infectivity has been widely studied. Georgis and Gaugler (1991) found that soil moisture was positively related to EPN efficacy in the control of white grubs in turfgrass. Studies such as those of Kung and Gaugler (1991), Grant and Villani (2003), Lacey and Unruh (1998) and Brown and Gaugler (1997) showed that soil moisture strongly influenced the survival, movement and pathogenicity of EPN. Temperature effects on EPN have similarly been the focus of a wide range of studies (Georgis and Gaugler, 1991; Griffin and Downes, 1994; Glazer *et al.*, 1996; Lacey and Unruh, 1998; Gouge *et al.*, 1999; Long *et al.*, 2000) that have found temperature has a major effect on the behaviour and infectivity of a wide range of EPN species. Lacey and Unruh (1998) found that mortality of host insects was widely variable between 10°C and 40°C for several EPN species.

1.5.10 <u>Influence of biotic factors on EPN</u>

Other organisms in soil can have positive or negative influences on the movement, dispersal and survival of EPN. Vertical dispersal of *Steinernema* spp. can be increased by earthworms (Shapiro *et al.*, 1993). In some cases insect hosts can be responsible for phoresis of EPN for up to two days post-infection (Lacey *et al.*, 1995; Downes and Griffin, 1996). This sort of phoresis by infected flying adult hosts could cause widespread dispersal further than the EPN would be capable of on their own (Kaya, 1990). Unintentional transport by humans is also thought to be responsible for long range dispersal of EPN in cargos and ships' ballast (Downes and Griffin, 1996).

Many soil organisms have antagonistic effects on EPN survival and dispersal. The impact of these natural enemies is largely unknown under field conditions (Kaya,

2002) but laboratory results suggest that antagonists such as nematophagous fungi may influence EPN dynamics (Koppenhoffer et al., 1996). Fungi have been the focus of most studies (Hominick, 1991; Timper and Kaya, 1992; Jaffee and Strong, 2005) with mites (Poinar, 1979; Ishibashi et al., 1987) and protozoa (Poinar and Hess, 1988) receiving relatively little attention. Nematophagous fungi are divided into two types based on the method they use to attack their nematode hosts. The nematode-trapping fungi usually consist of a network of hyphae in the soil with structures such as constricting rings, nets and adhesive traps used to catch a passing nematode (Barron, 1977). The hyphae then penetrate the body of the nematode. The endoparasitic type of nematophagous fungi do not utilise a network of hyphae but instead exist in the soil as spores that attach to or are ingested by a nematode. As IJs of EPN are non-feeding only those spores that attach to a host influence EPN.

Nematophagous fungi have been shown to reduce insect infection by EPN IJs (Timper and Kaya, 1992) but do not trap the majority of IJs emerging from infected insects (Jaffee and Strong, 2005) so are not thought to greatly impact on inundative applications of EPN to control pests.

1.6 <u>Anti-parasite behaviour</u>

Animals that act to avoid, remove or repel parasites have a greater chance of reducing the impact such parasites have on their fitness. Anti-parasite behaviours are well documented, especially in mammals such as the higher primates. Removal of ectoparasites is a very common anti-parasite behaviour. Many mammals have been studied to quantify the effect such behaviour has on fitness, wild impala for example (Mooring, 1995). Such behaviour also occurs in insects with studies showing behaviours such as grooming in bee colonies to remove mites (Zaitoun et al. 2001) and the removal of nematodes by worker termites (Mankowski et al., 2005). The latter study showed that soldier termites that were groomed by workers to remove EPN survived significantly longer than soldiers that were not groomed. Similar behavioural defences were observed in juvenile stages of *Popilla japonica* (Gaugler et al., 1994). If adult *H. abietis* displayed a similar grooming response it could reduce their susceptibility to entomopathogenic nematode attack.

Avoidance of fecally contaminated grazing areas by domestic animals has been widely studied and has been shown to reduce transmission of some endoparasites (Scantlebury *et al.*, 2004, Hutchings *et al.*, 2001). Avoidance of EPN treated areas by *H. abietis* would indicate EPN could be used as an adult deterrent as well as a biological control agent of immature stages.

1.7 <u>Assessing the impact of EPN application on native EPN populations</u>

The potential impact of inundative biological agents on the environment to which they are applied is one of main concerns of any biological control programme. The risks concerned with the release of non-native species for biological control of pests have been reviewed in several studies (Simberloff and Stiling, 1996; van Lenteren, 1997; van Lenteren *et al.*, 2003; Kimberling, 2004). The key issues that need to be addressed in a biological control programme are the effect that a control agent will have on non-target organisms and the possible dispersal and establishment capabilities of that control agent (van Lenteren *et al.*, 2003).

In the case of EPN dispersal and establishment of applied species is not as high a risk. Due to their small size and relatively low dispersal range EPN are unlikely to spread to new habitats and establish. Van Lenteren *et al.* (2003) proposed that agents such as EPN that dispersed less than 10 metres per season should not require further studies to assess the risk of dispersal of applied organisms. Establishment of applied exotic EPN is also not considered a high risk. Any establishment is usually at a low level and prevalence is often less than that of endemic EPN species (Millar and Barbercheck, 2001; Duncan *et al.*, 2003; Dillon *et al.*, 2008).

The effect applied EPN will have on non-target organisms is not as clear. Entomopathogenic nematodes have a wide host range in laboratory assays (Poinar, 1986) but this is more limited in field conditions as the conditions in the laboratory assays are not met, particularly the number of IJs reaching the host (Smits, 1996). As the populations of applied EPN drop to background levels within 5 years any impact on non-target insect populations is thought to be transient (Dillon *et al.*, 2008). Entomopathogenic nematodes and their symbionts are not considered to be a risk to

warm-blooded animals (Boemare *et al.*, 1996) possibly due to their high body temperatures being unsuitable for EPN colonisation (Schmiege, 1963), with the exception of *Photorhbdus asymbiotica* which has colonised warm blooded animals in a small number of cases (Gerrard *et al.*, 2004).

It is not safe to assume that applied species of EPN will not affect the background EPN populations as incidence of endemic species can be reduced after application of an exotic EPN species (Millar and Barbercheck, 2001). One of the risks associated with the reduction in endemic EPN species is the lessening of natural control of soil insect pests. Exotic species that impact on natural enemies of pests that are adapted to local conditions (Bennett, 1993) and this could have long term consequences on the local environment.

Inundative application of EPN can cause reductions in the diversity of the existing soil-dwelling nematode community. Somasekhar *et al.* (2002) showed that applications of *Heterorhabditis indica* and *H. bacteriophora* reduce the abundance and the number of genera of plant-parasitic nematodes. As the application of nematodes to control *H. abietis* involves the application of millions of IJs of one species to each stump it is possible that the makeup of the nematode community could be affected. The concern that the nematode populations in the soil could be drastically altered increases when exotic species are used and it is recommended that indigenous species should be used where possible (Ehlers and Hokkanen, 1996). It is also of concern when exotic strains of species already in the soil are applied. Different strains of the same EPN species can differ in virulence, reproductive capacity and tolerance to local environmental conditions (Somasekhar *et al.,* 2002).

Identification of different EPN species by morphological techniques is difficult and requires a high level of expertise and can often be carried out only on adults (Nguyen and Smart, 1996). Intraspecific morphological identification of strains is much more difficult and strains can be indistinguishable (Stock *et al.,* 2002). In recent years molecular methods for identification of EPN species and strains have been widely applied (Grenier *et al.*, 996; Reid *et al.*, 1997; Blouin *et al.*, 1999; Rolston *et al.*, 2006). Several of these methods also have the added advantage of enabling estimates of gene flow between native and applied strains. Dillon *et al.* (2008) showed that 60

months after application of a native and exotic strain of *S. feltiae* that EPN recovered from the application site were more similar to the genetic profile of the native strain.

1.7.1 <u>Molecular Methods for Analysis of Entomopathogenic Nematode Populations</u>

There are several methods that would allow an analysis of the genome of different EPN strains to assess gene flow between applied and indigenous populations. These include sequencing sections of the genome, randomly amplified polymorphic DNA (RAPD) analysis, microsatellite analysis and amplified fragment length polymorphism (AFLP) analysis. Sequencing sections of the genome was rejected for this project due to the high costs involved. RAPD analysis while simple to carry out has been shown to have a low reproducibility (Penner *et al.*, 1993; Jones *et al.*, 1997). The AFLP method was selected due to its relatively low costs, previous use on nematode populations and the fact that no knowledge of the nucleotide sequence is required in advance. This method has also been found to be highly reproducible (Huys *et al.*, 1996; Janssen *et al.*, 1996). The use of microsatellites is also highly reproducible (Jones *et al.*, 1997) but as the AFLP technique had been used successfully in similar tests of EPN samples (Rolston *et al.*, 2006; Dillon *et al.*, 2008) it was deemed the most suitable technique to use.

1.7.2 <u>The Amplified Fragment Length Polymorphism Method of Genetic Analysis</u>

Amplified fragment length polymorphism (AFLP) PCR was developed by Vos *et al.* (1995) and can be used to detect polymorphisms across the entire genome. The process consists of several stages. In the first stage two restriction enzymes are used to digest the DNA. The EcorI enzyme has a 6 bp recognition site and cuts more rarely than MseI which has a 4 bp recognition site. Then AFLP adaptors are ligated on to the ends of the DNA fragments in the presence of the restriction enzymes, thus ruling out fragment to fragment ligations. The adaptor end sequences of each fragment act as priming sites for the subsequent PCR reactions. The ligated fragments are then

amplified in the pre-selective amplification stage. Primers with a single selective nucleotide are used to generate a large number of fragments. The selective amplification stage uses two new primers with between one and three selective nucleotides. The EcorI primer is also labelled. The PCR products are then separated using a gel buffer in a glass capillary and the fluorescence of the labelled primer is read using a laser. The number and size of fragments that are selectively amplified is dependent on the frequency of cutting by the rare-cutting restriction enzyme and by the numbers of bases of the selective primers. The AFLP method has several advantages over other genetic analysis techniques. Primary among these is that no prior knowledge of the genome is required as the digestion is performed across the whole genome.

The AFLP technique is most commonly used to assess plant genomes but several studies have been carried out using this method on nematode populations. Many of these studies have investigated populations of plant-parasitic nematodes such as *Meloidogyne* spp. (Semblat *et al.,* 2000; Roze *et al.*, 2002; Fargette *et al.*, 2005) and *Heterodera* spp. (Kaplan *et al.,* 1999; Wang *et al.,* 2001). To date relatively few studies have used AFLP to analyse EPN populations (Hominick *et al.,* 1999; Rolston *et al.,* 2006; Dillon *et al.*, 2008).

Different strains of the same species would be expected to have similar coding regions of DNA. Non-coding regions in the model organism nematode, *C. elegans*, are thought to consist of up to 76% of the genome (Thomas, 2008). As mutations in these regions of the genome do not alter fitness they are thought to not be affected by selective pressure. This "Neutral Theory" of molecular evolution was first put forward by Kimura (1968). Under this model strains of nematodes whose populations are not interbreeding will develop different nucleotide sequences in their non-coding regions. By using the AFLP technique the different strains and hybrids should present distinct profiles. Hybrids should present sections of their profiles with similarities to both parent strains. Thus this method should allow us to identify strains and to determine if hybridisation is detectable.

1.8 Project Objectives

During large scale field trials of EPN against immature stages of *H. abietis* the level of parasitism achieved was higher than may have been predicted by studies of EPN foraging strategy in lab conditions. The cryptic nature of the stump habitat coupled with the peaty soil commonly found in northern European represent a difficult medium for successful control by EPN. The high levels of parasitism found during these trials suggest that the host-parasite interactions are more complex than previous behavioural studies would predict. It has also been suggested that damage caused by adults on site is greatly reduced after large-scale EPN application. In this thesis I will address the questions raised from previous field-based trials by addressing the following issues:

Assess the impact of EPN presence on adult *Hylobius* roosting and feeding by investigating the behavioural interactions between adult *H. abietis* and two species of entomopathogenic nematodes *Heterorhabditis downesi* and *Steinernema carpocapsae*. This will give an insight into the effect, if any, EPN have on adult weevil behaviour after EPN are applied to stumps.

Examine the relative susceptibility of the various life stages of *H. abietis* to infection by entomopathogenic nematodes. This will allow us to understand more clearly if high mortality found in field trials could be due to immature *Hylobius* becoming more susceptible to EPN infection over time as they metamorphose into the various insect life stages.

Assess the influence of substrate type and physical routes in the substrate on the infection by entomopathogenic nematodes of *H. abietis* larvae. Much of the expected behaviour and infectivity of EPN is based on simple substrate assays and it is possible that other factors in the field, such as roots and soil type, could alter the efficacy of EPN in infecting *Hylobius*.

Evaluate the efficacy of the amplified fragment length polymorphism method to assess hybridisation in *Steinernema feltiae*. This would illustrate how useful a method AFLP is in detecting whether applied strains of EPN become established in the soil post-application, one of the chief environmental concerns of biological control programs.

Chapter 2

General Materials and Methods

2.1 <u>Nematodes</u>

2.1.1 <u>Origin of Nematode Isolates</u>

The *Steinernema feltiae* used were the 4CFMO strain part of stock cultures in NUI Maynooth, isolated from a clear-fell forest site in Co. Mayo, Ireland by Dr A. Dillon; the UK 76 strain originally obtained from Microbio Ltd., Cambridge, England and the EN02 strain, obtained from Enema GmbH, Raisdorf, Germany. The *Heterorhabditis downesi* used were the K122 strain, part of the stock culture in NUI Maynooth, isolated from a sandy coastal site in Co. Wexford, Ireland by Dr C.T. Griffin. The *Steinernema carpocapsae* used were US-S-25 strain originally obtained from Koppert Ltd., Berkel en Rodenrijs, The Netherlands.

2.1.2 <u>Maintenance of Nematode Cultures</u>

The nematodes used on this project were reared and maintained through *Galleria mellonella* larvae obtained commercially from the Mealworm Company, Sheffield, UK and stored at 15°C. Approximately 20 *G. mellonella* larvae were placed in a 9cm Petri dish with 9cm filter paper (Whatman no. 1) in the lid and base. A suspension of EPN infective juveniles (1000 IJs/ml) was pipetted evenly on to both sheets of filter paper. The dishes were then incubated at 20°C.

2.1.3 <u>Harvesting and Storage of Nematodes</u>

Four days after application any insect cadavers displaying the characteristic colouration and consistency of EPN infection were placed into a 9cm Petri dish with damp tissue paper and incubated at 20°C. After 6 days for *Steinernema* and 12 days for *Heterorhabditis*, infected insects were transferred to White traps after White (1927). Traps consisted of a 15cm Petri dish containing a 5.5cm Petri dish lid covered with a sheet of 9cm filter paper (Whatman no. 1). The 15cm dish was filled to half the height of the 5.5cm lid with tap water. Infected insects were placed on the filter paper and emerging IJs were collected in the water.

Collected IJs were washed three times by sedimentation in tap water. This was repeated daily for four days after the first emergence. Harvested IJs were stored at concentrations of 2000 IJs/ml. Aliquots of 50ml were stored in 9cm plastic food containers with sealed lids at 9°C.

Plate 2.1 Infected *G. mellonella* on a White trap.

Filter paper

G. mellonella larva

5.5 cm Petri Dish lid

Tap Water

15 cm Petri dish base
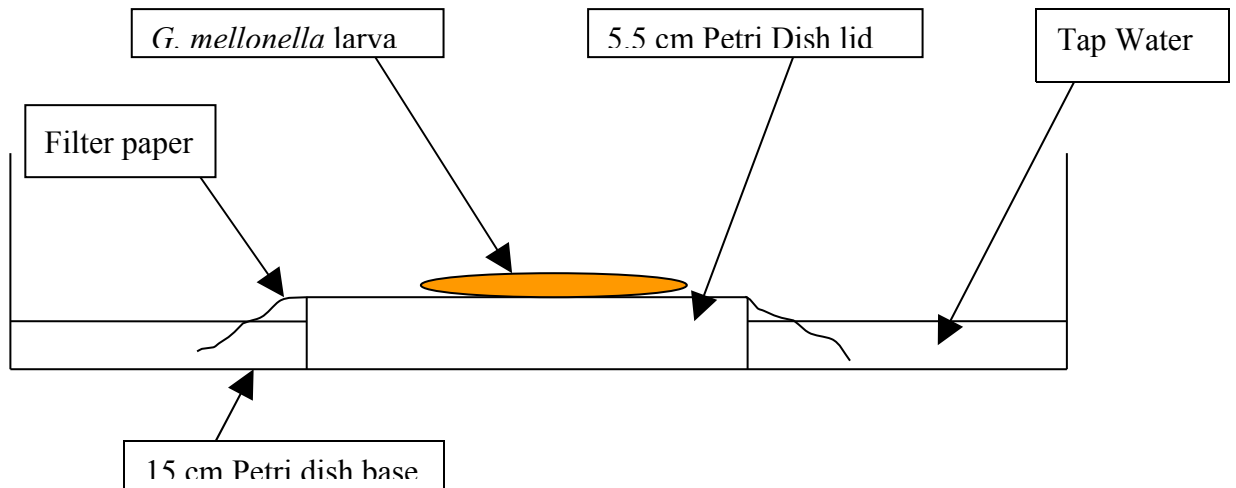
Fig 2.1: Diagram of White trap used to collect emerging nematodes

2.2 *Hylobius abietis*

2.2.1 Collection of Larvae and Pupae from Stumps

The *Hylobius* larvae and pupae used in experiments were collected from pine and Sitka Spruce stumps in Ireland. Sites between 12 and 18 months after felling were used. Soil from around the stumps was cleared away and the bark removed. Insects were collected from feeding galleries or pupal chambers using blunt-nosed forceps. Larvae and pupae were stored singly in wells of 24-well tissue culture plates. The lids of the plates were lined with moistened tissue paper and insects were stored at 9°C for up to one week until required.

2.2.2 Collection of Adult *Hylobius abietis*

Adult *H. abietis* were collected from replanted clearfell sites in Ireland. Insects were either directly collected while feeding on seedlings or caught in traps as they emerged from stumps. Traps consisted of a barrier of black cloth erected over the stump on bamboo poles. At the top of the barrier a pitfall trap collected the adult *H. abietis* as they emerged from the stumps (Dillon *et al.*, 2006). Adults were maintained in closed ventilated plastic boxes at 20°C with freshly cut conifer billets (short sections of

conifer trunk with the branches removed) and moist sand (8% w/w) to a depth of approximately 3 cm. Billets were replaced weekly.

Pitfall trap

Bamboo pole

Cloth barrier

Tree stump
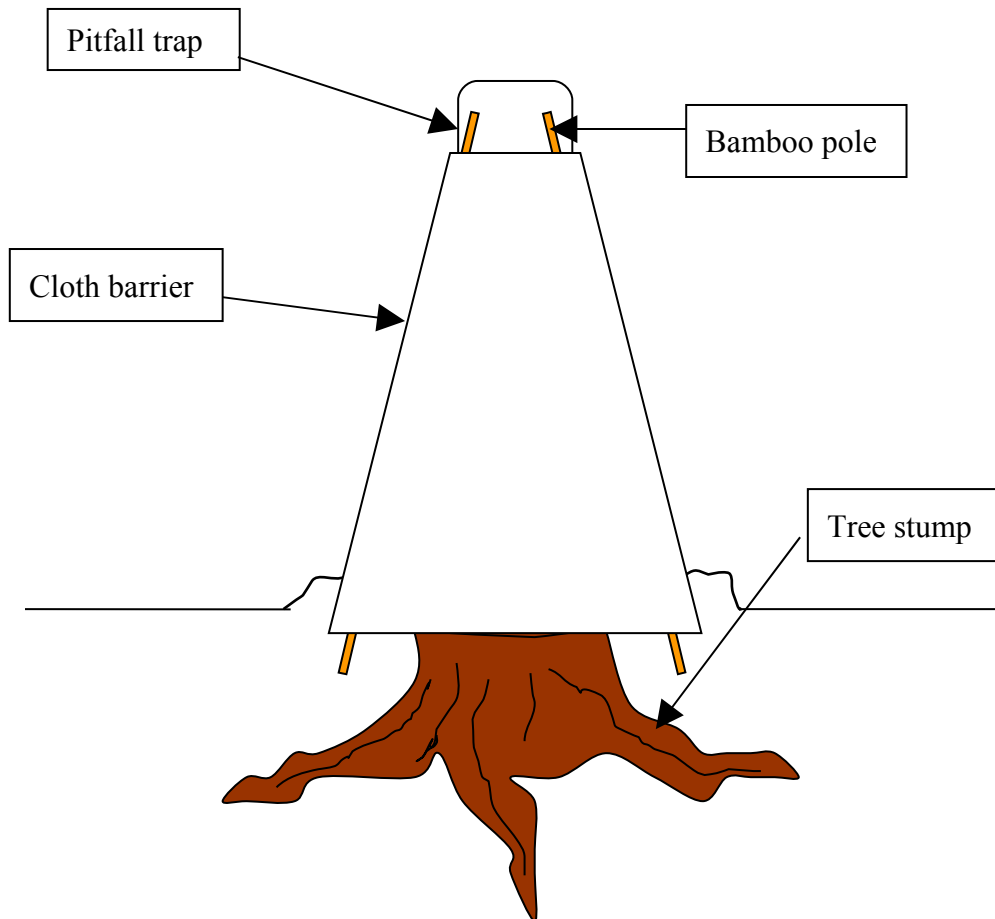
Fig 2.2: Diagram of a trap used to collect adult *Hylobius* emerging from stumps

Chapter 3.

BEHAVIOURAL INTERACTIONS BETWEEN ADULT *Hylobius abietis* AND EPN

3.1 <u>Introduction</u>

Coniferous forests in northern Europe are commonly replanted with seedlings soon after felling. The seedlings are routinely sprayed with insecticides (Chapter 2) to control populations of the large pine weevil, *Hylobius abietis*. Without control measures losses of seedling crops can be very severe with up to 100% loss in some cases (Heritage and Moore, 2001). In Ireland and the UK large scale trials of entomopathogenic nematodes against *H. abietis* are being carried out in an attempt to suppress overall populations and thus reduce damage. This control method does not attack the damage-causing adults but rather focuses on the immature stages developing in the stumps. As adult *H. abietis* are capable of migrating relatively long distances to find breeding sites (Solbreck, 1980) it is possible that significant damage could be caused by weevils entering the site from outside. Flight in adults is relatively rare in Ireland as the climatic conditions required for flight, temperatures exceeding 18°C combined with wind speeds of less than 4 m/s, are rare (Met Eireann Annual Report, 2007). However it is likely that changing climate conditions could make the required temperatures more common. Adult *H. abietis* are relatively insusceptible to EPN infection (Pye and Burman, 1985) and are unlikely to visit the stumps to which EPN are applied so it is unlikely that EPN applications would control adults migrating on to the site as they would not be expected to visit the stumps to oviposit as they would be too degraded by the time of EPN application. It is likely that large scale applications of EPN to stumps will infect weevils as they develop and emerge from stumps as callow adults are highly susceptible to infection (Chapter 4). However it is well documented that even parasites at sublethal doses can have diverse effects on animal behaviour. Anti-parasite behaviours are well documented, especially in

mammals such as the higher primates (Hutchins and Barash, 1976; Waal and Roosmalen, 1979; Franz 1999). Some insects have aggressive defences against potential parasites such as stemborers attacking and sometimes killing the braconid parasitoid wasp *Cotesia sesamiae* (Potting *et al.,* 1999). Removal of ectoparasites is a very common anti-parasite behaviour. Many mammals have been studied to quantify the effect such behaviour has on fitness, wild impala for example (Mooring, 1995). Such behaviour also occurs in insects with studies showing behaviours such as grooming in bee colonies to remove mites (Zaitoun *et al.* 2001) and the removal of nematodes by worker termites (Mankowski *et al.*, 2005). The latter study showed that soldier termites that were groomed by workers to remove EPN survived significantly longer than soldiers that were not groomed. Similar behavioural defences were observed in juvenile stages of *Popilla japonica* with the insect larvae seen to brush their bodies to remove nematodes. This activity significantly reduced the rate of parasitism by EPN (Gaugler *et al.,* 1994). If adult *H. abietis* displayed a similar grooming response it could reduce their susceptibility to entomopathogenic nematode attack. As the different species of EPN have different movement and prey-seeking behaviours two different species were tested and both the EPN and *H. abietis* behaviour was monitored.

The two EPN species used in these experiments, *S. carpocapsae* and *H. downesi*, have different behavioural patterns. The foraging strategies of EPN have been defined by Lewis *et al.* (1992) as being along a continuum between a "sit and wait" ambushing strategy or a more active cruising one *S. carpocapsae* is thought to adopt an ambush strategy in its host finding while *H. downesi* is considered a more active host-seeker. There is some evidence that any behavioural strategy is subject to the age and lipid reserves of the IJ (Lewis *et al.*, 1995; Dempsey and Griffin, 2002; Haas *et al.,* 2002). It is thought that ambushing strategies are subject to a trade off and with increasing time and decreasing energy reserves the IJ is more likely to switch to actively seek out a host. Successful large scale parasitism by *S. carpocapsae* of hosts like *H. abietis* larvae and pupae that occupy cryptic habitats (Dillon *et al.*, 2006, Brixey *et al.*¸ 2006; Torr *et al.*, 2007) also suggest that the foraging strategies are subject to conditions and that species classified as ambushers may act like cruise foragers under certain circumstances. Torr *et al.* (2004) showed that *S. carpocapsae, S. feltiae* and *H. megidis*, three EPN species classified as an ambusher, a cruiser and an intermediate

strategist respectively, all moved through sand towards vibrations. The different host-finding and behavioural strategies displayed by the EPN would affect how they and the adult *H. abietis* interact.

One of the most common anti-parasite behaviours is for the potential host to avoid areas of high parasite populations. Avoidance of fecally contaminated grazing areas by domesticated animals has been widely studied and has been shown to reduce transmission of some endoparasites (Scantlebury et al., 2004, Hutchings et al., 2001). *Popilla japonica* larvae migrate away from the application point of EPN in soil (Schroeder *et al.,* 1993). Slugs have also been shown to avoid soils containing juveniles of *Phasmarhabditis*, a slug parasitic nematode species (Wilson *et al.*, 1999). Avoidance of EPN treated areas by *H. abietis* would indicate EPN could be used as an adult deterrent as well as a biological control agent of immature stages. As the economic damage caused by the adults is to the replanted seedlings, and those seedlings are commonly replanted in amongst the EPN treated stumps any deterrent effect would be desirable. It is unlikely that the seedlings provide the sole food source for the adult weevils and crowns of nearby mature trees and other sources could be utilised causing no significant economic losses (Orlander *et al.* 2000). Infection by EPN could also influence feeding behaviour of pine weevils. Parasitism by a parasitoid, *Apophua simplicipes*, caused a significant decrease in feeding by the banded leafroller (Cossentine *et al.,* 2004). This is a desirable consequence of any biological control system as the amount of damage caused by the pest is decreased before the control agent has reduced pest populations to the desired level. Conversely other studies have shown that parasitism can actually increase the level of feeding by the pest species in the short-term such as in the case of insects infected with a gregarious parasitoid (Slanky, 1986).

In this chapter the behaviours of both EPN and adult *H. abietis* are investigated. Attention is focused on how the two interact with each other. How *H. abietis* reacts to the two different nematode species and any differences in those reactions are addressed. Several key questions are asked:

(1) Do *H. abietis* notice the presence of nematodes on their bodies and if so do they alter their behaviour in response to EPN presence?

(2) Do *H. abietis* react differently to different species of EPN on their bodies? If so, do *S. carpocapsae* and *H. downesi* have different behaviour patterns while on the body of a *H. abietis* host that would make them more likely to be noticed by the weevil?

(3) Does EPN presence in the substrate cause a change in the amount of time spent by the insect on that substrate?

(4) Does EPN presence in the substrate influence *H. abietis* feeding patterns? Will the EPN presence cause a reduction in *H. abietis* feeding?

(5) Do EPN exposed *H. abietis* have different feeding patterns? Does being infected by EPN change the amount they feed? Do different exposure concentrations or different EPN species have differing effects on *H. abieitis* feeding?

(6) Can *H. abietis* mount an immune response to EPN invasion and does this immune response influence feeding behaviour?

3.2 <u>Materials and Methods</u>

3.2.1 *Nematodes*

Two species of nematodes were used, *S. carpocapsae* and *H. downesi*. Nematodes were cultured through *G. mellonella* larvae as described in Chapter. IJs were stored at 9°C at a concentration of 2000 IJs/ml. All nematodes used were between three and five weeks after emergence.

3.2.2 *Insects*

Adult *Hylobius abietis* used were either collected in traps as they emerged from stumps or collected from seedlings on replanted clearfell sites in Ireland as described in Chapter 2. Weevils were stored in ventilated boxes at 20°C and fed with fresh conifer billets for up to three weeks before use. Crickets used were 4th instar *Achaeta domestica* supplied by Livefoods Ltd., UK. Crickets were stored in ventilated boxes at 20°C and fed with carrot and apple slices for up to one week before use.

3.2.3 Hylobius abietis *and* Acheta domestica *grooming reactions in response to the presence of nematodes on the head region*

A suspension of either *S. carpocapsae* or *H. downesi* was concentrated by sedimentation to 200 IJs/µl. An adult *H. abietis* then had 5 µl of one of the suspensions pipetted onto its head region at the point closest to the top of the rostrum with a Gilson pipette. Control insects had 5 µl of water pipetted to the same area. Insects were assigned to different treatments randomly. An insect was then placed in a 15cm Petri dish and observed continuously for 20 minutes. The insects' behaviour was assigned to several different classes and the number of incidences and the total length of time spent in each class was recorded using The Observer® for Windows software package (Noldus). The behavioural classes were:

(1) Grooming Head – The insect was grooming its head region with one or more of its legs.

(2) Grooming Antennae – The insect was grooming its antennae with one or more of its legs.

(3) Grooming Elytra – The insect was grooming its wing cases with one or more of its legs.

(4) Grooming Legs – The insect was grooming one leg with another leg.

Each treatment consisted of 50 insects over the course of 3 separate experiments. In the first two experiments each treatment consisted of 20 insects and in the third experiment each treatment consisted of 10 insects. Each experiment used a different batch of nematodes reared on different dates.

As a comparison, adult brown crickets were observed as above. The total number of crickets tested was thirty over the course of three experiments, n = 10 for each treatment.

3.2.4 Nematode behaviour on adult Hylobius abietis

An adult *H. abietis* was restrained from moving its legs by wrapping its abdomen and thorax regions in several layers of Parafilm. A suspension of either *S. carpocapsae* or *H. downesi* was concentrated by sedimentation to 100 IJs/µl. One microlitre of one of these suspensions was pipetted onto the head region of the restrained insect at the point nearest to the top of the rostrum. The insect was placed in a 9 cm Petri dish and the head region was observed under a dissecting microscope at X20 magnification. Every two minutes for twenty minutes the behaviour of the IJs that left the initial application point was observed and recorded in four different classes using The Observer software package for Windows. These classes were:

(1) Standing: This was where an IJ had lifted over half of its body off the surface of the insect.
(2) Wriggling: Any IJ that still had more than half its body length on the surface of the insect and was moving.
(3) Climbing: Any IJ that had climbed off the surface of the insect on top of another IJ.
(4) Still: The IJ was not moving on the surface of the insect.

Each treatment consisted of 30 insects over the course of three experiments with 10 insects per treatment in each experiment. Each experiment used different batches of nematodes reared on different dates.

3.2.5 Effect of EPN presence in the substrate on adult Hylobius abietis *roosting behaviour*

A 9cm Petri dish with a dividing central wall (Sarstedt Ltd., P/N 82.1195) was filled to a depth of approximately 1cm with moss peat compost. Twenty-five thousand IJs of either *S. carpocapsae* or *H. downesi* were applied to the top of the soil on one side of the dish in 1ml of water. One millilitre of water was applied to the other side of the dish. The dish was then left for one hour to allow the liquid to soak into the compost. One adult weevil was randomly assigned to one side of the dish, facing away from the dividing wall. The amount of time taken to leave that half of the dish was recorded up to a maximum of twenty minutes. The weevil was then picked up using a forceps and the dish was turned by 90° before the weevil was again placed in the centre of one side of the dish, randomly assigned by a random number generator, and timed again as above. When the weevil had been timed 10 times for each side this was repeated for another weevil. Each treatment consisted of 30 weevils in three separate experiments with 10 weevils per treatment in each experiment. Each experiment used different batches of nematodes reared on different dates and freshly collected weevils stored for no longer than 4 weeks.

3.2.6 Effect of EPN presence in the substrate on adult Hylobius abietis *feeding behaviour*

Twenty-four arenas were set up using plastic plant propagators measuring 240 mm x 380 mm. These were filled with moss-peat compost to a depth of approximately 20 mm. A 5.5 cm Petri dish was placed at both ends 10 mm in from each end. These were filled with compost and a suspension of 25,000 IJs was applied to the surface of the compost in one of the dishes in 1ml of water. Twelve of the arenas were treated with *S. carpocapsae* and twelve with *H. downesi*. One milliliter of water was applied to the other dish. Discs of bark with a diameter of 10 mm were punched from freshly cut Sitka Spruce billets using a metal corer. Two of these were placed on top of the compost in each of the Petri dishes (Fig. 3.1). One adult *H. abietis* was placed into each propagator and a plastic lid was used to cover the propagator. The bark discs were replaced every two days for 6 days. Feeding scars were traced onto transparent acetate sheets and the images digitized using a scanner and measured using ImagePro

software for Windows. This experiment was run three times with a total n of 36 for each treatment. Each experiment used insects collected at different dates and nematodes reared on different dates. The experiment was conducted in a controlled temperature room at 20°C under a L16:8D light regime.
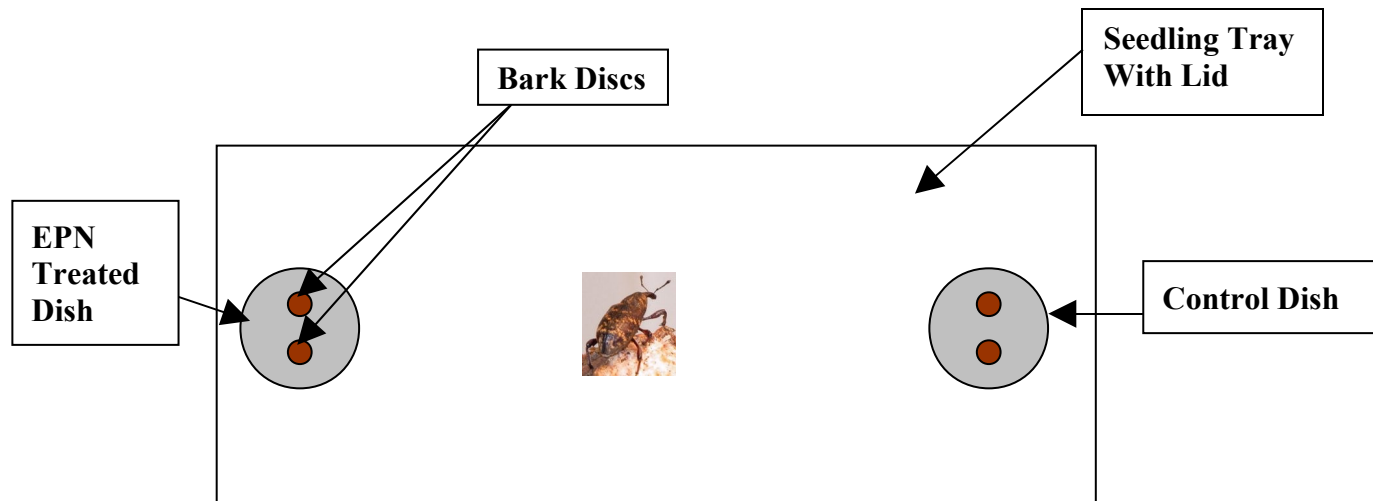
Figure 3.1: Arena for studying the effect of EPN presence on adult *Hylobius abietis* roosting behaviour

3.2.7 Effect of EPN infection on adult H. abietis *feeding.*

Infection: Fifteen millimeter filter paper discs were placed in each well of a 24-well multiwell plate and were then treated with one of five treatments:

(1) Low *S. carpocapsae* concentration of 400 IJs in 50 µl of water, total n = 100.

(2) High *S. carpocapsae* concentration of 2000 IJs in 50 µl of water, total n = 120.

(3) Low *H. downesi* concentration of 2000 IJs in 50 µl of water, total n = 90.

(4) High *H. downesi* concentration of 10,000 IJs in 50 µl of water, total n = 120.

(5) Control of 50 µl of water, total n = 75.

The concentrations were chosen on the basis of experiments conducted by Dr. Robbie Girling (NUIM, unpublished data) that showed these concentrations would infect and kill a low proportion of adult weevils for the low concentration and a high proportion at the high concentration.

One adult *Hylobius abietis* was then placed in each well for 24 hours with no access to food. Weevils were then stored in ventilated plastic boxes (205 mm X 140 mm X 100mm) with snap-on lids with fresh Sitka spruce billets as a source of food on a substrate of 8% w/w sand. Weevils from the same treatments were stored together in ventilated plastic boxes (205 mm X 140 mm X 100mm) with snap-on lids without food for one day 1, 7 or 14 days after infection until used in the feeding assay.

Feeding Assay 1 – One day post-infection: Plastic food containers (9 cm diameter 5 cm high) with snap-on lids were filled with approx. 50g of dry sand. Each container had one weevil that had been removed from the storage described above (and had no access to food for the preceding 24 hours) placed in it, with a total of twenty weevils for each treatment. A disc of bark (10 cm diameter) freshly punched from a Sitka spruce trunk was placed in each container as a food source for the weevils. Each disc was weighed before being placed in the container. Discs were changed daily and removed discs were weighed again and checked for signs of feeding by the weevils on days 1, 2 and 6. After day six all weevils, alive and dead, were dissected to check for the presence of nematodes. The head of the weevil was removed using a scalpel and forceps and it was placed in a Petri dish filled with water. The rostrum was then removed and the oesophagus dissected to check for nematode presence. The elytra and hindwings were removed using a fine forceps and placed in another Petri dish filled with water and the presence and number of nematodes was recorded. The body of the weevil was placed in a 5 cm Petri dish filled with approx. 2 ml of water. The remaining body parts were dissected in water and the internal organs broken up and examined under a dissecting microscope. Any presence of nematodes and their condition, i.e. alive, dead or encapsulated, was noted.

Assay 2 – Seven days post-infection: A second group of 20 weevils for each treatment were used in a feeding assay 7 days after the initial infection. Bark discs were

weighed on days 2 and 5. After day six weevils were again dissected as described above to check for the presence of EPN.

Assay 3 – Fourteen days post-infection: A third group of 20 weevils, or all surviving weevils if less than 20 remained, per treatment were used and discs were again weighed on days 2 and 5 before the weevils were dissected on day 6.

The experiment was conducted in a controlled temperature room at 20°C under a L16:8D light regime.

3.2.8 *Statistical Analyses*

All statistics in this chapter were carried out using the Minitab 14® software package for Microsoft Windows®. Data of three or more test groups were analysed using ANOVA where data could be normalised, or when not normally distributed, a Kruskal-Wallis analysis was carried out. ANOVA was followed by Tukey's post-hoc, pair-wise, comparison where $P < 0.05$. Proportional data was analysed using a Chi-square test. Data compared between two groups was analysed using an unpaired t-test or a Mann-Whitney where data could not be normalised. Non-normally distributed data was transformed using a square-root or natural log transformation where it was possible to normalise by transformation. Significance levels were taken to be where $P < 0.05$.

3.3 RESULTS

3.3.1 Hylobius abietis *grooming reactions in response to the presence of nematodes on the head region.*

The presence of 1000 IJs on the head region of an adult *H. abietis* significantly increased the time spent grooming by weevils exposed to *S. carpocapsae* compared to those exposed to *H. downesi* and water controls (Fig. 3.3.1a). (Tukeys following ANOVA, F = 19.82, df = 2, P = 0.000). Weevils that had *S. carpocapsae* applied to their head region groomed almost four times longer on average (44.16 seconds) than those that had *H. downesi* applied (11.33 seconds). There was a difference across the treatments in the number of grooming events that took place (P = 0.000, Kruskal-Wallis, df = 2, H = 37.14) with weevils in each nematode treatment grooming more than ten times as often on average than the control (Fig. 3.3.1b). Insects treated with *S. carpocapsae* groomed their head and legs most often on average, 15.96 and 25.36 seconds respectively (Fig. 3.3.1c). Grooming of the antennae, 2.97 seconds, and elytra, 0.31 seconds, was less common. Weevils treated with *H. downesi* groomed their heads on average, 7.34 seconds, followed by antennae, 2.16 seconds. They groomed their legs for an average of 1.72 seconds and, similar to *S. carpocapsae*, grooming of the elytra was least common at an average of 0.1 seconds. Control insects had very little grooming on average with all grooming categories averaging less than one second (Fig. 3.3.1c).

Mean Grooming Time

Fig 3.3.1a

Mean Grooming Events

Fig 3.3.1b
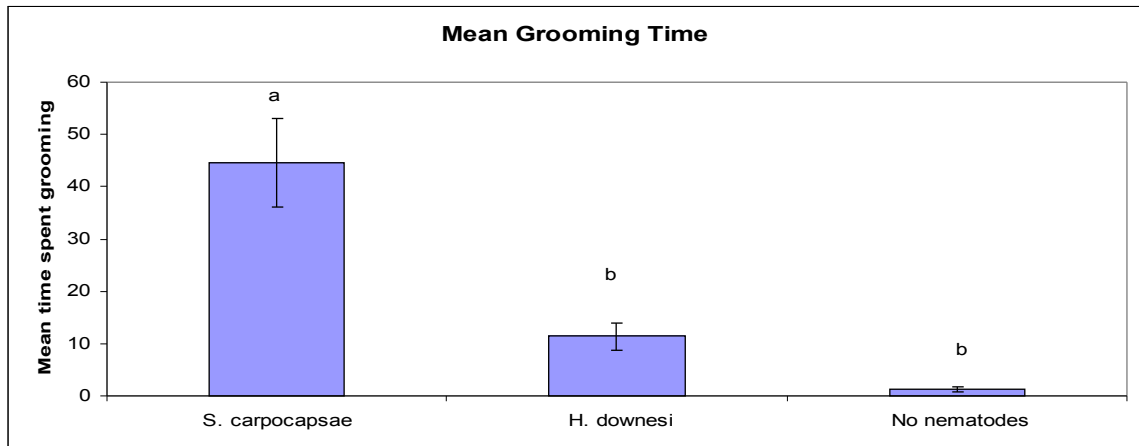
Grooming Categories - Mean Time
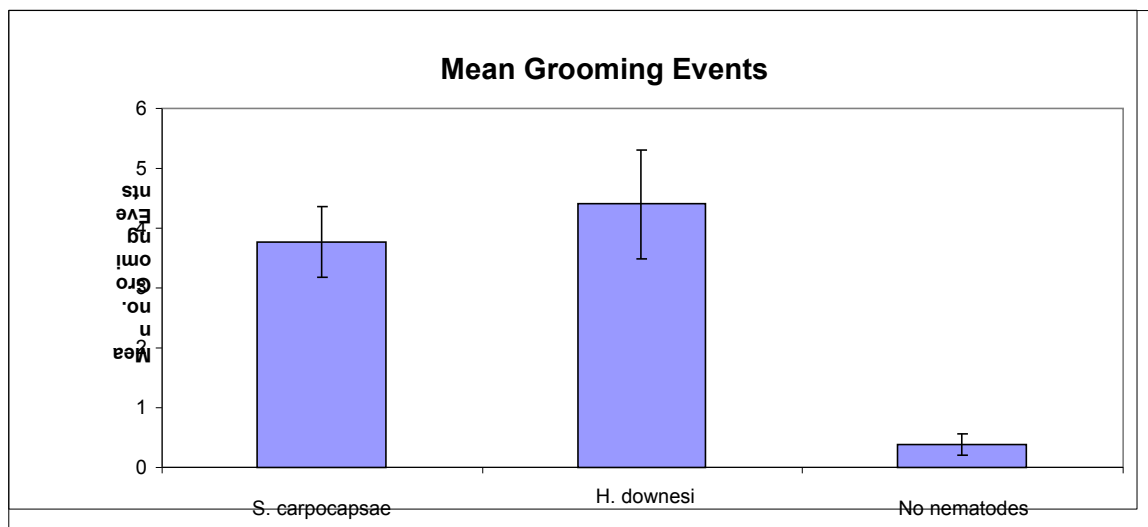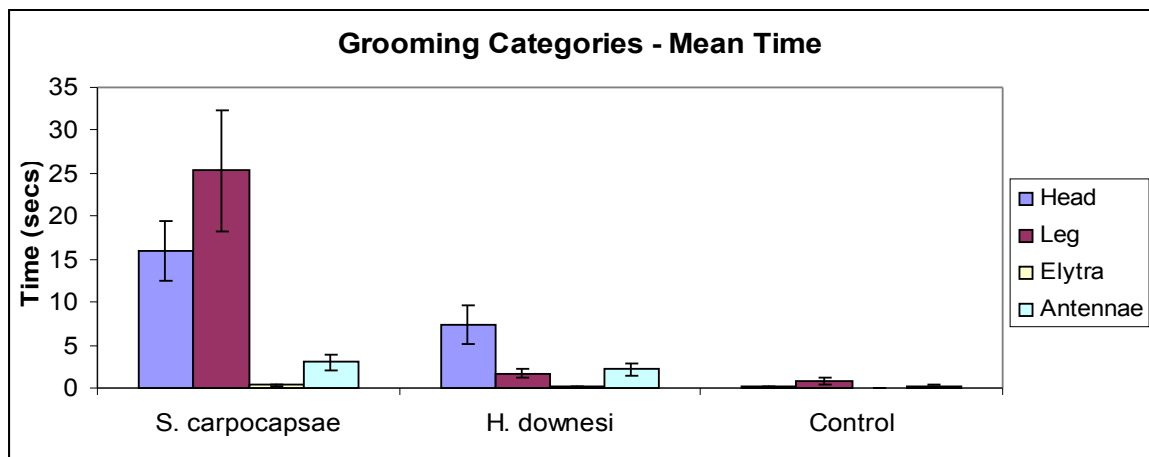
Fig 3.3.1c

Fig. 3.3.1: Mean (+/- SE) time spent grooming (Fig.3.3.1a; Upper Figure), number of grooming events (Fig. 3.3.1b; Middle Figure) and time spent in grooming categories (Fig. 3.3.1c; Lower Figure) by adult weevils with or without nematodes applied to their head region. Weevils were observed for 20 minutes and their behaviours recorded. Data shown are a mean of three experiments with a total of 50 weevils for each treatment. Different letters indicate a significant difference between treatments (Tukey's at P = 0.05, following ANOVA, $P < 0.05$).

3.3.2 Acheta domestica *grooming reactions in response to the presence of nematodes on the head region.*

In order to see if the presence of EPN elicited a grooming response in other insects, the same experimental setup was employed using crickets, *Acheta domestica,* instead of *H. abietis*. Under the same conditions as described for *H. abietis* above, crickets treated with EPN also groomed for significantly longer over a twenty minute period than corresponding controls (P = 0.043, ANOVA, F = 3.37) (Fig. 3.3.2a). Insects treated with each of the two nematode species groomed their legs for significantly longer than water-treated controls (Tukey's after ANOVA, P = 0.0000, F = 23.74). There was a difference across the treatments in how long they spent grooming their heads (Kruskal-Wallis, P = 0.000, d.f. = 2, H = 16.67) and elytra (Kruskal-Wallis, P = 0.004, d.f. = 2, H =11.11). There was no significant difference in how long they spent grooming their antennae (ANOVA, P = 0.352, F = 1.07) (Fig. 3.3.2b). There was no significant difference in the number of grooming events across the treatments (P>0.05, Kruskal-Wallis, df = 2)(Data not shown).
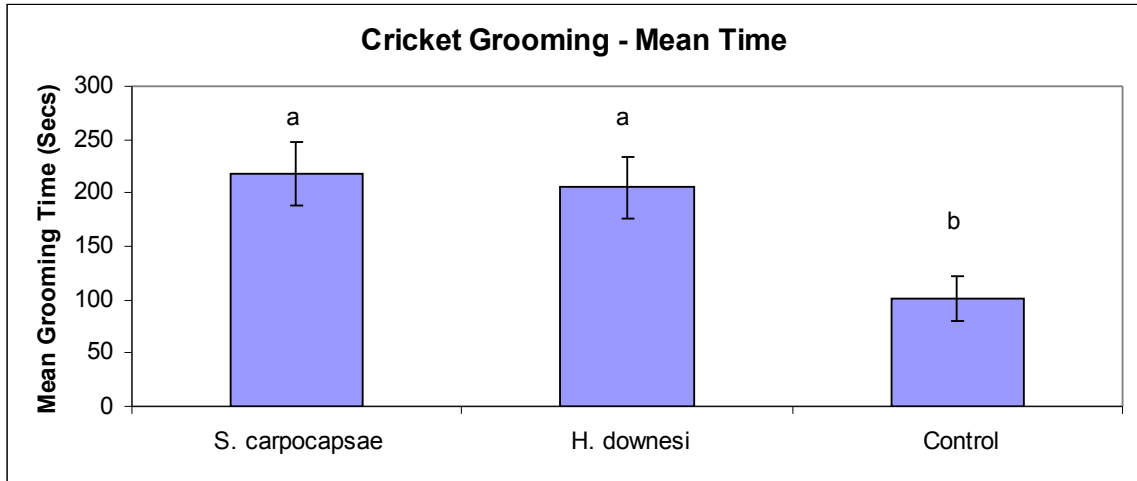
Fig. 3.3.2a: Mean (+/- SE) time spent grooming by adult brown crickets, *Acheta domestica*, after the application of nematodes or a water control to their head region. Crickets were observed for 20 minutes and their behaviours recorded. Different letters indicate a significant difference between treatments (Tukey's $P < 0.05$, ANOVA, $P = 0.043$).
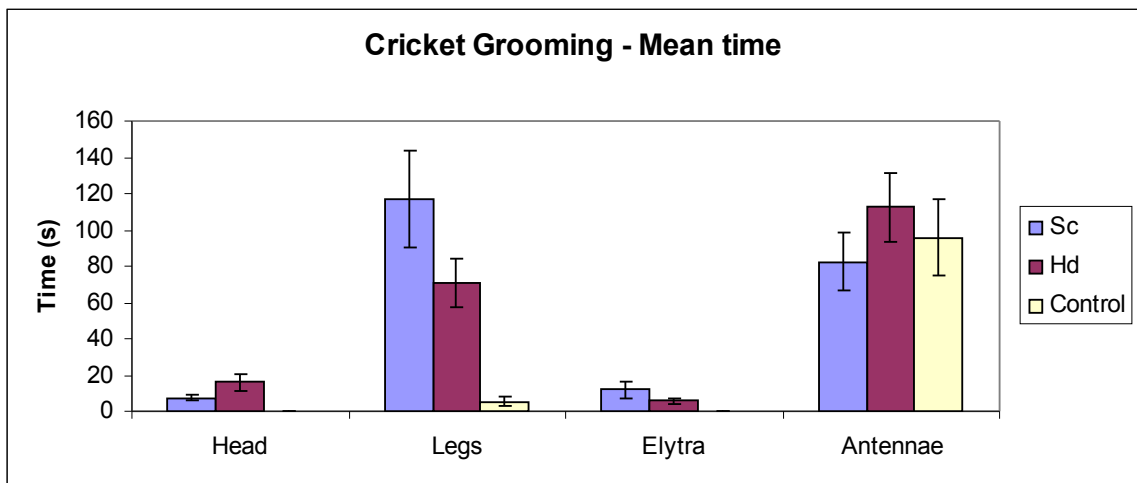
Fig. 3.3.2b: Mean (+/- SE) time spent grooming in four categories by *A. domestica* after the application of two different species of nematode or a water control to the head region. Insects were observed for 20 minutes and behaviours were recorded. Data shown are a mean of three experiments with a total n of thirty.

3.3.3 Nematode behaviour on adult H. abietis

The behaviours of any of the 100 nematodes that left the point of application on the head region of an adult *H. abietis* were recorded every two minutes. The numbers of nematodes displaying the various behaviours changed over time. Nematodes had begun to disperse from the application point after 2 minutes (Fig. 3.3.3a). The number of nematodes of both species leaving the application point on the rostrum increased steadily until the 14 minute observation point. From fourteen to twenty minutes after application the number of *S. carpocapsae* leaving the point of application remained relatively steady while the number of *H. downesi* continued to slowly increase. At each time interval there were more IJs of *S. carpocapsae* than *H. downesi* outside of the application point (Fig. 3.3.3a).

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Over time the proportion of IJs displaying the different behaviours changed (Fig. 3.3.3b). Initially the majority of *S. carpocapsae* IJs that left the application point displayed the wriggling behaviour. The proportion of nematodes engaged in the standing and climbing behaviours increased steadily until the ten minute point when the wriggling and climbing behaviours were displayed by an approximately equal number of IJs. After this point the proportion of nematodes displaying the standing behaviour remained relatively constant while the numbers exhibiting wriggling and climbing continued to increase at approximately the same rate until 14 minutes before levelling off (Fig. 3.3.3 b).

Observations of *H. downesi* showed that nematodes that left the point of application were initially most likely to display the wriggling, climbing or still behaviours. These three behaviours were displayed by approximately even numbers of IJs in the first four minutes. After this the climbing behaviour became prevalent and increased steadily throughout the 20 minute observation period to become the most common behaviour exhibited. The numbers engaged in the still behaviour increased less rapidly and stayed level after 14 minutes. Standing was relatively rare in *H. downesi* and remained at a low level throughout the observation period (Fig. 3.3.3b).

At ten minutes (Fig. 3.3.3c) the proportion of nematodes displaying the standing behaviour was significantly higher in *S. carpocapsae* than in *H. downesi* (P = 0.001, Chi-square =12.054, df =1). There was no significant difference in the proportion of *S. carpocapsae* displaying the wriggling behaviour than *H. downesi* at the ten minute point (P = 0.353, Chi- square = 0.857, df = 1). There was no significant difference between species in nematodes that left the application point and displayed the climbing behaviour (P = 0.141, Chi-square = 2.170, df = 1). Significantly more *H. downesi* IJs showed the still behaviour than *S. carpocapsae* (P = 0.018, Chi-square 5.604, df = 1).

At the end of the observation period of 20 minutes a similar pattern of differences was observed (Fig. 3.3.3d). *S. carpocapsae* had proportionately more IJs displaying the standing behaviour (P = 0.004, Chi-square = 3.497, df = 1) while *H. downesi* had more IJs displaying the climbing (P = 0.022, Chi-square = 5.255, df = 1) and still behaviours (P = 0.041, Chi-square = 4.190, df = 1). No significant difference was found between the two species for the wriggling behaviour (P = 0.061, Chi-square =3.497, df = 1).

By the end of the 20 minute observation period the number of nematodes that had left the application point varied widely across the different replications. In the *S. carpocapsae* treated insects the range was from 8 to 50 nematodes and in the *H. downesi* treated insects it ranged from 3 to 39. The mean number outside the application point at this observation time was 15.8 and 13.26 for *S. carpocapsae* and *H. downesi* respectively.
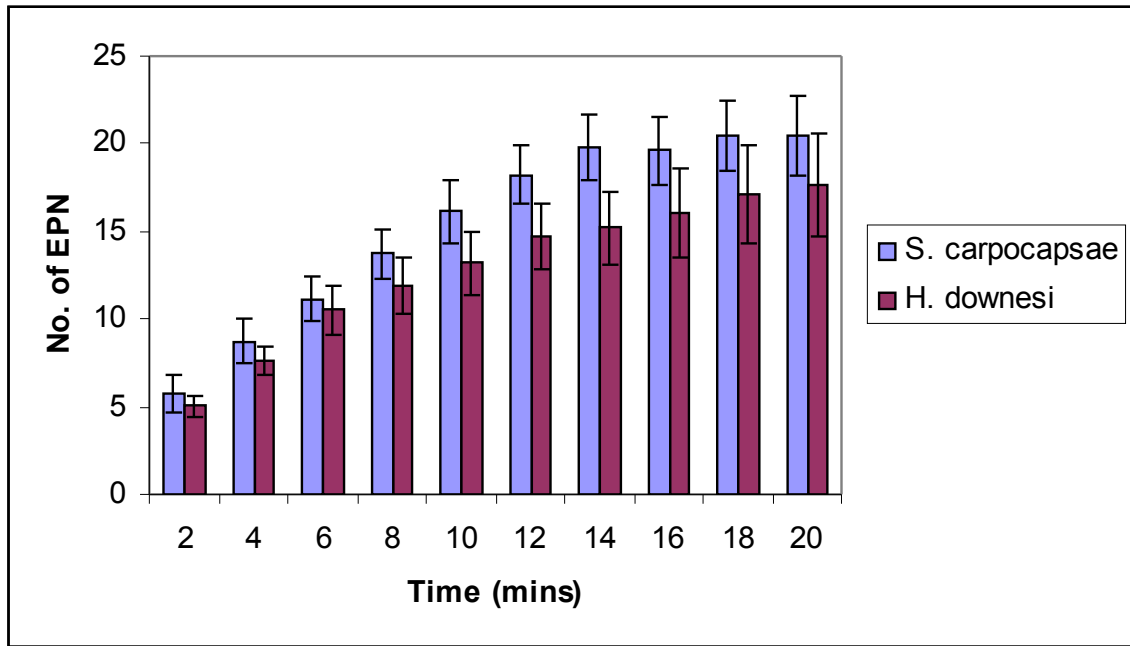
Fig. 3.3.3a: Mean (+/- SE) number of nematodes leaving the area of application on the head region of an adult *H. abietis* during a twenty minute observation period. Each bar represents the mean of data from 3 experiments with n = 10 in each experiment; 100 nematodes were applied to each weevil.

S. carpocapsae
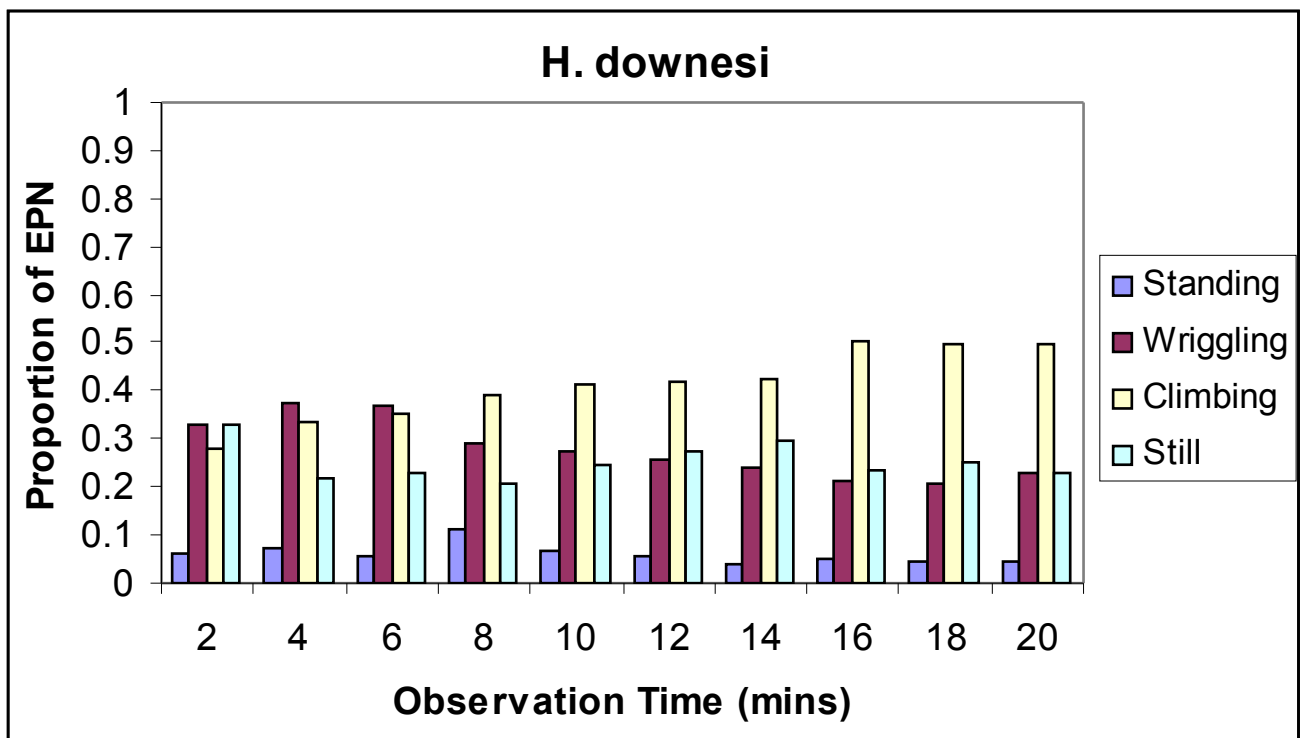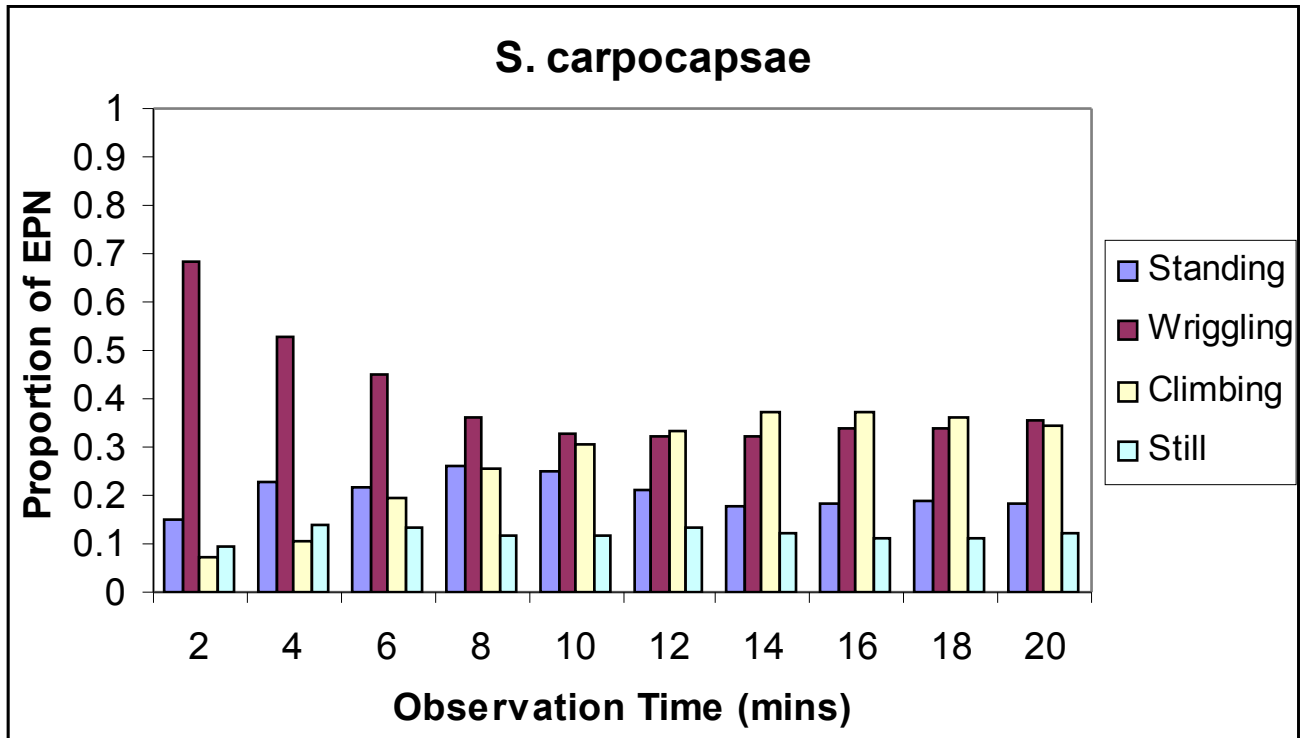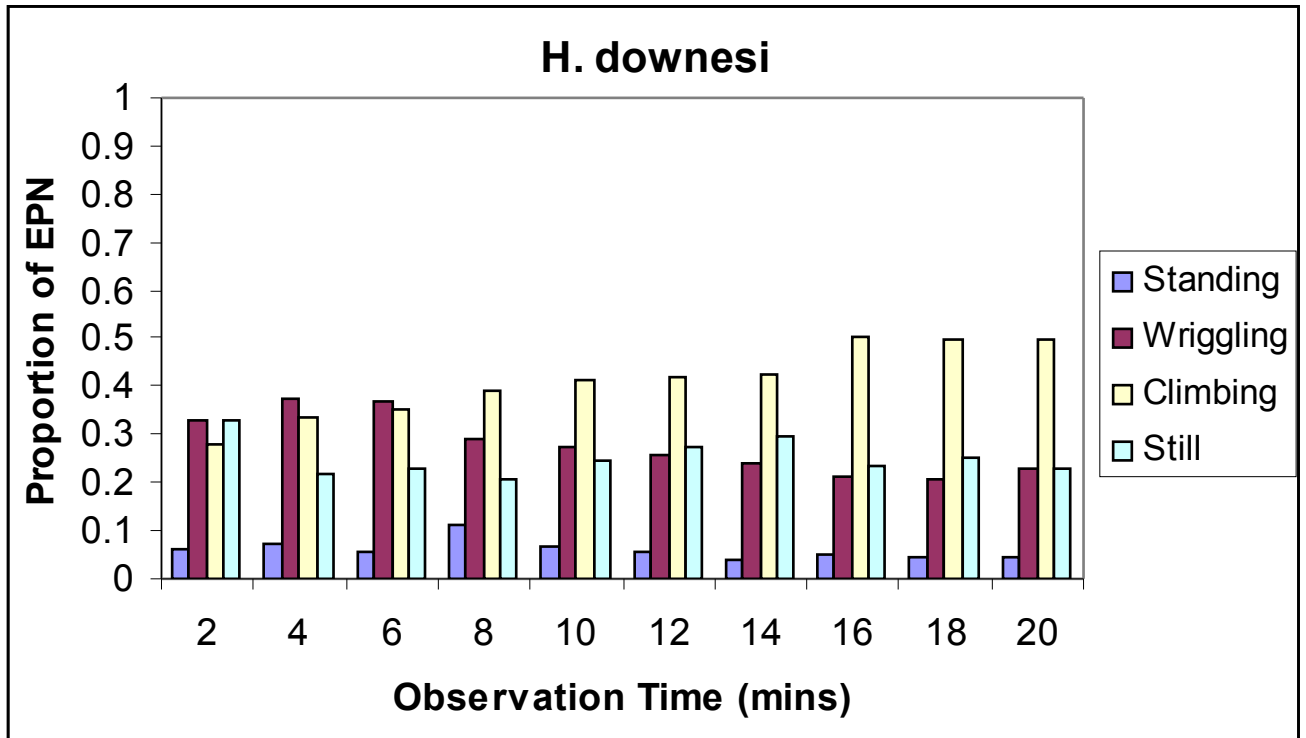
H. downesi

H. downesi

Fig. 3.3.3b: Proportion of EPN per weevil engaged in behaviours recorded for IJs of *S. carpocapsae* (Upper Figure) and *H. downesi* (Lower Figure) at two minute intervals over a twenty minute observation period. Data from three experiments with a total n of 30 per treatment.

EPN Behaviour @ 10 mins
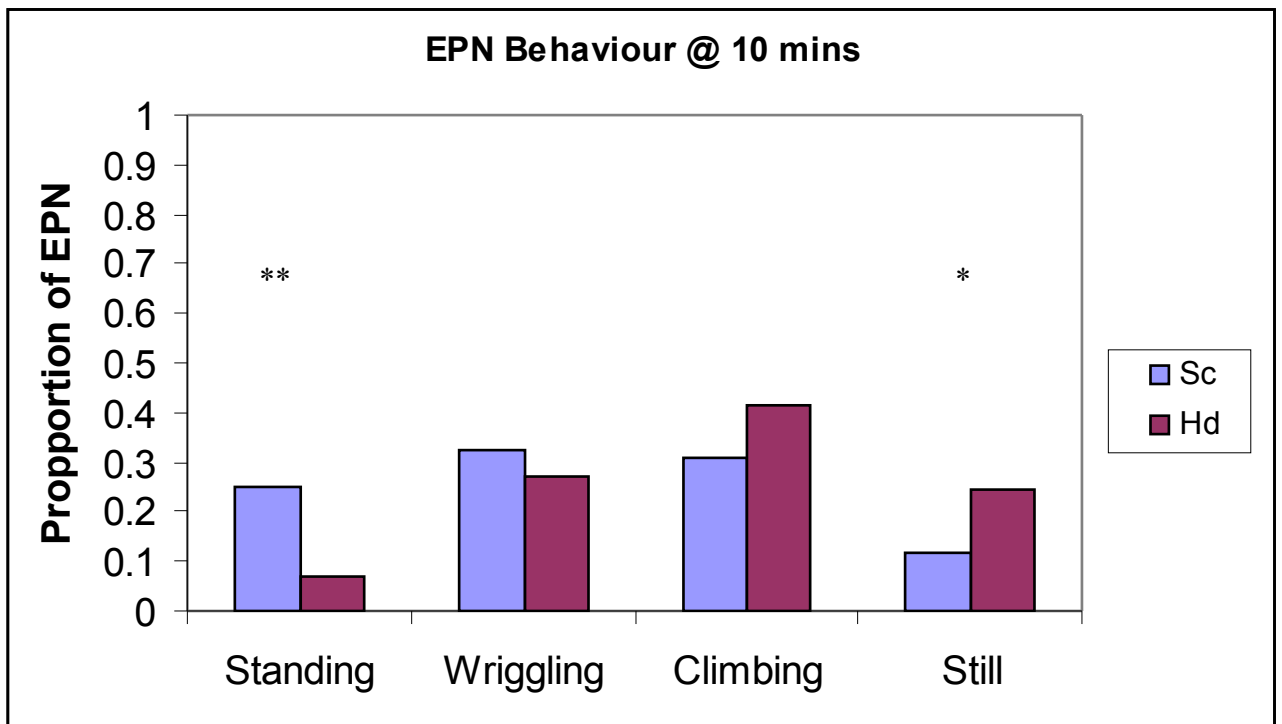
Fig. 3.3.3c: Behavioural classification after 10 minutes of IJs that left the application point on the head region of *Hylobius abietis*. Bars represent proportion of EPN displaying a behaviour at the observation point ten minutes post-application. Data are from three experiments with a total n of 30 for each treatment. Asterisks indicate a

significant difference between treatments (* = P < 0.05, ** = P < 0.01). Sc = *Steinernema carpocapsae,* Hd = *Heterorhabditis downesi.*
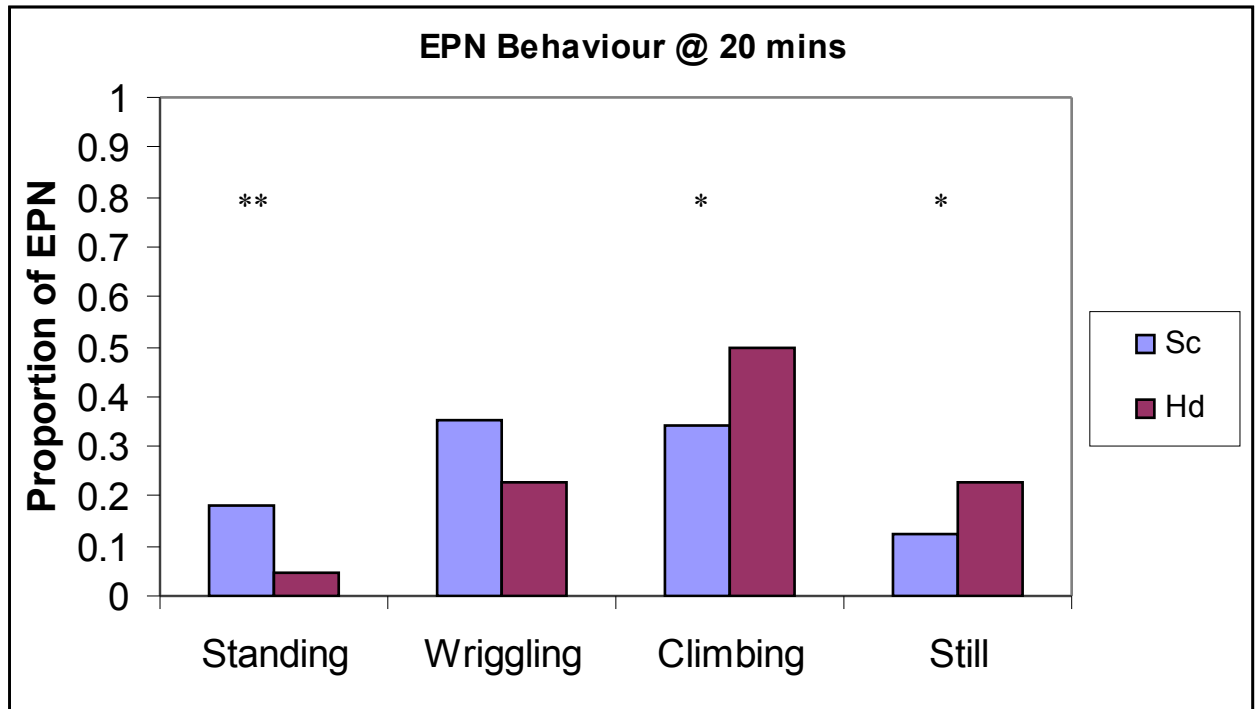
Fig. 3.3.3d: Behavioural classification after 20 minutes of IJs that left the application point on the head region of *Hylobius abietis*. Bars represent mean numbers +/- SE of three experiments with a total n of 30 for each treatment. Asterisks indicate a significant difference between treatments. (* = P < 0.05, ** = P < 0.01) Sc = *Steinernema carpocapsae,* Hd = *Heterorhabditis downesi.*

3.3.4 Effect of EPN presence in the substrate on adult H. abietis roosting behaviour

The amount of time taken for a *H. abietis* to leave an area where the substrate contained a high concentration of IJs did not significantly differ from the amount of time taken to leave a similarly sized area with no nematodes on the substrate. This was true for both *S. carpocapsae* (P = 0.654, paired t-test) and *H. downesi* (P = 0.521, Mann-Whitney). Weevils on substrates treated with both nematode species took approximately four minutes to leave either side of the dish. Weevils in the *S. carpocapsae* treated dishes took an average of 254.75 and 268.62 seconds to leave the treated and untreated sides respectively. Weevils in *H. downesi* treated dishes took 243.04 and 248.6 seconds to leave the treated and untreated sides respectively (Fig.

3.3.4). Weevils were incubated for two weeks after the experiment at 20°C and all dead insects were dissected. Of the total 120 insects used in this experiment 28 died in the two week incubation period. None had any evidence of nematode infection and of the 28 dead insects 4 had become infected with fungus.
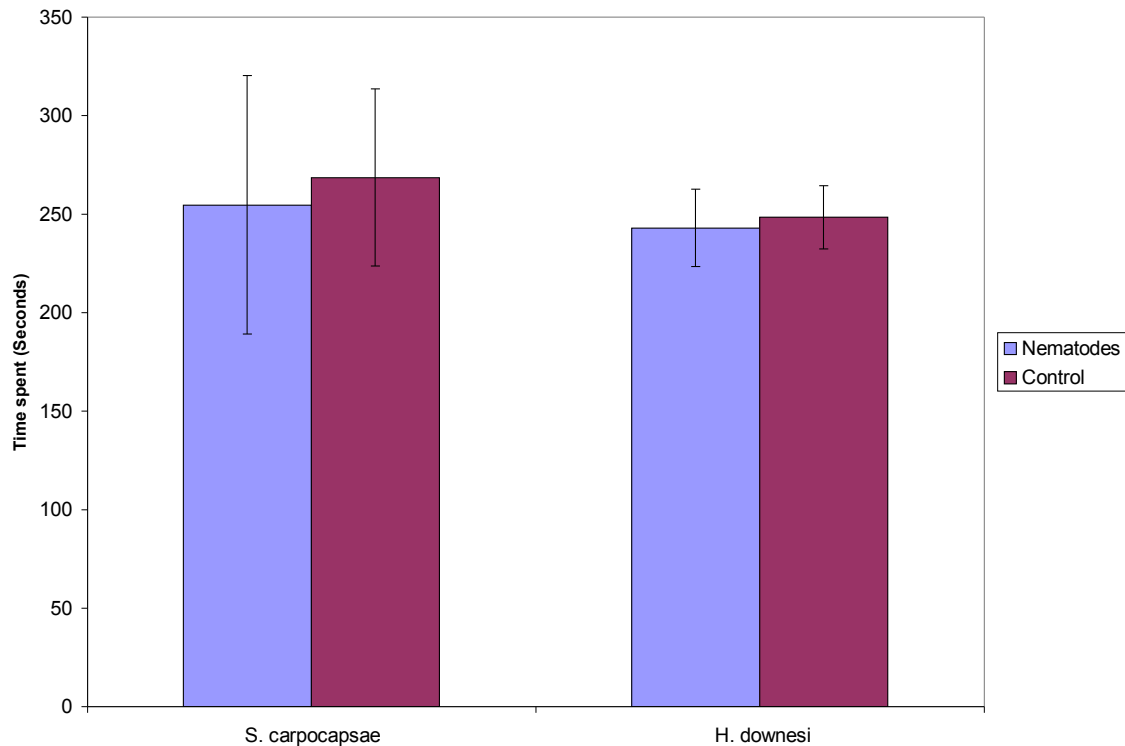
Fig. 3.3.4: Mean (+/- SE) time spent by adult *H. abietis* on a substrate that had been treated with nematodes or a water control. Data shown are the means of three experiments. *n* = 10 *H. abietis* for each treatment in each experiment. Differences between treatments (nematode and control) are not significant for either nematode species.

3.3.5 *Effect of EPN presence in the substrate on adult* **H. abietis** *feeding.*

Adult *H. abietis* were offered a choice of bark discs to feed on. The discs were on two different substrates; one that had EPN applied to it and one to which only water had been applied. The area of bark removed by feeding of adult *H. abietis* from discs of Sitka spruce on a substrate to which 25,000 *S. carpocapsae* IJs had been applied two days earlier differed significantly to the area removed from discs on a substrate with no nematodes (Mann-Whitney, P = 0.044, W = 490.0) (Fig. 3.3.5). This difference was not found in the *H. downesi* treatments when compared to their corresponding controls (Mann-Whitney, P = 0.749, W = 640.0). There was also no significant

difference found in the area of bark removed from discs on substrates for either nematode species after 4, 6 or 8 days compared to their corresponding controls.
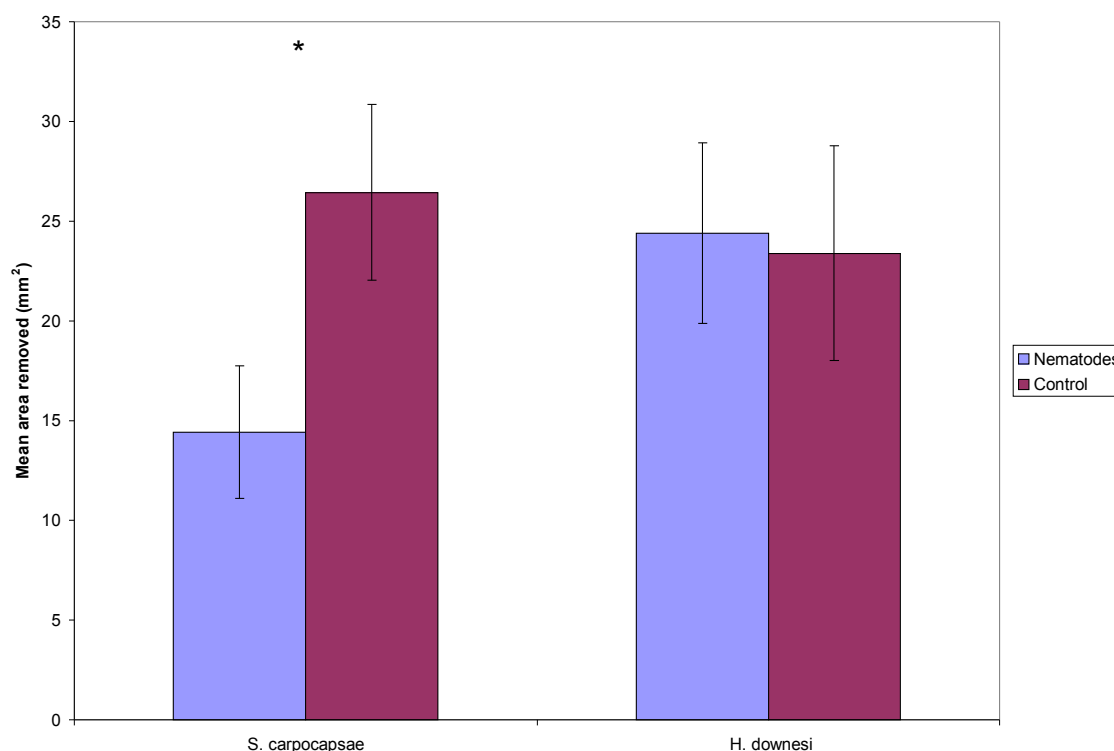
Fig. 3.3.5: Mean (+/- SE) area of bark removed by adult *H. abietis* from bark discs on a substrate that had been treated with nematodes or a water-treated control. Data shown are the mean of two experiments. n = 12 *H. abietis* for each nematode species in each experiment. Asterisks indicate a significant difference between treatments. (* = P < 0.05).

3.3.6 *Effect of EPN infection on adult* H. abietis *feeding.*

To assess the impact of EPN infection on adult *H. abietis* feeding, three assays were carried out. One was carried out immediately after exposure, a second one week after exposure and the third two weeks after exposure to EPN. In order to explore the effect of nematode infection on feeding rate, data from the three feeding assays were combined. Infection by EPN increases *H. abietis* feeding activity as discs exposed to weevils that had nematodes in their tissues upon dissection lost significantly more weight than those exposed to weevils in which no nematodes were found (Fig. 3.3.6a). Differences were significant for each of the nematode treatments; *S. carpocapsae* Low Concentration (Mann-Whitney, P = 0.0002, W = 2766.0), *S.*

carpocapsae High Concentration (T-test, P = 0.000, T-value = 4.22), *H. downesi* Low Concentration (T-test, P = 0.000, T-value = 4.42) and *H. downesi* High Concentration (T-test, P = 0.000, T-value = 6.04).

There was no difference in the mean amount of weight lost by the bark discs across all of the treatments including the no weevil control (P = 0.13, df = , F = 1.72, ANOVA) (Fig. 3.3.6c). There was also no difference between the insect treatments including the no nematode control (P = 0.949, df = 4, F = 0.18, ANOVA) (Fig. 3.3.6c).

There were no significant differences between the nematode treatments in the numbers of dead weevils in Assay 1 (P = 0.190, Chi-square = 4.765, DF = 3). The number of dead weevils ranged from 3 to 9 out of twenty. In assay 2 there was a significant difference in the number of dead weevils (P = 0.004, Chi-square = 13.577, DF = 3) , which ranged from 2 out of 20 in the *S. carpocapsae* low concentration to 12 out of 20 in the *H. downesi* low concentration (Table 3.3.6a). In Assay 3 it was not possible to perform statistics on mortality as the number of insects was too low in the *S. carpocapsae* treatments due to a higher mortality of insects in storage for these treatments (Table 3.3.6b). When the numbers of weevils that died during Assay 3 was compared for the *H. downesi* treatments only there was no difference found (Chi-square = 1.908, df = 1, P = 0.167).

Dissections of all weevils each week yielded information on numbers and condition of nematodes invading each insect (Fig. 3.3.6d – Fig. 3.3.6h) (Appendix). In Assay 1, of the insects treated with *S. carpocapsae* 35% had no nematodes on dissection for both concentrations (Fig. 3.3.6d and Fig. 3.3.6e). Insects treated with *H. downesi* had nematodes in 40 and 35% of insects for the low and high concentrations respectively (Fig. 3.3.6d and Fig. 3.3.6f). The proportion of weevils with live nematodes found in their bodies ranged from 20% in insects treated with the high concentration of *H. downesi* to 55% in the *S. carpocapsae* low concentration treatment. Across all treatments a total of 17 weevils were found to have encapsulated nematodes upon dissection. Of these only one insect was dead at the time of dissection, a significant difference (Chi-square = 5.61, d.f. = 1, P = 0.018).

In Assay 2 no live nematodes were found in any of the treatments (Fig. 3.3.6d, Fig. 3.3.6g and Fig. 3.3.6h). In the *S. carpocapsae* treated weevils only 12 of the 40 insects had nematodes on dissection (Fig. 3.3.6g). In the *H. downesi* treated insects 34 out of 40 had no nematodes found (Fig. 3.3.6h). No encapsulated nematodes were found in Assay 2. No nematodes were found in any insects during Assay 3. The data from the Assay 1 and Assay 2 were examined for evidence of associations.

Weevils carrying nematodes under the elytra were more likely to have nematodes in their bodies (11 out of 12 insects) than those without nematodes under their elytra (39 out of 68 insects) (P = 0.024, Chi-square = 5.124, DF = 1). There was no significant difference between the number of dead or alive weevils that had nematodes inside their bodies (P = 0.518, Chi-square = 0.418, DF = 1).
.

There was a significant difference in the amount of weight lost from bark discs exposed to weevils that died during the assays compared in two treatments, the no nematode control (Two sample t-test, P = 0.004, T = 3.13, df = 25) and *H. downesi* Low Concentration (Two sample t-test, P = 0.022, T = 2.38, df = 41) (Fig. 3.3.6b). This difference was not found in the other treatments: *H. downesi* High Concentration (Two sample t-test, P = 0.913, T = 0.11, df = 56), *S. carpocapsae* Low Concentration (Two sample t-test, P = 0.433, T = -0.79, df = 29) or *S. carpocapsae* High Concentration (Two sample t-test, P = 0.669, T = 0.43, df = 19).
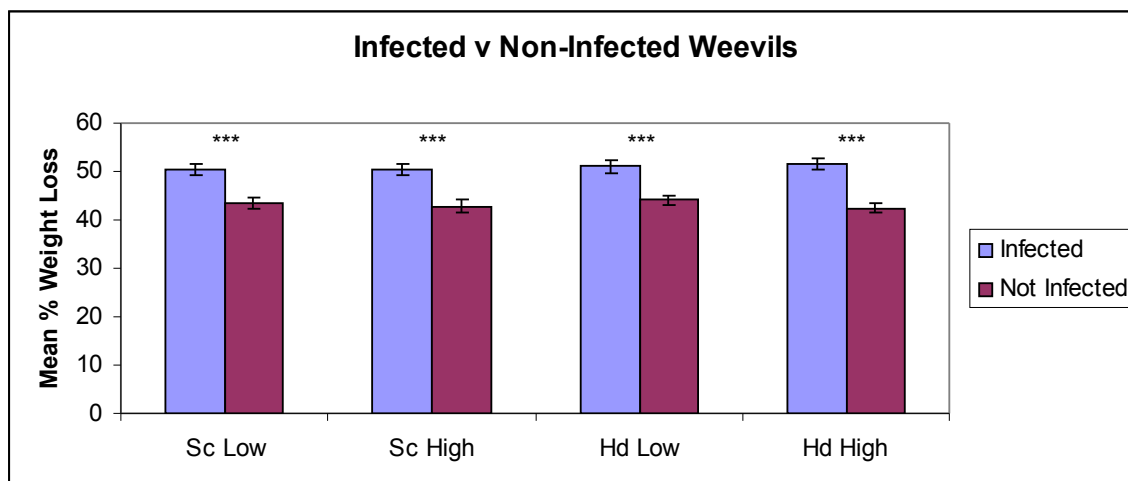
Fig. 3.3.6a: Mean (+/- SE) % weight lost by bark discs exposed to infected and non-infected adult weevils. Data shown are a mean of 3 feeding assays of one experiment. First assay consisted of 3 sets of measurements of each treatment with the second and third assays consisting of 2 sets of measurements. "Hd Low" and "Hd High" bars represent data from weevils exposed to a low (2000) and high concentration (10,000) of *H. downesi* respectively. "Sc Low" and "Sc High" bars represent data from weevils exposed to a low (400 IJs) and high (2000) concentration of *S. carpocapsae* respectively. Asterisks indicate a significant difference between treatments (*** = P < 0.001).
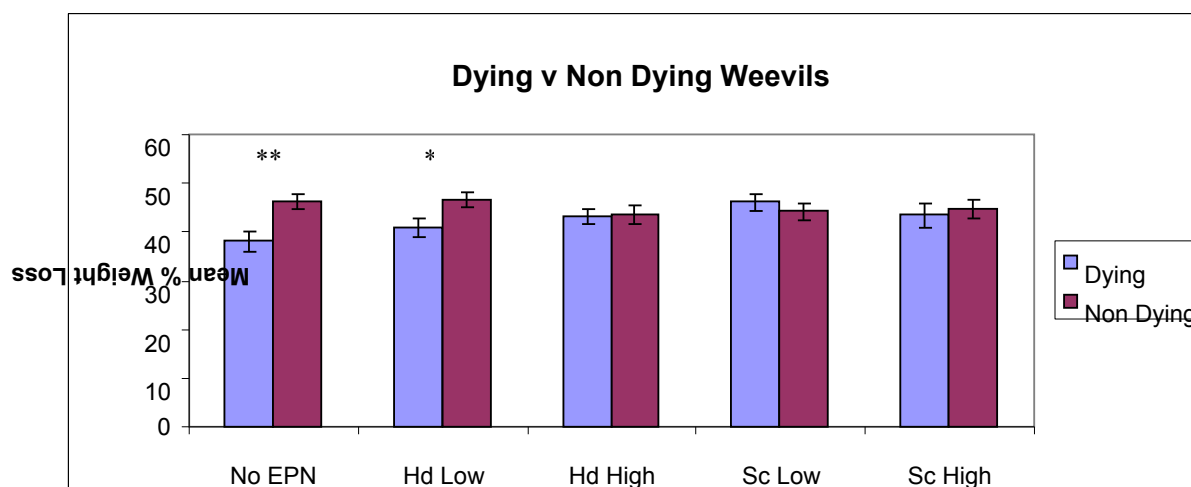
Fig. 3.3.6b: Mean (+/- SE) % weight lost by bark discs exposed to both adult weevils that died and those that did not die during the course of a 6 day feeding trial. Data shown are a mean of 3 feeding assays of one experiment. First assay consisted of 3 sets of measurements of each treatment with the second and third assays consisting of 2 sets of measurements. The "No EPN" bars represent data from weevils not exposed to EPN. "Hd Low" and "Hd High" bars represent data from weevils exposed to a low (2000) and high concentration (10,000) of *H. downesi* respectively. "Sc Low" and "Sc High" bars represent data from weevils exposed to a low (400 IJs) and high (2000) concentration of *S. carpocapsae* respectively. Asterisks indicate a significant difference (* = P < 0.05, ** = P < 0.01)
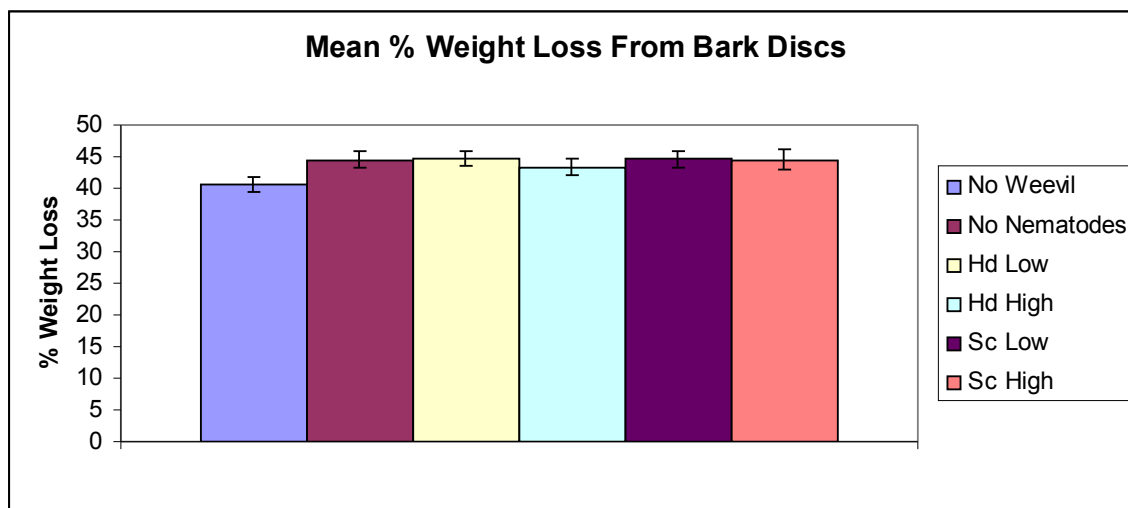
Fig. 3.3.6c: Mean (+/- SE) % weight lost by bark discs during a weevil feeding assay. Data shown are a mean of 3 assays of one experiment. First assays consisted of 3 sets of measurements of each treatment with the second and third assays consisting of 2 sets of measurements. The "No Weevil" treatment consisted of weight lost by discs in a no weevil control. "No Nematodes" = weevils not exposed to nematodes. "Hd Low" and "Hd High" = weevils treated with a low (2000 IJs) and high (10,000 IJs) concentration respectively of *Heterorhabditis downesi*. "Sc Low" and "Sc High" = weevils treated with low (400 IJs) and high (2000 IJs) concentrations respectively of *Steinernema carpocapsae*.

Table 3.3.6a: Number of weevils dead (n) after exposure to two concentrations of *H. downesi* (2000 and 10,000 IJs) and *S. carpocapsae* (400 and 2000 IJs) and a no EPN control. All insects were infected on the same date. Assay 1 ran from day 1 to day 6, Assay 2 from days 7 to 13 and Assay 3 from days 14 to 20.

No. of dead weevils (No. Used)

	No EPN	Hd Low	Hd High	Sc Low	Sc High
Assay 1	1 (20)	5 (20)	9 (20)	5 (20)	3 (20)
Assay 2	6 (20)	12 (20)	10 (20)	2 (20)	5(20)
Assay 3	6 (20)	5 (20)	8 (20)	4 (7)	3 (3)

Table 3.3.6b: Number of stored weevils dead (n) over the course of the experiment. Weevils were those not used in the series of assays described in table 3.3.4a, above.

	No EPN	Hd Low	Hd High	Sc Low	Sc High
Week 1	1 (55)	3 (70)	7 (70)	52 (80)	49 (100)
Week 2	1 (34)	15 (47)	12 (43)	34 (41)	40 (43)
Week 3	0 (13)	9	5 (11)	0	0

EPN Found in Dissections - Assay 1

Legend:
- Live & Dead & Encap
- Live & Dead & Elytra
- Live & Encap & Elytra
- Live & Encap
- Live & Elytra
- Elytra Only
- Encap Only
- Dead Only
- Live Only
- No EPN

EPN found in Dissections - Assay 2

Legend:
- No EPN
- Elytra Only
- Dead Only
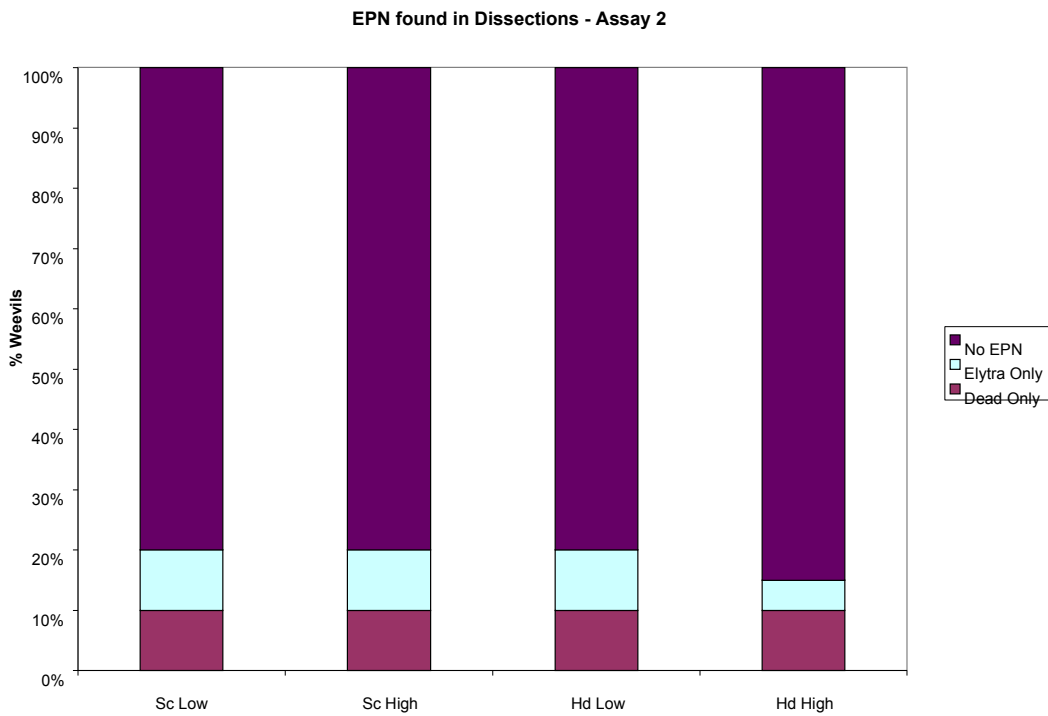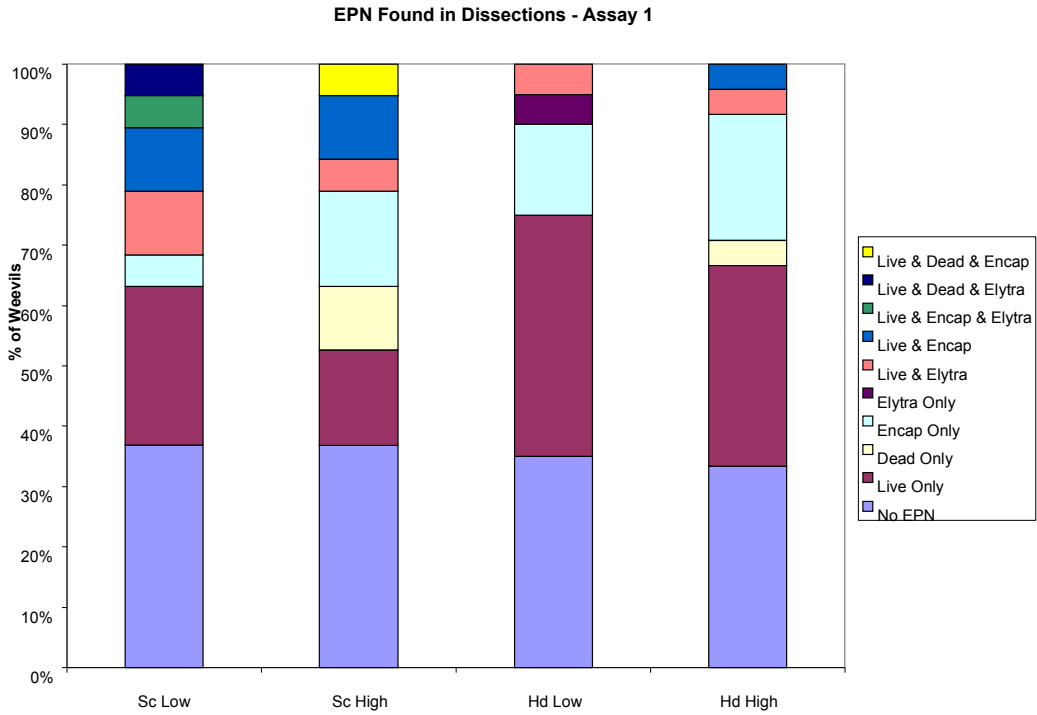
Fig. 3.3.6d: Dissections one and two weeks after infection with EPN. For each treatment n = 20. "No EPN" were weevils that no nematodes were found upon dissection. "Elytra Only" had nematodes under the wing cases only. "Encap" were weevils found with encapsulated nematodes. "Dead" had dead nematodes on dissection and "Live" had live nematodes in the body tissues.

S. carpocapsae Low Dose - Week 1 Dissections

S. carpocapsae High Dose - Week 1 Dissections
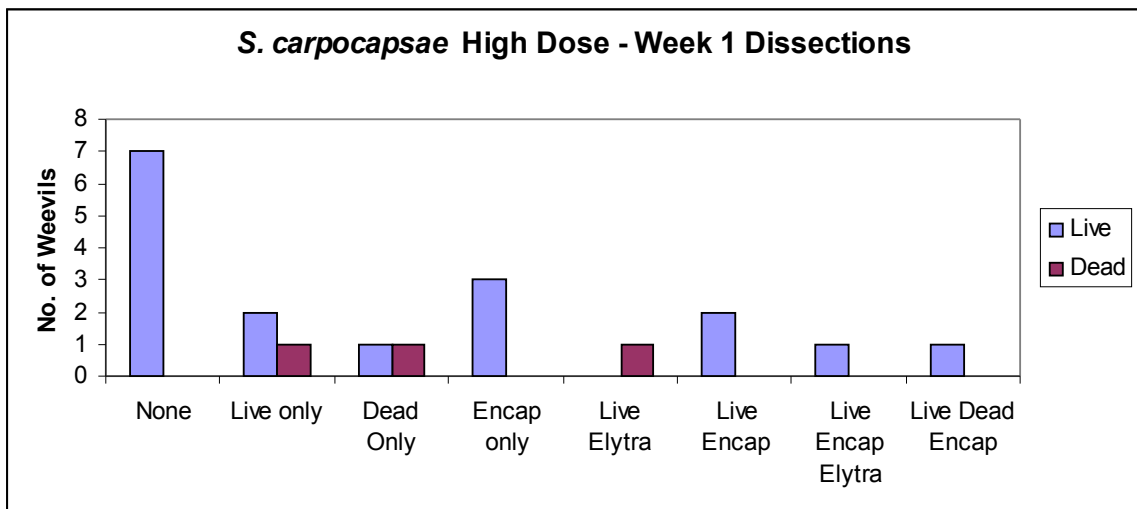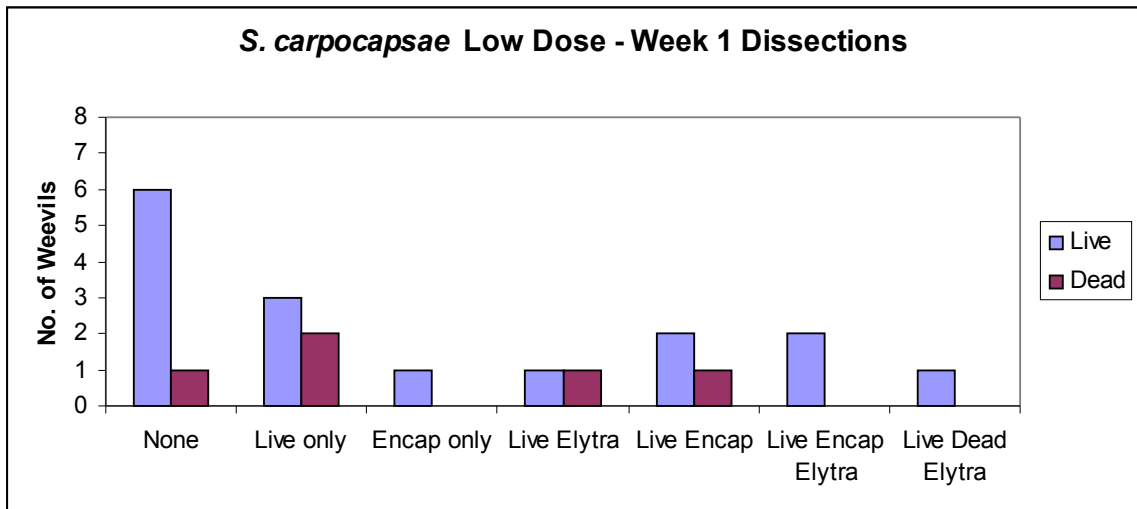
Fig. 3.3.6e: Number of live and dead weevils with nematodes of various states upon dissection 7 days after exposure to a low concentration (upper figure) and a high concentration (lower figure) of *Steinernema carpocapsae*. A total of 20 weevils were exposed at each concentration. The "Live" data series represents data from weevils that were alive at the time of dissection. The "Dead" data series represents data from weevils that were dead at the time of dissection. "None" data points represent weevils that had no nematodes upon dissection. "Live Only" bars represent weevils that only had live nematodes in their body tissues. "Encap Only" bars represent weevils that had only encapsulated nematodes upon dissection. "Dead Only" bars represent weevils that only had dead nematodes in their body tissues upon dissection. "Live Elytra" bars represent weevils that had both live nematodes in their body tissues and nematodes under the elytra. "Live Encap" bars represent weevils that had both live and encapsulated nematodes in their body tissues. "Live Encap Elytra" bars represent weevils that had live and encapsulated nematodes in their body tissues and under the

elytra. "Live Dead Encap" bars represent weevils that had live, dead and encapsulated nematodes in their body tissues on dissection.
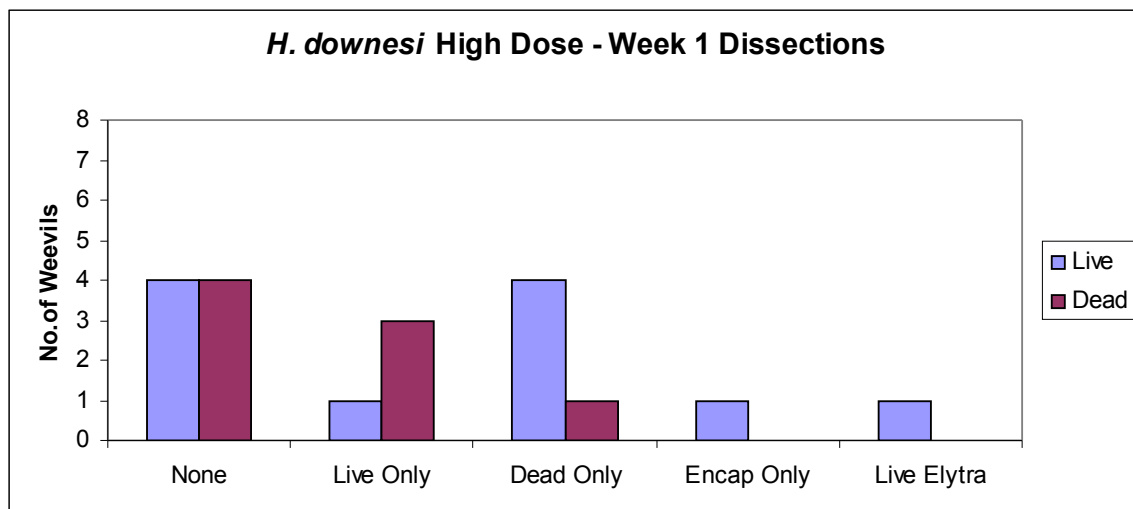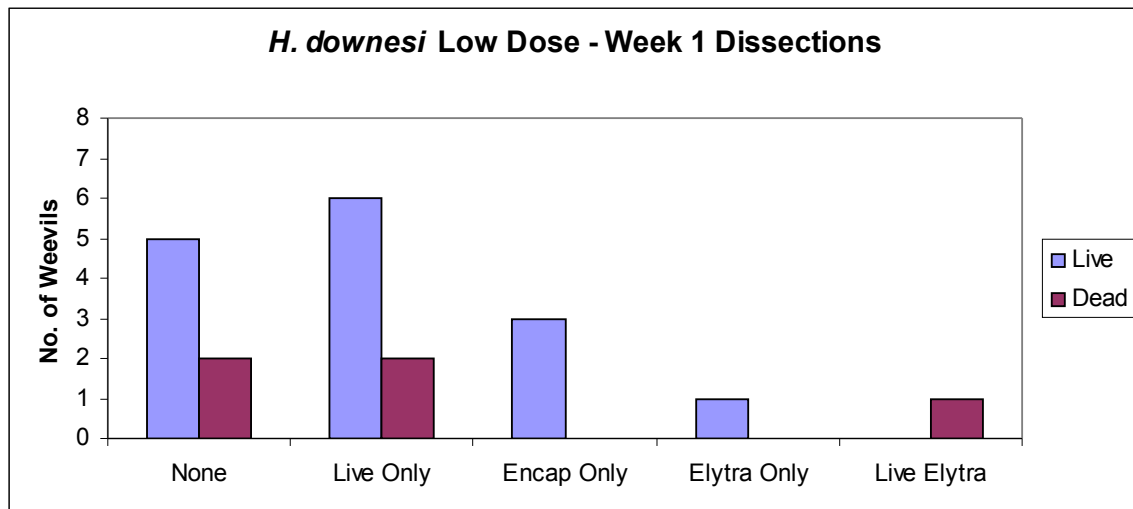
Fig. 3.3.6f: Number of weevils with nematodes of various states upon dissection 7 days after exposure to a low concentration (upper figure) and a high concentration (lower figure) of *Heterorhabditis downesi*. A total of 20 weevils were exposed at each concentration. "None" represents weevils with no nematodes after dissection. "Live Only" represents weevils with live nematodes in their body tissues. "Dead Only" represents weevils with dead nematodes in their body tissues. "Encap Only" represents weevils with encapsulated nematodes in their body tissues. "Elytra Only" represents weevils with nematodes under their elytra. "Live Elytra" represents weevils that have live nematodes in their body tissues and under their elytra.
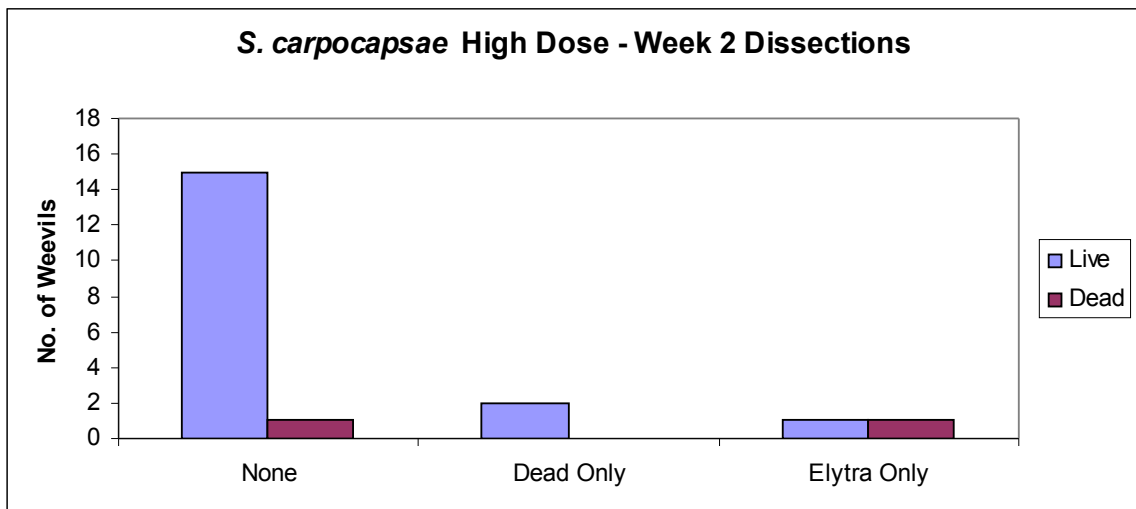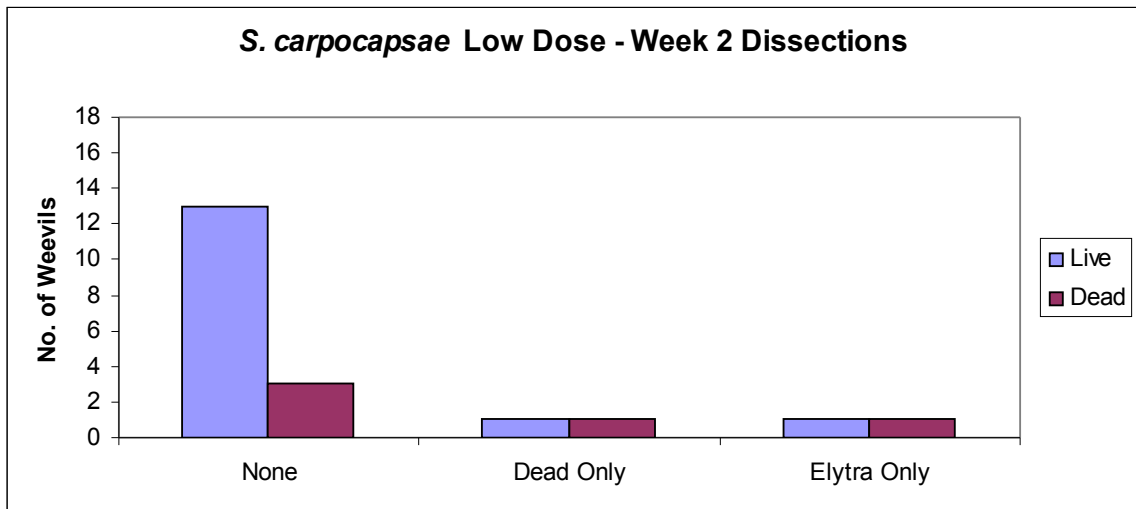
Fig. 3.3.6g: Number of weevils found with nematodes in various states 2 weeks after exposure to a low concentration (upper figure) and a high concentration (lower figure) of *Steinernema carpocapsae*. A total of 20 weevils were exposed at each concentration. The "Live" data series represents weevils that were alive at the time of dissection. The "Dead" data series represents weevils that were dead at the time of dissection. "None" represents weevils that harboured no nematodes upon dissection. "Dead Only" represents weevils that had only dead nematodes in their body tissues. "Elytra Only" represents weevils that only had nematodes under their elytra.
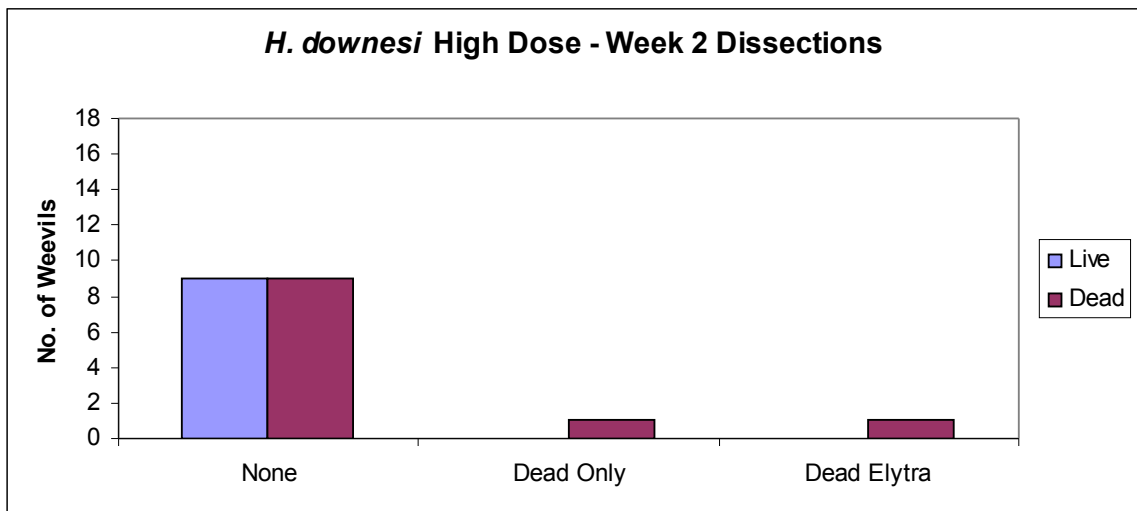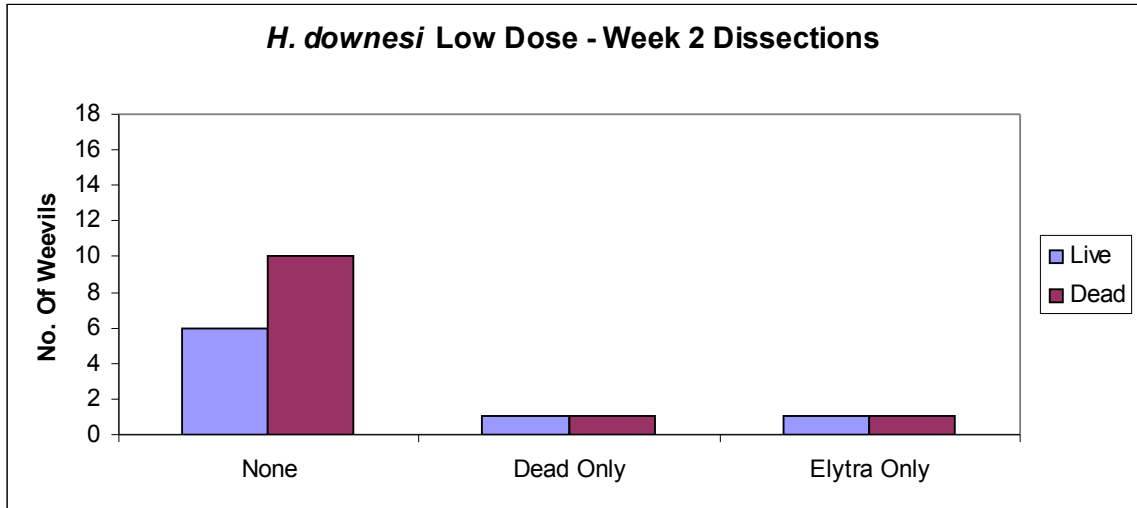
Fig. 3.3.6h: Number of weevils found with nematodes in various states 2 weeks after exposure to a low concentration (upper figure) and a high concentration (lower figure) of *Heterorhabditis downesi*. A total of 20 weevils were exposed at each concentration. "None" represents weevils that harboured no nematodes upon dissection. The "Dead" data series represents weevils that were dead at the time of dissection. "Dead Only" represents weevils that had only dead nematodes in their body tissues. "Elytra Only" represents weevils that only had nematodes under their elytra. "Dead Elytra" represents weevils that had dead nematodes in their body tissues and nematodes under their elytra.

3.4 <u>Discussion</u>

Adult *H. abietis* are the least susceptible life stage of the insect to EPN attack (See also Chapter 4). Part of this insusceptibility is presumably due to the hardened exoskeleton making the adult body more difficult to penetrate. The fact that adult *H. abietis* are much more mobile than the immature stages and that they live above ground would also make them a more difficult target for EPN. Another factor that makes insects less likely to be invaded by EPN is defensive or evasive behaviours. Removal of nematodes from the body can significantly reduce infection by EPN (Mankowski *et al.*, 2005). The experiment on the effect of nematode presence on weevil grooming shows that adult *H. abietis* react to the presence of nematodes in high concentrations on their bodies. It also shows that different species of EPN produce significantly different reactions. *Steinernema carpocapsae* elicit a more prolonged time spent grooming over a twenty minute observation period than *H. downesi* and water controls. This suggests that differences in the behaviour of the applied nematode species can cause varying host responses. With different insects, *Acheta domestica*, there was again a significant difference between the nematode treatments and the control but no difference was found between the two EPN treatments. This is different to what was observed in *H. abietis* where there was a significant difference in the reactions caused by *S. carpocapsae* and *H. downesi*. *Acheta domestica* are very active insects compared to *H. abietis*. They have much longer antennae than the relatively short antennae of the pine weevil and the *A. domestica* groom their antennae very frequently. It is likely that any applied nematodes on the head region would be moved around by this regular grooming activity and this would impact on any effect of active dispersal difference between the nematode species.

The difference observed between weevil reactions to *S. carpocapsae* and *H. downesi* suggests a difference in the behaviour of the two species on the insect. It is thought that different EPN species employ different behavioural strategies when seeking out their hosts. These vary from a sit and wait ambush strategy to an active cruising one

with differing levels of intermediate strategies (Lewis *et al.,* 1992). As these nematodes were applied directly to the insect it was uncertain how this would affect their behaviour as this may vary depending on the age of the nematodes (Lewis *et al.*, 1995; Dempsey and Griffin, 2002), substrate they're applied (Shapiro *et al.*, 2000) on and the presence or absence of host cues (Lewis *et al.*, 1992; Lewis *et al.*, 1993). The area of the insect they were applied to, i.e. the head region contains a concentration of the weevil's sensory organs including the eyes and the antennae. If there were large differences in the behaviour of the two EPN species then it is likely that this would affect the insect's reactions to them as was observed in *H. abietis*.

Observations of the nematodes themselves in a similar setup showed that they displayed different behaviours over the course of the twenty minute period that supports the reasoning that differing behaviour patterns caused the different grooming reactions mentioned above. *S. carpocsae* IJs were more likely to actively move about on the surface of the insect than *H. downesi* IJs, with significantly more of them displaying this behaviour. They were also more likely to raise themselves on their tails and wave in the air, a behaviour known as nictating. This behaviour type is commonly practiced by ambush foragers (Kaya and Gaugler, 1993; Ishibashi and Kondo, 1990) and is used to attach to a passing host and perhaps a reaction to volatile cues released by the host. *Heterorhabditis downesi* IJs were significantly more likely to be motionless than those of *S. carpocsae*. This would make them less likely to travel across a sensitive section of the weevil and thus less likely to trigger a grooming reaction.

Sensitivity to the presence of nematodes could cause a change in behaviours other than grooming. If the sensitivity to the presence of nematodes on their bodies extended to nematode presence in the substrate then EPN applications could cause weevils to leave the treated area. An evasive reaction like this has been noted in *Popilla japonica* (Gaugler *et al.*, 1994) and would be highly desirable in *H. abietis* as it would reduce damage caused to seedlings treated with EPN. The roosting experiment showed that the presence of EPN on the substrate did not affect the length of time weevils spent on that substrate when compared to a water control. This suggests that either the nematodes were not detected by the weevils or that they did not initiate a repellent response if they were detected. As the nematodes were applied

directly onto the head region of the weevil in the grooming experiment the nematodes were more likely to cross a sensitive sensory organ. Nematodes on the substrate would only come into contact with the legs and or underside of the insect. This would reduce the likelihood of the weevil detecting the presence of the nematodes and lower any possibility of a reaction. As the number of EPN applied to the relatively small amount of soil was very high, 25,000 IJs and the depth of soil for them to disperse through, approx. 15 mm, was quite shallow and there were still no deterrent effects it is unlikely that field applications of EPN around seedlings would stop weevils crossing the point of application. Were there to be a highly deterrent response to applied EPN then weevils in the field could climb seedlings to avoid the applied nematodes on the soil. This would be highly damaging as it has been shown that even a single weevil can kill a one year old seedling within a few days if the insect feeds on it exclusively (Aoife Dillon, NUIM, Pers. Comm.).

One of the commonest ways animals can become infected with parasites is to ingest them with their food or water. Several trematode parasites are transmitted by ingestion. *Fasciola hepatica* is transmitted to its final host, usually a ruminant grazer, with ingested grass. *Paucivitellosus fragilis* in fish (Rhode, 1984), *Trichostrongylus tenuis* in red grouse (Hudson and Dobson, 1997) and *Giardia lamblia* (Ortega and Adam, 1997) also rely on active ingestion in order to infect their hosts. Insects are also susceptible to contaminated food with many bacterial, viral and fungal pathogens being transmitted through ingestion (Engelhard *et al.*, 1994; Washburn *et al.*, 1998; Pijlman *et al.*, 2003). EPN are thought to primarily invade their hosts through natural openings (Poinar, 1979). It was observed during the grooming experiments that some of the weevils after removing a large number of the nematodes from their head region then ingested the IJs. It was also seen in the route of entry investigations (Chapter 4) that nematodes could invade adult weevils and be found alive in the upper digestive tract within two hours of exposure. This suggests that adult weevils could become infected by active ingestion of EPN. All weevils that were observed ingesting EPN survived for at least two weeks after being seen to do so. As the number of weevils seen ingesting nematodes was small, 6 in total, this does not rule out the possibility of infection by ingestion.

The grooming reactions experiment shows that weevils can detect the presence of EPN on their head region, and so it is possible that in some situations EPN could repel adult weevils if the nematodes could reach sensitive regions like the head in sufficient numbers to cause a reaction. In a field situation this would be desirable as large scale applications of EPN could, in the short term at least, reduce adult feeding on transplanted seedlings. Alternatively if *H. abietis* were to feed in areas with high EPN concentrations they could become infected by ingesting the IJs along with their food. The experiment investigating the effect of the presence of EPN on weevil feeding showed that large numbers of EPN in the area immediately surrounding a food source can reduce weevil feeding if an alternative food source is available. It is likely that weevils would also feed on brash or the crowns of nearby mature trees (Orlander *et al.*, 2000; Orlander *et al.*, 2001). The amount of bark removed by a single *H. abietis* in a day coupled with the extremely high densities of weevils in some clearfells, up to 65,000 per hectare in Irish forests has been estimated by Ward (1991), suggests that seedlings are not the only food source utilised. As *H. abietis* cause most damage on replanted sites it is likely that seedlings planted near stumps would be in an area of very high nematode population density. This high nematode density would be temporary as sampling studies after large scale applications have shown a drastic reduction in nematode populations in the months after application (Dillon *et al.*, 2008). It is also possible that seedlings could be planted some months after the application of EPN to the site; a short-term repellent effect could be missed entirely. The data from the experiment shows a reduction in feeding in the *S. carpocapsae* treatment only and this reduction only occurred for two days after application. The differences in behavioural strategies of the nematode species are likely to be the reason why this reduction was not seen in the *H. downesi* treatments. As *S. carpocapsae* is more inclined to nictate on the surface of a substrate than *H. downesi* the likelihood of its presence being detected by the weevil is increased. As the high of nematode concentration necessary to cause this reduction in feeding would only be on site for a short period and the planting of seedlings could be some time after EPN application, any reduction in feeding on seedlings is likely to be over a very short time period. It is unlikely that this would cause any real reduction in damage in the field. Given the high humidity levels in the experimental setup and the shallow depth of soil in the treated area migration down into the soil and establishment is likely to be much less than that which would be seen in the field (Koppenhöffer *et al.*, 1995). With

nematode application targeted at the summer months it is likely that high soil surface concentrations would occur only fleetingly as the EPN would migrate down into the soil profile to avoid desiccation and UV radiation at the surface. This would make any impact the EPN would have on adult weevil feeding behaviour to be slight.

An alternative strategy that offers a possibility of damage reduction by adults is direct treatment of the seedlings themselves. It has been shown in previous trials by Pye and Pye (1985) that seedlings dipped in suspensions of *S. carpocapsae* prior to planting were more likely to survive. Seedling mortality was reduced from 64 to 21% and *H. abietis* populations were reduced by 89%. It was also shown that spraying seedlings with *S. carpocapsae* almost doubled the proportion of undamaged seedlings from 27 to 52%. Spraying also reduced the proportion of seedlings killed, from 66 to 33%. It is possible that a large number of nematodes on the seedling would be detected by a weevil approaching to feed and cause it to utilize another food source. If the short term repellent effect caused by nematodes (Chapter 4) was increased in longevity in the field then this would be a very effective way of reducing weevil damage. It is also likely that such large concentrations of nematodes would infect and kill any weevils that were in the soil or leaf litter overnight (Buried mortality assay, Chapter 4).

Should the EPN infect the adult weevils, either emerging from or ovipositing on the stumps, while at high post-application concentrations this could have a more significant impact on damage caused. Animals infected by parasites can drastically alter their behaviours. Some hosts that are intermediate in their parasites' life cycle can become more conspicuous to predators that are the parasites' final host, for example acanthocephalan parasites caused increased predation by fish in infected *Gammarus spp.*, the parasites' final host (Bakker *et al.*, 1997). Others can drastically alter their feeding habits.

Many infected animals increase their feeding to compensate for nutritive costs of their parasite load. Some parasites however cause their host to reduce their feeding (Tien *et al.*, 2001; Cossentine *et al.*, 2004) and this scenario is most beneficial in a biological control program. It has been recorded in the United Kingdom that *H. abietis* emerging from EPN treated stumps did not feed on seedling replants on the site (Stuart Heritage, UK Foresty Commission, pers. comm.). In the investigation into effect of

EPN infection on feeding, a large proportion of all the EPN treated weevils were killed by the nematodes especially in the *S. carpocapsae* treatments. There was no significant difference in the amount of feeding done by any of the EPN treated weevils compared to the non-exposed controls. When weevils that died during the course of each assay were compared to those that survived there was also no difference. This shows that even those insects that were going to die within 6 days continued feeding at a similar rate to those that were going to survive. On the contrary it was shown that weevils that were infected with EPN, including some that died and some that did not, fed significantly more than those in which no EPN were found. This data indicate that should field applications of EPN could temporarily increase the feeding rate of infected insects. Damage would eventually be reduced as some proportion of the infected adults would be killed by EPN infection but even a short-term increase in feeding on seedlings could cause widespread seedling deaths as the amount of feeding required to kill a young seedling is low (Aoife Dillon, NUIM, Pers. Comm.). This is an unlikely scenario however as the overall increase in feeding by infected adults was small and any increase in feeding by infected individuals would be offset by the reduction in numbers of insects emerging from stumps post-application.

It is unlikely that the exposure to EPN would occur under conditions as suitable to nematode infection as those carried out in the lab. Exposure of adults in more realistic conditions shows that high mortality of adult weevils under field conditions is unlikely unless the insects were to overnight in an area of high nematode density as limited exposure times at low EPN concentrations are not capable of infecting adult *H. abietis* (Chapter 4). As adult weevils migrating on to clear-felled sites are unlikely to visit stumps after EPN have been applied applications of EPN to seedlings may infect adults sheltering at the base of seedlings over night after feeding.

Weevils were able to mount an immune response to both species as encapsulated nematodes were found in the first feeding assay. Encapsulated EPN were often found in a state of degradation so it is likely, especially by the second and third assays, that some weevils had degraded invading nematodes completely. Encapsulation is an important part of the insect immune response (Lackie *et al.*, 1985;Pech and Strand, 1996; Strand, 2008). Encapsulation first requires haemocytes to recognise the target as foreign (Pech and Strand, 1996; Strand, 2008). Failure to be recognised the target

as a foreign body or an ability by that foreign body to suppress the insect's humoral immune response may contribute to differences of nematode pathogenicity (Li *et al.*, 2007). However, there was no obvious difference in the encapsulation rate of *H. downesi* and *S. carpocapsae* in this experiment.

In the first assay live nematodes were found to have invaded weevils in all treatments and all treatments also had some weevils with live IJs under the elytra. There was a statistical association between high numbers of nematodes under the elytra and the number of nematodes invading the insect. This suggests that the wing cases offer an environment that increases the chances of successfully establishing in the insect. It is likely that the space under the elytra offers a sheltered position in which the nematodes are at reduced risk of desiccation or physical removal from the body of the insect. Nematodes that shelter under the elytra would be able to invade the insect when conditions were more suitable for a successful infection. This "sit and wait" approach would allow a nematode to wait for earlier invading nematodes to weaken the insect's defences before invading. The sheltered area beneath the elytra would also allow the EPN to survive low humidity environments to emerge when the insect moved to more suitable conditions such as under the leaf litter or in the top layers of the soil where *H. abietis* overnight. In the second feeding assay live nematodes were found under the elytra two weeks after exposure. This data shows that nematodes are able to survive on the body of a weevil for at least two weeks in very dry conditions not well suited to nematodes. This would make it possible for weevils to transport live nematodes across a site or from one site to another as has been shown in previous studies (Lacey *et al*., 1995; Parkman and Smart, 1996). Transported nematodes could establish at the new location either by leaving or reproducing in their phoretic host.

Chapter 4

Nematode host-finding and *Hylobius abietis* life stage susceptibility

4.1 <u>Introduction</u>

Current methods employed to control *Hylobius abietis* in replanted Irish forests are centred on applying chemical pesticides to seedlings. The target stage of the insect in this approach is the adult. The pesticide is ingested along with bark from the seedlings so a certain level of damage is accepted by the grower. Due to the cryptic nature of the habitat of the immature stages, i.e. under the bark of stumps on site, reduction of *Hylobius* populations in the stump using chemical pesticides would be difficult. One of the advantages of using entomopathogenic nematodes in biological control is that they can actively seek out their hosts. Various factors influence the behaviour of entomopathogenic nematodes including distant host cues, the substrate and the lipid reserves and age of the nematodes (Burman and Pye, 1980; Lewis *et al*, 1992, Cohen *et al.*, 2002). A lack of understanding of how differing factors alter the host-finding behaviour of EPN can contribute to poor control results in field applications (Georgis and Gaugler, 1991).

The different behavioural strategies employed by entomopathogenic nematode species have been classified by Lewis *et al.* (1992) as being along a continuum between sedentary ambushers to more active cruising strategists. *Steinernema carpocapsae,* classed as an ambush strategist, is thought to stay near the soil surface (Georgis and

Poinar, 1983) and lift its body into the air in order to attach to a passing host. Ambush strategists are considered to be more successful in infecting more mobile, surface dwelling hosts; while cruisers are expected to infect less mobile hosts underground (Campbell and Gaugler, 1993). The evidence for this is mostly based on laboratory experiments that were often carried out on artificial substrates such as agar or sand columns (Glazer & Lewis, 2000) and are unlikely to be closely mirrored in a more complex substrate such as would be found in a field application. The use of *S. carpocapsae* to control insects in cryptic soil habitats has shown that the nematodes can penetrate through the soil, up to 50 cm in field trials against *Hylobius abietis* (Dillon *et al.*, 2006).

The presence of roots can influence the movement of entomopathogenic nematodes through soil. The physical presence of the surface of the root could also offer a route along which the IJs could travel. The presence of roots in soil has been shown to both positively and negatively affect the rate of host infection by EPN. Choo and Kaya (1991) showed that low density roots in a humus soil can significantly increase the infection of *Galleria mellonella* larvae by *H. bacteriophora.* In trials against *H. abietis* in Ireland *S. carpocapsae* performed better against this insect host than its expected foraging strategy would predict (Dillon *et al.*, 2006; Dillon *et al.*, 2007). They suggested that the applied nematodes may use the roots of the stump as a physical routeway to travel along. This routeway could assist the IJs movement as they would avoid moving through the more complex forest soil. One of the aims of this chapter is to investigate the influence that physical routeways in the soil have on host-finding in *S. carpocapsae* and *H. downesi.*

Soil composition and texture can have a strong influence on the efficacy of EPN in killing insects. Soils with a large pore size tend to favour dispersal (Georgis and Poinar, 1983) and survival of EPN (Kung *et al.,* 1990). Prevalence of EPN in soil sampling surveys is higher in organic or sandy soils compared to clays and loams (Miduturi *et al.,* 1996; Dillon *et al.*, 1999). This suggests that soils with a smaller pore size and higher water retention are less suitable for nematode movement and survival. Water content (Kaya, 1990) and pH (Fisher and Fuhrer, 1990) of soil have been shown to influence EPN dispersal and host finding abilities so the texture of the substrate should have an impact on infection rates. In Ireland much of the planting of

coniferous plantations is on peat soils with high organic content. Where soils are more pure mineral the soil is regularly overlain with a layer of peat which gets mixed in during planting. Field trials of EPN against *H. abietis* have been conducted in Ireland in deep and shallow peats (Dillon *et al.*, 2006; Dillon *et al.*, 2007) and in mineral soils (Dillon *et al.*, 2008). In all of these trials *H. downesi* proved to be more effective than *S. carpocapsae*. An objective of this chapter is to investigate the impact of soil type on host finding in *S. carpocapsae* and *H. downesi*.

While a greater understanding of the behaviour of the EPN is desirable it is also important to assess which of the life stages of the target insect is most susceptible. *Hylobius abietis* have five main developmental stages that occur in the stump. The first stage, the egg, is of little interest as insect eggs are not significantly more attractive to IJs than plastic beads of a similar size and shape (Pollard, unpublished data). The larva has the longest duration in the stump, between ten and 24 months and goes through several instars. This is followed by the pupal stage that can last from several weeks or if overwintering several months, then a brief callow adult stage and finally a hardened adult that exits the stump. Many factors influence the length of each of the developmental stages. Time of oviposition and temperature are the most important factors (Christiansen, 1971; Lekander *et al.*, 1985) though other factors such as host tree species play a part (Bakke and Lekander, 1965; Thorpe and Day, 2002). Development in the UK from egg to adult takes from 12-36 months (Leather *et al.*, 1999). If one of these stages were to prove more susceptible than the others then timing application to when the most susceptible stage was present would be extremely beneficial. In previous field trials of EPN against *H. abietis* populations in stumps it was found that that the various insect life stages had different susceptibility to EPN infection. Dillon *et al.* (2006) found that 45% of larvae, 32% of pupae and 30% of adults in the stumps were killed by EPN. Brixey *et al.* (2006) found that 14% of larvae, 13% of pupae and 44% of callow adults were infected by EPN after application to stumps. In laboratory trials on a cotton substrate with *S. carpocapsae* Pye and Burman (1978) found that between 80 and 95% of larvae were killed by EPN with 0% and 5% of pupae and adults killed respectively.

The objectives of this chapter were:

1. To assess the contribution that physical routeways have on host finding by *S. carpocapsae* and *H. downesi* and the impact of substrate type (peat or sand/peat mix) on host finding by *S. carpocapsae.*

2. To investigate any impact, if any, that *H. abietis* larval feeding has on susceptibility to EPN infection.

3. To assess the susceptibility of various life stages of *H. abietis* to EPN infection. Larvae, pupae and adult *H. abietis* are indefinitely exposed to various concentrations of *S. carpocpasae* and *H. downesi* on filter paper to investigate the effect of EPN concentration on mortality.

4. To ascertain the route by which EPN enter adult *H. abietis*.

4.2 Materials and Methods

4.2.1 *Nematodes*

Three species of nematodes were used, *Steinernema carpocapsae* (All strain)*, Heterorhabditis downesi* (K112 strain) and *Steinernema feltiae* (4CFMO strain). Nematodes were cultured as described in Chapter 2. IJs were stored at 9°C at a concentration of 2000 IJs/ml. All nematodes used were between three and five weeks after emergence.

4.2.2 *Weevils*

Hylobius abietis adults used were either trapped as they emerged from the stumps (See section in General Methods) or directly removed from seedlings on replanted conifer sites in Ireland (Chapter 2). The adults were then stored in ventilated boxes at 20°C and fed with fresh conifer billets for up to three weeks until use. Larvae, pupae and callow adults were gathered from conifer tree stumps between 18 and 24 months after felling. Soil surrounding the stumps was removed to a depth of approximately 40cm. The bark was then removed with a chisel and immature *H. abietis* removed with a blunt forceps. Insects were stored in 24-well multi-well tissue culture plates. The lids of the plates were lined with damp tissue paper. Immature insects were stored at 9°C for up to two weeks until required.

4.2.3 *The effect physical routeways and feeding by* Hylobius abietis *larvae on infection by entomopathogenic nematodes.*

A sand substrate was prepared by heat sterilising sand at 120°C for 12 hours. The sand was then allowed to cool to room temperature before 8% water by weight was added. A sand/peat mix substrate was prepared by mixing 8% w/w sand, prepared as

above, with milled moss peat compost in 1:1 ratio by volume. Cylindrical plastic pots with a diameter of 100 mm and a height of 120 mm were filled to a depth of 80 mm with one of the substrates. Late instar *H. abietis* larvae were placed in cages of wire mesh. Each cage was made from a section of aluminium mesh measuring approximately 60 mm by 40 mm, rolled into tubes approximately 15 mm in diameter. The edge strands of the mesh were then intertwined and one end of the tube pinched to form a roughly cylindrical shape with one open end. Once the insect was placed in each cage the open end was pinched closed and four of the cages were placed equidistant along the bottom circumference of the pot. Each substrate (sand or sand/peat mix) was used for each of four treatments. These consisted of:

(1) Routeways and Feeding: Spruce twigs measuring 60 mm in length with a diameter of approximately 8 mm were placed in the substrate pointing from the centre diagonally down to each insect. The end of the twig was placed alongside the insect inside the wire mesh in such a way that the insect could feed on it.

(2) Routeways Only: As (1) above except the conifer twig was placed outside the wire mesh of each insect.

(3) Feeding Only: A 3 mm section of conifer twig was placed in the wire mesh with each insect.
(4) No Routeway or Feeding (= Neither): A 3mm section of conifer twig was placed outside the wire mesh of each insect.

At the top of the substrate in each pot a small depression 5 mm in diameter was made in the centre using the handle of a mounted needle. A suspension of 5000 IJs of either *S. carpocapsae* or *H. downesi* was applied in 1ml of water to this depression. Pots were then covered with snap-on plastic lids and incubated for 8 hours at 20°C in the first experiment and 12 hours in the subsequent experiments. All insects were removed and washed with water. The insects were placed in wells of 24-well multiwell plates and incubated at 20°C for 2 weeks. Dead insects were dissected and the number of nematodes found recorded.

In the first experiment only the sand substrate was tested for both species of nematode with a total of 30 insects for each treatment. In the second, third and fourth experiments only *S. carpocapsae* was used for both substrates with a total of 20 insects for each treatment in these three experiments. A different batch of nematodes reared on different dates was used for each experiment.

4.2.4 *Susceptibility of immature* Hylobius abietis *larvae and pupae to infection by entomopathogenic nematodes on filter paper.*

Late instar larvae and pupae of *Hylobius abietis* were placed in wells of 24-well tissue culture plate that had been lined with one 1 cm diameter disc of filter paper. Each insect was then treated with either *S. carpocapsae* or *H. downesi* applied to the filter paper in 50 µl of water. For larvae the concentrations were 25, 50, 100, 200, or 500 IJs. For pupae the concentrations were 25, 50, 100 or 200 IJs. Control treatments for both larvae and pupae had 50 µl of water applied to the filter paper. The insects were then incubated for 2 weeks at 20°C. Insects were checked daily for mortality and the life stage at time of death was noted. The total number of larvae and pupae tested at each concentration was 24.

4.2.5 *Susceptibility of adult* Hylobius abietis *to infection by entomopathogenic nematodes on filter paper*

Discs of filter paper 1 cm in diameter were placed in 20 wells of 24-well tissue culture plates. Nematodes were applied to the filter paper in 50 µl of water. Treatments were 50, 100, 500 or 1000 IJs of *S. carpocapsae* or 100, 500, 1000 or 4000 IJs of *H. downesi.* A control treatment consisted of 50 µl of water. A single adult weevil was placed in each of the 20 wells and the lid was put on the plate. Plates were sealed with Parafilm laboratory tape and were stored at 20°C. Insects were checked for mortality daily and dead insects were dissected and the presence of nematodes counted. Each treatment consisted of 20 weevils.

4.2.6 *The effect of exposure time on susceptibility of the various life stages of* Hylobius abietis *to infection by* Heterorhabditis downesi *on filter paper.*

Single larvae, pupae, callow adults and adults of *Hylobius abietis* were placed in wells of 24-well tissue culture plates that had been lined with one 1 cm disc of filter paper. Two hundred IJs of *H. downesi* were applied to each well in 200 µl of water. Lids were put on the tissue culture plates and they were incubated at 20°C for 1, 8, 24 or 48 hours. Insects were removed and washed and were then placed in wells of clean 24-well tissue culture plates. The insects were then incubated at 20°C for 12 days. Pupae and adults were treated at all exposure times. Callow adults were exposed for 1, 8 and 24 hours only. Larvae were exposed for 8 and 24 hours only. All treatments consisted of 10 insects.

4.2.7 *Susceptibility of adult* Hylobius abietis *to three nematode species in a peat medium.*

Single adult weevils were buried in milled peat compost inside a bijou screw cap tube measuring 50 mm height and 17 mm diameter. The compost was packed into the tube so that the weevil was surrounded at all times. Suspensions of 500, 1000, 2000 or 4000 IJs in 500µl of water were applied to the top of the compost and the lid was then screwed on. The tubes were then left at room temperature for 12 hours. Weevils were then removed from the tubes and placed singly in wells a 24-well tissue culture plate. The lid of the plate was lined with moistened tissue paper and the plates were incubated at 20°C. Insect mortality was checked daily for 12 days. Ten weevils were treated for each nematode concentration and the experiment was repeated three times. In the first experiment *S. feltiae, S. carpocapsae* and *H. downesi* were applied, in the second and third experiments only *S. carpocapsae* and *H. downesi* were used. A different batch of nematodes reared on different dates was used for each experiment.

4.2.8 *Route of entry of entomopathogenic nematodes into adult* Hylobius abietis.

One hundred thousand IJs of *S. carpocapsae* were applied in 2 ml of water to a 9 cm disc of filter paper in the base of a 9 cm Petri dish. Twenty-five adult *H. abietis* were then placed in the dish which was incubated at 20°C. Five insects were removed after 60, 90, 120, 180 and 240 minutes. The insects were removed and placed in a 500 ml conical flask. Five millilitres of chloroform was applied to a ball of cotton wool approx. 5 cm in diameter and this was then placed in the conical flask. The top of the flask was then sealed with a rubber bung and left for ten minutes. The bung was then

removed and the flask placed in a fume hood and the chloroform allowed to evaporate. The insects were removed and placed singly in 5 cm Petri dishes of water for dissection. Dissection was carried out as described in Section 3.2.7 and the presence and location of IJs was noted.

4.2.9 *Statistical analyses*

All statistics in this chapter were carried out using the Minitab 14® software package for Microsoft Windows®. Data of 3 or more test groups were analysed using a Kruskal-Wallis analysis as data of this kind could not be normalized in this chapter. Proportional data was analysed using a Chi-square test. Data compared between two groups was analysed using an unpaired t-test or a Mann-Whitney where data could not be normalised. Lethal concentrations and lethal exposure times were calculated using Probit analysis. Non-normally distributed data was transformed using a square-root or natural log transformation where it was possible to normalise by transformation. Significance levels were taken to be where $P < 0.05$.

4.3 Results

4.3.1 *The effect of routeways, feeding and substrate type on infection of* Hylobius abietis *larvae with entomopathogenic nematodes.*

In the first experiment, both *S. carpocapsae* and *H. downesi* were applied to test the impact of routeways and insect feeding on infection of *H. abietis* larvae in sand (Table 4.3.1a). When all 8 treatments (Routeways & Feeding, Routeway Only, Feeding Only and Neither for both *S. carpocapsae* and *H. downesi*) are compared there is a significant difference in *H. abietis* mortality (Chi-square = 45.923, df = 7, P = 0.000) (Table 4.3.1b). When all *S. carpocapsae* treatments are pooled there is no significant difference across the treatments (Chi-square = 6.726, df = 3, P = 0.081), however this is significantly different at the 10% level. When all *H. downesi* treatments were pooled the treatments were found to have significant differences in the number of insects killed (Chi-square = 10.062, df = 3, P = 0.018). The presence of twigs in an 8% w/w sand substrate did not significantly effect the number of insects killed by either *H. downesi* (Chi-square = 0.745, df =1, P = 0.388) or *S. carpocapsae* (Chi-square = 1.690, df = 1, P = 0.194) after an 8 hour exposure to EPN. Insects that were able to feed were not significantly more likely to be killed by *H. downesi* (Chi-square = 7.194, df = 1, P = 0.666) but significantly more insects were killed by *S. carpocapsae* when they could feed (Chi-square = 4.174, df = 1, P = 0.041). In all treatments *H. downesi* killed 92 out of a total of 120 insects while *S. carpocapsae* killed 49 out of 120, a highly significant difference (Chi-square = 31.790, df = 1, P = 0.000).

Table 4.3.1a: Experiment 1: The effect of routeways, feeding and EPN species on mortality of *H. abietis* larvae.

Experiment 1			
	Dead	**Alive**	**% Dead**
H. downesi			
Routeways + Feeding	20	10	66.7
Routeways Only	28	2	93.3
Feeding Only	25	5	83.3
Neither	19	11	63.3
Total	**92**	**28**	**76.7**
S. carpocapsae			
Routeways + Feeding	12	18	40
Routeways Only	9	21	30
Feeding Only	18	12	60
Neither	10	20	33.3
Total	**49**	**71**	**40.8**

Table 4.3.1b: Results of Chi-square tests for Experiment 1.

Species	Treatment	Chi-square	df	P
H. downesi	Roots v No Roots	6.726	1	0.388
	Feeding v No Feeding	7.194	1	0.666
S. carpocapsae	Roots v No Roots	1.690	1	0.194
	Feeding v No Feeding	4.174	1	0.041
Both	*H. downesi v S. carpocapsae*	31.790	1	0.000

In experiment 1, two EPN species, *S. carpocapsae* and *H. downesi,* were compared in a sand substrate. The total percentage of insects killed was higher in *H. downesi* treated insects, 76.7%, than insects in *S. carpocapsae* treatments, 40.8% (Table 4.3.1a). The presence of twigs in the substrate ("Routeways Only" and "Routeways and Feeding" treatments pooled) did not significantly change the mortality of insects by either EPN species (Table 4.3.1b). The number of insects killed that were able to feed ("Feeding Only" and "Routeways and Feeding" treatments pooled) differed significantly from the treatments in which the insect could not feed in *S. carpocapsae* treatments only (Table 4.3.1b).

In experiments 2-4 one EPN species, *S. carpocapsae*, was tested in two different substrates, sand and a sand/peat mix. The exposure time was also increased from 8 to 12 hours. When results from both substrate types are pooled there was a highly significant difference in *H. abietis* mortality across all treatments (Chi-square = 64.128, df = 7, P < 0.001)/ In order to investigate what factors were responsible for these differences in mortality further Chi-square analyses were carried out. When all treatments are assessed separately for the two different substrates there are significant differences across the treatments for both the sand substrate (Chi-square = 37.511, df = 3, P = 0.000) and the sand/peat mix (Chi-square = 26.599, df = 3, P = 0.000). The number of insects killed by *S. carpocapsae* with twigs in the substrate (145 out of 240) was significantly different to the number of insects killed when there were no twigs in the substrate (76 out of 240) (Chi-square = 39.925, df = 1, P = 0.000). Twigs in the substrate caused significantly different mortality in both the sand (Chi-square = 35.753, df = 1, P = 0.000) and the sand/peat mix (Chi – square = 18.438, df = 1, P = 0.000). Mortality of insects that were able to feed did not differ significantly from those that could not feed in the sand substrate (Chi-square = 1.690, df = 1, P = 0.194) but did differ in the mixed substrate (Chi-square = 6.112, df = 1, P = 0.013). The two different substrates, 8% w/w sand and a sand peat mix, had no effect on the numbers of insects killed by *S. carpocapsae* (Table 4.3.1c) when data from all treatments in the three experiments was pooled (Chi-square = 0.008, df = 1, P = 0.927).

Table 4.3.1c: Experiments 2-4: The effect of routeways, feeding and substrate on mortality of *H. abietis* larvae to *S. carpocapsae*.

Experiment 2			
	Dead	**Alive**	**% Dead**
Sand			
Routeways + Feeding	11	9	55
Routeways Only	11	9	55
Feeding Only	4	16	20
Neither	6	14	30
Total	**32**	**48**	**40**
Sand/Peat Mix			
Routeways + Feeding	11	9	55
Routeways Only	10	10	50
Feeding Only	8	12	40
Neither	5	15	25
Total	**34**	**46**	**42.5**
Experiment 3			
	Dead	**Alive**	**% Dead**
Sand			
Routeways + Feeding	15	5	75
Routeways Only	10	10	50
Feeding Only	9	11	45
Neither	4	16	20
Total	**38**	**42**	**47.5**
Sand/Peat Mix			
Routeways + Feeding	15	5	75
Routeways Only	5	15	25
Feeding Only	5	15	25
Neither	1	19	5
Total	**26**	**54**	**32.5**
Experiment 4			
	Dead	**Alive**	**% Dead**
Sand			
Routeways + Feeding	14	6	75
Routeways Only	15	5	50
Feeding Only	5	15	45
Neither	2	18	20
Total	**36**	**44**	**47.5**
Sand/Peat Mix			
Routeways + Feeding	16	4	80
Routeways Only	12	8	60
Feeding Only	7	13	35
Neither	10	10	50
Total	**45**	**35**	**56.25**

Table 4.3.1d: Results of Chi-square tests for Experiments 2-4.

Substrate	Treatment	Chi-square	df	P
Sand	Roots v No Roots	26.599	1	0.000
	Feeding v No Feeding	1.690	1	0.194
Sand/peat Mix	Roots v No Roots	18.438	1	0.000
	Feeding v No Feeding	6.112	1	0.013
Both	Sand v Sand/peat Mix	0.008	1	0.927

4.3.1b *Nematodes found upon dissection of insects.*

The mean number of nematodes invading the *H. abietis* larvae in the first experiment ranged from 10 IJs in the *S. carpocapsae* "Neither" treatment to 14.56 IJs in the *H. downesi* "Food only" treatment (Fig 4.3.1a). There was no significant difference due to treatment in the number of invading nematodes in the insects killed by *H. downesi* (Kruskal-Wallis, P = 0.736, H = 1.27, df = 3) or *S. carpocpasae* (Kruskal-Wallis, P = 0.663, H = 1.58, df = 3). Twigs in the substrate did not significantly alter the number of EPN invading killed insects for *H. downesi* (Mann-Whitney, W = 2343.0, P = 0.3878) or *S. carpocapsae* (Mann-Whitney, W = 543.0, P = 0.896) treatments. There was similarly no significant difference in the number of invading EPN in killed insects that could or could not feed for both *H. downesi* (Mann-Whitney, W = 2162.0, P =0.5899) and *S. carpocapsae* (Mann-Whitney, W = 892.5, P = 0.2424) treatments.

In the experiments with different substrates (Experiments 2-4) the mean number of invading nematodes ranged from 1.63 IJs in the "Neither" treatment of the sand substrate to 7.23 IJs in the "Route & Food" treatment of the mixed substrate (Fig 4.3.1b). The number of IJs invading across the treatments differed significantly in a mixed substrate (Kruskal-Wallis, P = 0.005, df = 3). There was a similar trend in the sand only substrate, significant at the 10% level (Kruskal-Wallis, P = 0.073, df =3). In both substrates the highest mean number of invading nematodes was found when both a routeway was present and the insects were allowed to feed (Fig 4.3.1b). In the sand substrate the number of invading nematodes found on dissection of insects was not significantly different due to twigs in the substrate (Mann-Whitney, W = 3908.5, P = 0.8689). Insects that were able to feed had a significantly different number of

invading nematodes compared to those that could not feed in this substrate (Mann-Whitney, W = 3378.0, P = 0.0295). In the mixed substrate the presence of twigs in the substrate did not significantly affect the numbers of invading EPN in killed insects (Mann-Whitney, W = 3554.0, P = 0.0599), though the difference is significant at the 10% level. Killed insects that were able to feed differed significantly in the number of invading EPN (Mann-Whitney, W = 3468.0, P = 0.0021). The numbers of invading EPN from all treatments in each substrate was also significantly different (Mann-Whitney, W = 9470.0, P = 0.0048).
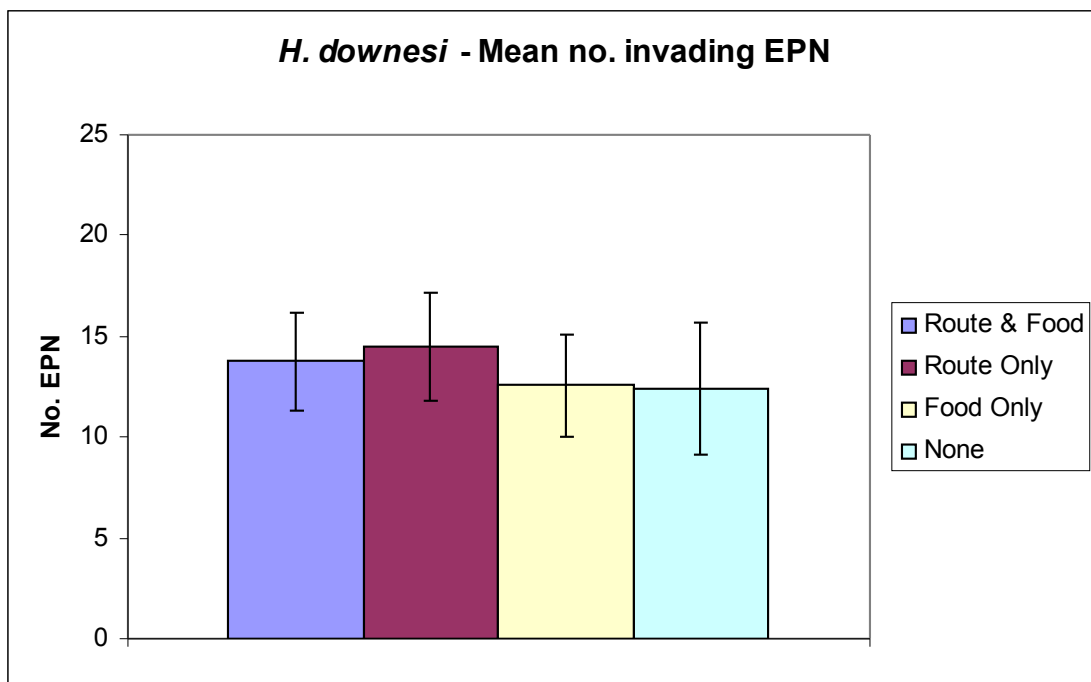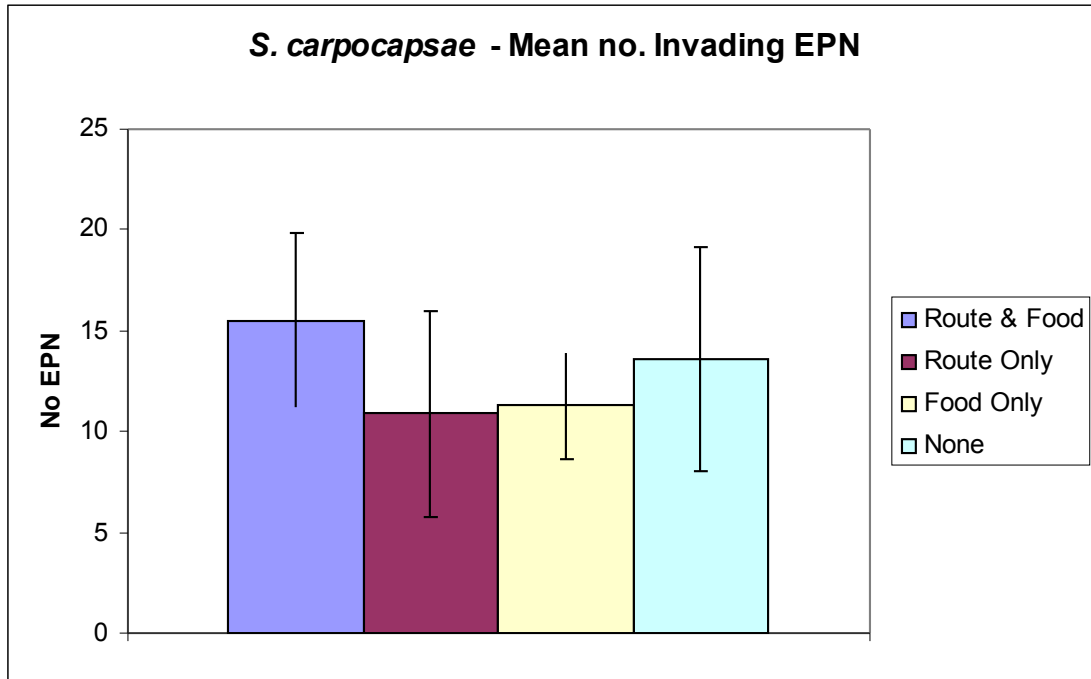
Fig 4.3.1a: Mean number (+/- SE) of IJs of *S. carpocapsae* (Upper Figure) and *H. downesi* (Lower Figure) found upon dissection of *H. abietis* larvae after exposure for 8 hours in an 8% w/w sand substrate and incubation at 20°C. Route & Food = Larvae were adjacent to a conifer twig that they could feed on. Route only = Larvae were adjacent to a conifer twig that they could not feed on. Food only = Larvae were adjacent to a small piece of conifer bark that they could feed on. Neither = Larvae were adjacent to a small piece of conifer twig that they couldn't feed on.

No. Invading EPN - Mixed Substrate

No. Invading EPN - Sand Substrate
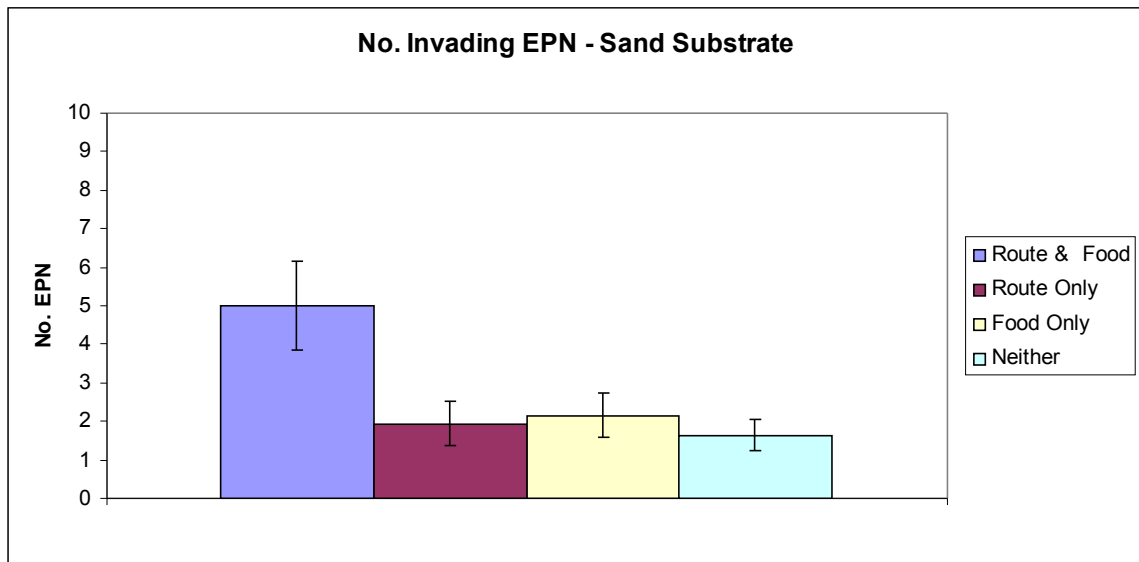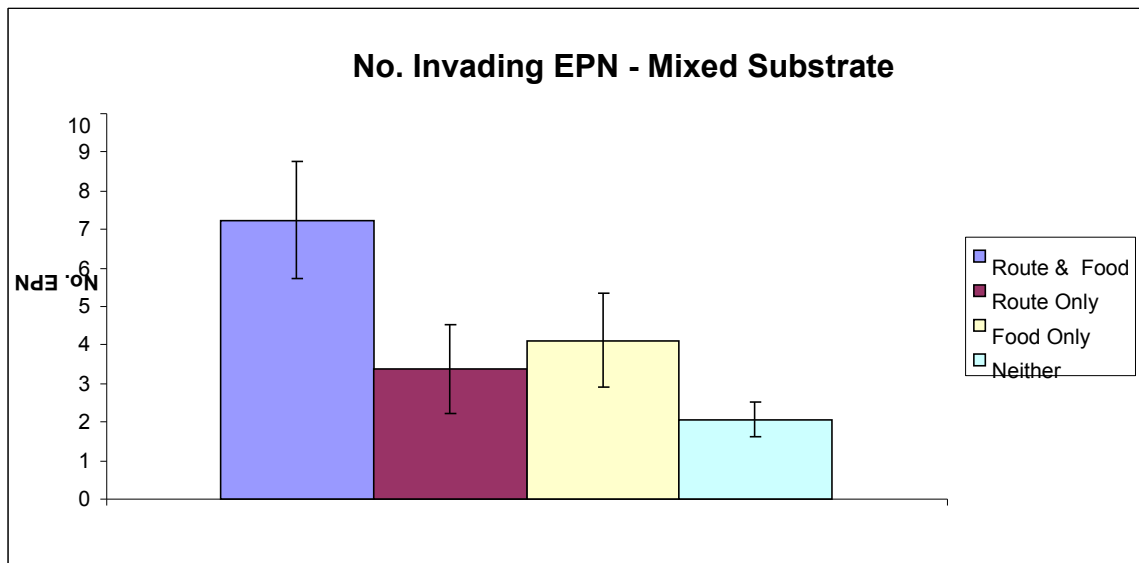
Fig 4.3.1b: Mean number (+/- SE) of *S. carpocapsae* found upon dissection of *H. abietis* larvae after exposure in a peat and sand mixed substrate (upper figure) and a sand substrate (lower figure) for 12 hours and incubation at 20°C. Route & Food = Larvae were adjacent to a conifer twig that they could feed on. Route only = Larvae were adjacent to a conifer twig that they could not feed on. Food only = Larvae were adjacent to a small piece of conifer bark that they could feed on. Neither = Larvae were adjacent to a small piece of conifer twig that they couldn't feed on. Data shown are a mean of three experiments (Experiments 2-4) with n = 20 for each treatment in each experiment.

4.3.2 *The susceptibility of* Hylobius *larvae, pupae and adults to entomopathogenic nematodes on filter paper.*

The effect of nematode concentration on filter paper on *H. abietis* larvae and pupae

Larvae and pupae were exposed to four common concentrations of EPN on filter paper (25, 50, 100 and 200 IJs) and larvae were also exposed to one higher concentration (500 IJs) of *H. downesi* and *S. carpocapsae*. In three of the four common concentrations the proportion of *H. abietis* larvae killed by both *S. carpocapsae* and *H. downesi* (Table 4.3.2a) was higher in three of the four common concentrations than the proportion of pupae killed. When the data from all the common concentrations were pooled significantly more larvae were killed than pupae by *H. downesi* (Chi-square = 35.230, P = 0.000, DF =1) and *S. carpocapsae* (Chi-square = 9.299, P = 0.002, DF = 1). During the larval assay 15 insects pupated. Of these 8 died due to nematode infection, a small proportion of the total insects killed (Fig 4.3.2a). During the pupae assay 118 of the 192 insects developed into callow adults. Of these callow adults 81 died due to nematode infection, representing a relatively large proportion of the killed insects (Fig 4.3.2b). At all concentrations of both EPN species at least 20 of the 24 the larvae were killed (Fig 4.3.2c and Fig 4.3.2d). This made any accurate LC_{50} calculation impossible. The expected concentration response was not seen in pupae, in particular in the case of those insects treated with *H. downesi* (Table 4.3.2a) (Fig 4.3.2d). The LT50 and LT90 values were calculated for both *S. carpocapsae* and *H. downesi* for both larvae and pupae (Table 4.3.2b).

Table 4.3.2a: Mortality of *H. abietis* larvae and pupae to a range of concentrations of *S. carpocapsae* and *H. downesi*. For all treatments a total of 24 insects were exposed to EPN.

Larvae		
Species	**Concentration**	**No. Killed**
H. downesi	25	21
	50	23
	100	24
	200	24
	500	24
S. carpocapsae	25	20
	50	21
	100	23
	200	22
	500	23
Control	0	1
Pupae		
Species	**Concentration**	**No. Killed**
H. downesi	25	12
	50	22
	100	12
	200	11
S. carpocapsae	25	16
	50	13
	100	15
	200	24
Control	0	0

S. carpocapsae - Larvae Assay - Life Stage of Dead insects

Bar chart showing percentage of dead insects by life stage (Pupae, Larvae) at concentrations 25, 50, 100, 200, 500. Numbers on bars: 20, 21, 23, 22, 23.

H. downesi - Larvae Assay - Life Stage of Dead Insects

Bar chart showing percentage of dead insects by life stage (Pupae, Larvae) at concentrations 25, 50, 100, 200, 500. Numbers on bars: 21, 23, 23, 24, 24.

Fig 4.3.2a: Life stage of *H. abietis* at the time of death after exposure as larvae to various concentrations of *Steinernema carpocapsae* (Upper Figure) and *Heterorhabditis downesi* (Lower Figure) on filter paper in the wells of a 24-well tissue culture plate. Data are presented as a percentage of dead insects in that treatment. Numbers on bars indicate total number of dead insects in that treatment.

S. carpocapsae - Pupae Assay - Life Stages of Dead Insects

H. downesi - Pupae Assay - Life Stages of Dead Insects
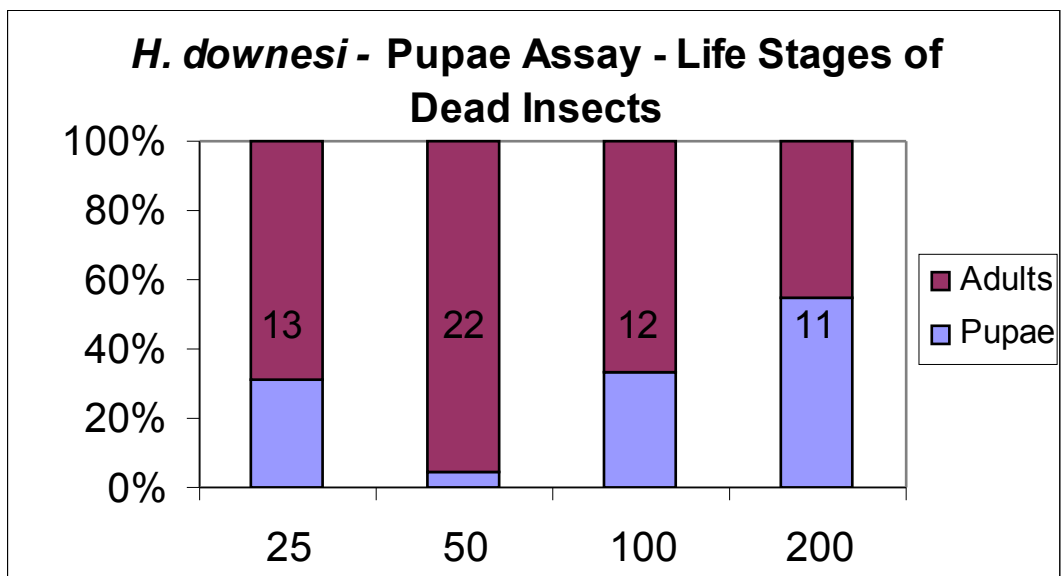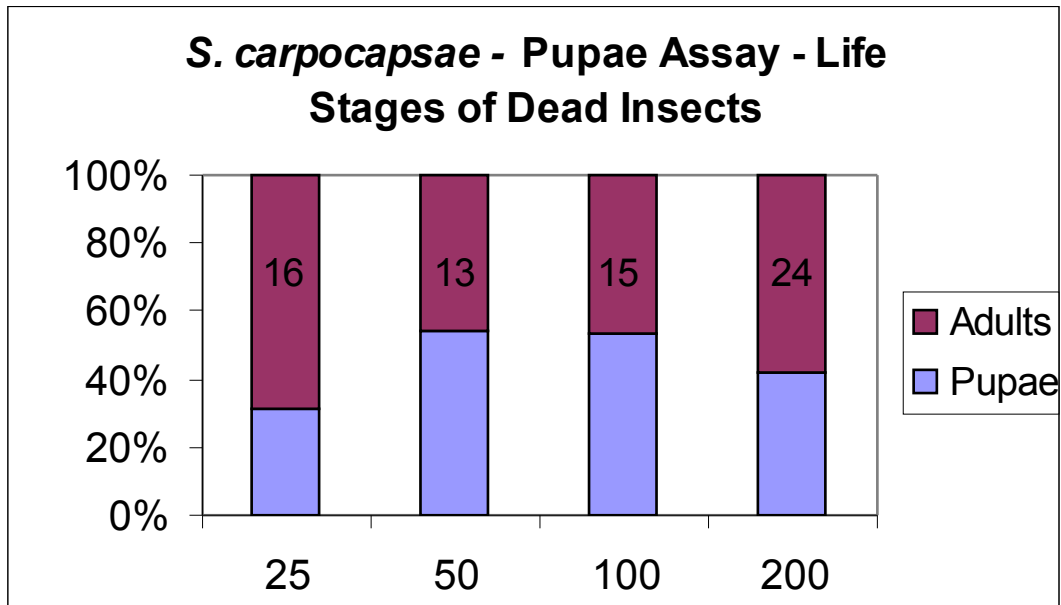
Fig 4.3.2b: Life stage of *H. abietis* at the time of death after exposure of pupae to various concentrations of *Steinernema carpocapsae* (Upper Figure) and *Heterorhabditis downesi* (Lower Figure) on filter paper in the wells of a 24-well

tissue culture plate as a percentage of dead insects. Numbers on bars indicate total number of dead insects in that treatment.

Table 4.3.2b: LT_{50} values (in days) and 95% CI values for different concentrations of two EPN species applied to larvae and pupae of *H. abietis.*

Larvae			
Species	**EPN Concentration**	**LT_{50} +/- SE (days)**	**Lower Fc – Upper Fc**
H. downesi	25	5.43 +/- 0.466	4.06 – 6.16
	50	4.19 +/- 0.467	2.90 – 4.81
	100	4.91 +/- 0.272	4.08 – 5.36
	200	3.71 +/- 0.410	2.10 – 4.02
	500	3.35 +/- 0.128	-1.04 – 2.01
S. carpocapsae	25	5.45 +/- 0.617	4.62 – 6.62
	50	5.43 +/- 0.466	4.67 – 6.31
	100	4.42 +/- 0.408	3.92 – 5.23
	200	5.28 +/- 0.441	4.65 – 6.14
	500	4.24 +/- 0.646	3.80 – 5.59
Pupae			
Species	**EPN Concentration**	**LT50 +/- SE (days)**	**Lower Fc – Upper Fc**
H. downesi	25	12.11 +/- 0.648	11.09 – 13.28
	50	7.83 +/- 0.277	7.21 – 8.80
	100	12.03 +/- 1.34	9.70 – 12.87
	200	14.46 +/- 1.584	10.78 – 12.70
S. carpocapsae	25	9.75 +/- 0.449	8.95 – 10.70
	50	12.39 +/- 1.071	9.92 – 11.73
	100	10.81 +/- 0.662	10.14 – 13.48
	200	6.09 +/- 0.189	5.19 – 6.73
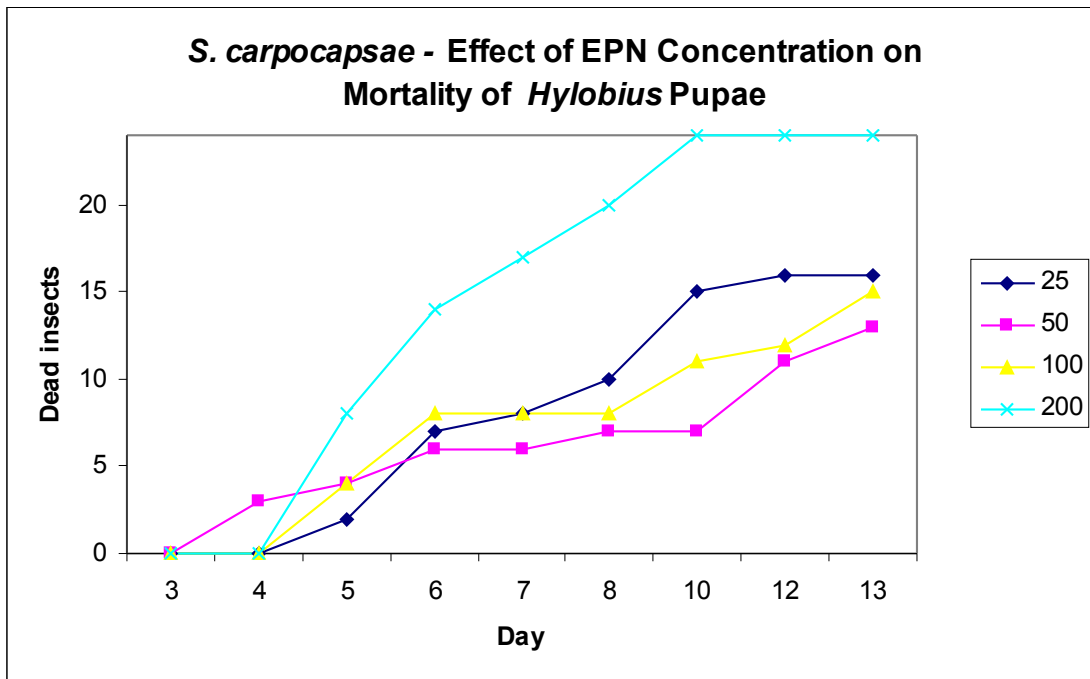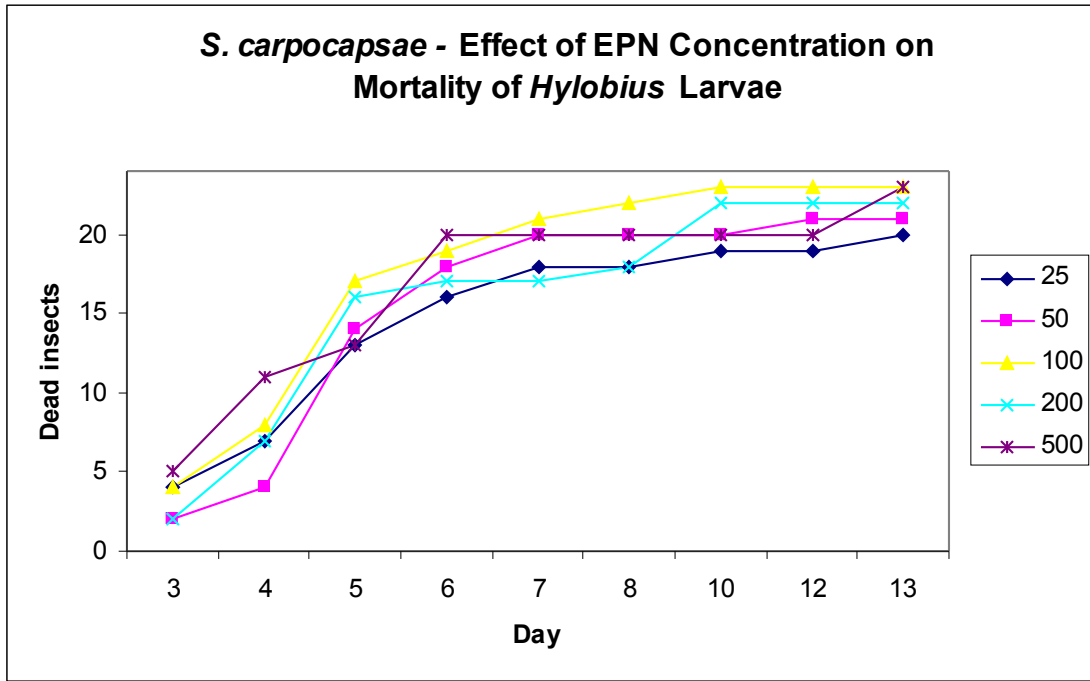
Fig 4.3.2c: Cumulative number of *H. abietis* larvae (Upper Figure) and pupae (Lower Figure) killed by various concentrations of *Steinernema carpocapsae* over time, after

continuous exposure on filter paper in wells of a multi-well tissue culture plate. For each treatment n = 24.

H. downesi - Effect of EPN Concentration on Mortality of Hylobius Larvae

H. downesi - Effect of EPN Concentration on Mortality of Hylobius Pupae
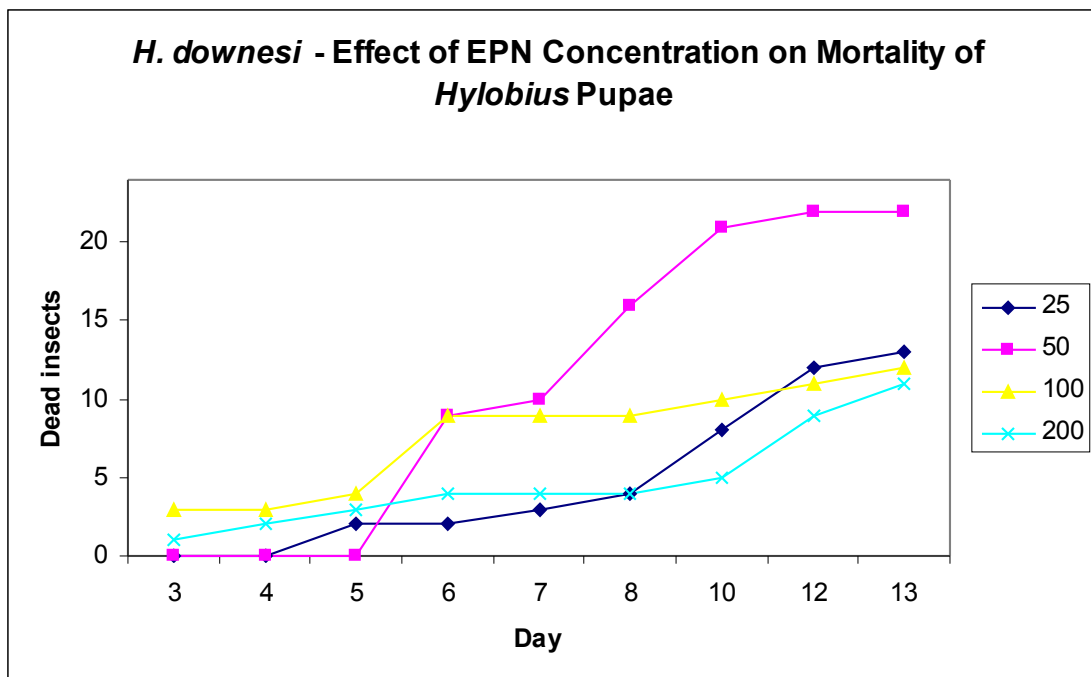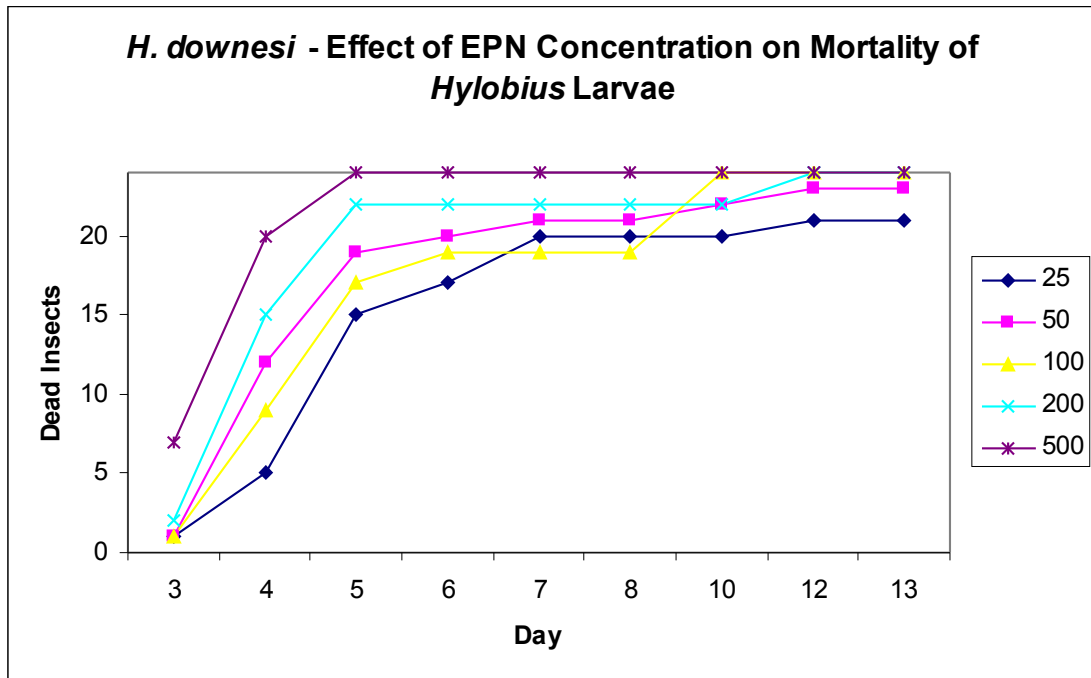
Fig 4.3.2d: Cumulative number of *H. abietis* larvae (Upper Figure) and pupae (Lower Figure) killed by various concentrations of *Heterorhabditis downesi* over time after continuous exposure on filter paper in wells of a multi-well tissue culture plate. For each treatment n = 24

Adult *Hylobius* susceptibility to entomopathogenic nematodes

Five days after exposure to nematodes on filter paper (Fig. 4.3.2e, Table 4.3.2c) *S. carpocapsae* killed significantly more adult *H. abietis* than *H. downesi* at concentrations of 100 (Chi-square = 8.64, df = 1, P = 0.003) and 500 IJs (Chi-square = 10.101, df = 1, P = 0.001). There was no difference found between the two species at a concentration of 1000 IJs at that time (Chi-square = 1.29, df = 1, P = 0.256). Ten days after exposure there was no significant difference in the number of *H. abietis* killed by 100 IJs of *S. carpocapsae* and *H. downesi* (Chi-square = 3.75, df = 1, P = 0.053). At a concentration of 500 IJs *S. carpocapsae* killed significantly more weevils than *H. downesi* (Chi-square = 7.059, df = 1, P = 0.008). At a concentration of 1000 IJs all insects were killed by both nematode species 10 days after exposure.

Table 4.3.2c: Mortality of adult *Hylobius abietis* 5 and 10 days after exposure to 4 concentrations of *S. carpocapsae* or *H. downesi* on filter paper.

	S. carpocapsae			
	50	**100**	**500**	**1000**
Day 5	9	12	14	17
Day 10	13	15	20	20
	H. downesi			
	50	**100**	**500**	**1000**
Day 5	3	4	14	19
Day 10	9	14	20	20

S. carpocapsae - Effect of Dose on Adult *Hylobius abietis* Mortality

H. downesi - Effect of Dose on Adult *Hylobius abietis* Mortality
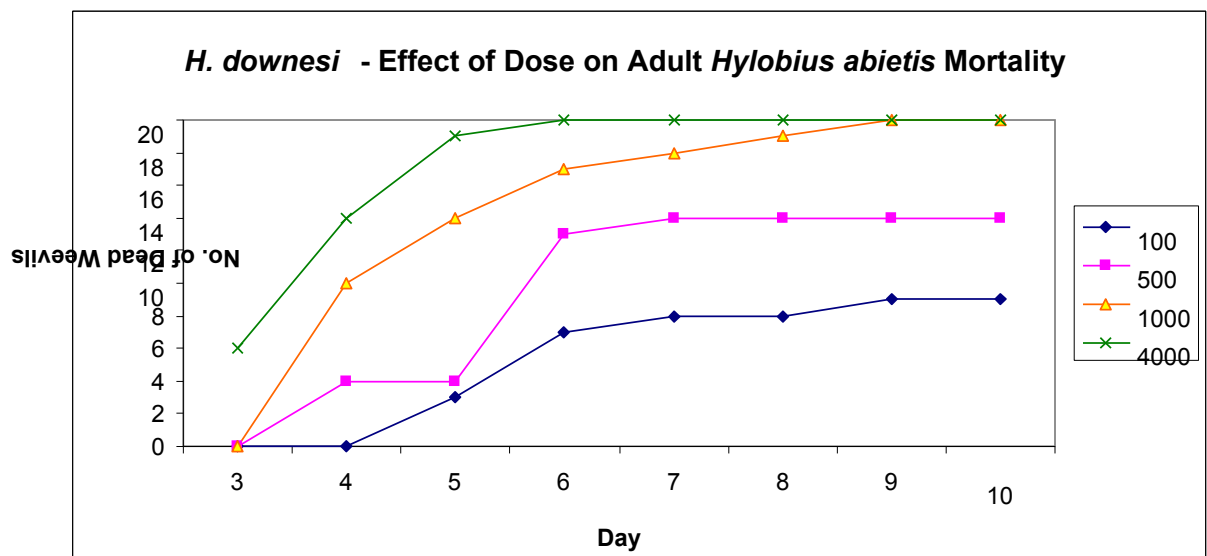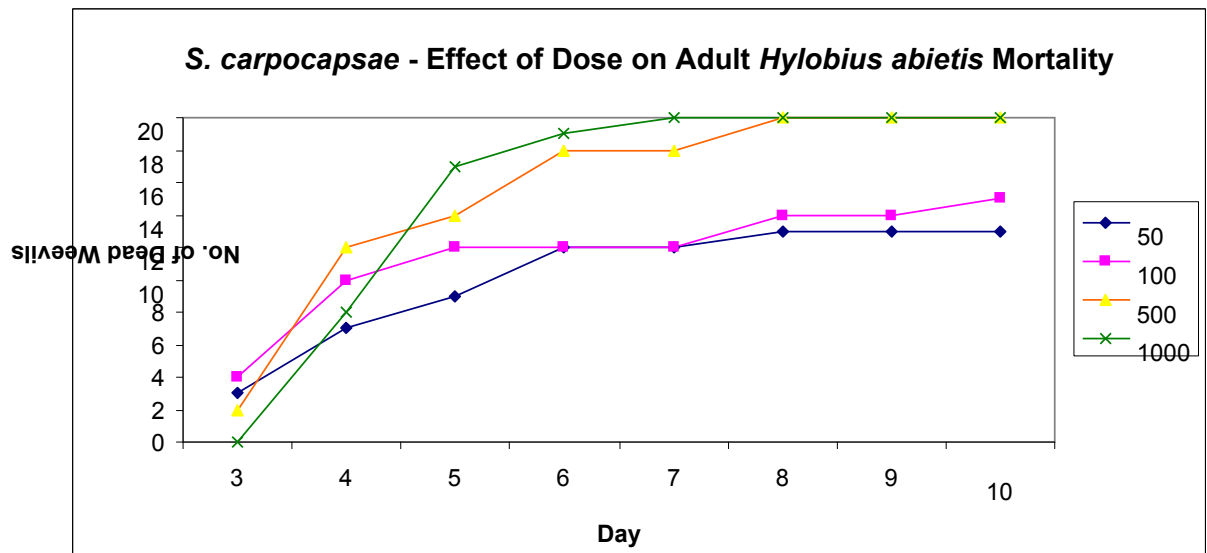
Fig 4.3.2e: Number of dead adult *Hylobius abietis* after exposure to various concentrations of either *Steinernema carpocapsae* (Upper Figure) or *Heterorhabditis downesi* (Lower Figure). For each treatment n = 20.

4.3.3 *The effect of exposure time on susceptibility of the various life stages of* **Hylobius abietis** *to infection by* **Heterorhabditis downesi** *on filter paper.*

Larvae, pupae, callow adults and adult *H. abietis* were tested to investigate the impact of exposure time on infection by *H. downesi*. The callow adult stage had the highest mortality for all exposure times in which it was tested while the full adult stage had no mortality. When the data for the exposure times tested on all of these life stages (8 and 24 hours) is pooled (Table 4.3.3) there is a highly significant difference across the mortality of the different life stages (Chi-square = 39.192, df = 3, P = 0.000). There was no difference in the mortality of the larval and pupal stages when these two exposure times are pooled (Chi-square = 0.960, df = 1, P = 0.327).

Table 4.3.3: Number of dead *H. abietis* of varying life stages 12 days after exposure to 200 IJs of *Heterorhabditis downesi* for 1, 8, 24 or 48 hours. Larvae were tested at the 8 and 24 hour exposure times only. Pupae were tested for all exposure times. Callow adults were tested at the 1, 8 and 24 hour exposure times only. Adults were tested for all exposure times. For each treatment n = 10.

Life Stage	Exposure Time				
	1 Hour	8 Hour	24 Hour	48 Hour	Control
Larvae	-	4	7	-	0
Pupae	4	6	8	10	1
Callow	7	9	10	-	0
Adults	0	0	0	0	0

4.3.4 *Susceptibility of adult* Hylobius abietis *to three nematode species in a peat medium.*

In order to examine an exposure to EPN that is more consistent with conditions in the field, adult *H. abietis* were buried in a vial of peat compost. After exposure for 8 hours buried in compost, comparisons of total cumulative adult mortality at day ten for all three experiments pooled showed *S. carpocapsae* killed significantly more *H. abietis* than *H. downesi* at all nematode concentrations: 500 IJs (Yates' χ^2=10.55, df=1, *P*=0.001); 1000 IJs (Yates' χ^2=4.93, df=1, *P*=0.03); 2000 IJs (Yates' χ^2=10.31, df=1, *P*=0.001); and 4000 IJs (Yates' χ^2=6.90, df=1, *P*=0.009) (Table 4.3.4). Within

S. carpocapsae treatments highest mortality occurred at the 2000 IJs concentration and lowest at the 500 IJs concentration, although there were no significant differences between concentrations (Fig 4.3.4a). Within *H. downesi* treatments highest mortality occurred at the 4000 IJs concentration and lowest at the 500 IJs concentration, but again none of the within treatment differences were significant (Fig 4.3.4a). 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*. The majority of insects that died were killed within 6 days of exposure (Fig 4.3.4a and Fig 4.3.4b). No insects were killed until 3 days after exposure to any of the EPN species.

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 the following nematode concentrations: 500 IJs (Pearsons χ^2=20.5, df=17, *P*=0.25); 1000 IJs (Pearsons χ^2=17.2, df=17, *P*=0.44); and 4000 IJs (Pearsons χ^2=18.9, df=17, *P*=0.33), indicated that in all cases the Weibull distribution did not significantly differ from that of the data and therefore it was reasonable to assume that it fitted the data accurately. This was not the case for the 2000 IJ concentration (Pearsons χ^2=27.76, df=17, *P*=0.05), which significantly differed and therefore suggested that the Weibull distribution did not fit the data adequately; however, the Weibull distribution was the best fit to the data of all available distributions. In tests for equal slopes, between EPN species, the 500 IJs (χ^2=3.8, df=1, *P*=0.052), 2000 IJs (χ^2=2.3, df=1, *P*=0.13) and 4000 IJs (χ^2=1.3, df=1, *P*=0.26) concentrations were not significantly different 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 (χ^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. Given these tests on the data, the 4000 IJ concentration was the most statistically valid for use in comparisons of LT_{50} and LT_{90} values for the two EPN species.

Overall, LT_{50} and LT_{90} values for all concentrations ranged between 7.07-8.42 days and 15.06-18.71 days respectively for *H. downesi*, and 3.54-5.15 days and 7.78-10.81 days respectively for *S. carpocapsae* (Table 4.3.4b). For all concentrations of EPN 95% confidence intervals of LT_{50} and LT_{90} values did not overlap between *S.*

carpocapsae and *H. downesi*, suggesting that at every concentration *S. carpocapsae* had significantly lower LT_{50} and LT_{90} values than *H. downesi*. The relative potency of *H. downesi* vs. *S. carpocapsae* varied from 0.48-0.68, which suggests that, at the tested concentrations, *S. carpocapsae* was 48-68% more efficient at killing *H. abietis* than was *H. downesi*. Comparing LT_{50} and LT_{90} values for each concentration, within each nematode species, the only significant differences were for *S. carpocapsae* between the 500 and 2000 IJs and the 1000 and 2000 IJs concentrations, for both LT_{50} and LT_{90} values, in both cases with the higher concentration having significantly lower values.

Table 4.3.4: No. of dead weevils 10 days after exposure to various concentrations of EPN in a peat medium. For each treatment n = 10.

No. of Dead Weevils - Day 10						
EPN Species	Concentration	Exp. 1	Exp. 2	Exp. 3	Total	% Mortality
S. feltiae	500	4	-	-	4	40
	1000	4	-	-	4	40
	2000	5	-	-	5	50
	4000	2	-	-	2	20
H. downesi	500	2	6	5	13	43.33
	1000	3	6	7	16	53.33
	2000	1	7	8	16	53.33
	4000	1	6	10	17	56.67
S. carpocapsae	500	9	9	8	26	86.67
	1000	8	8	9	25	83.33
	2000	10	9	9	28	93.33
	4000	9	8	10	27	90.00
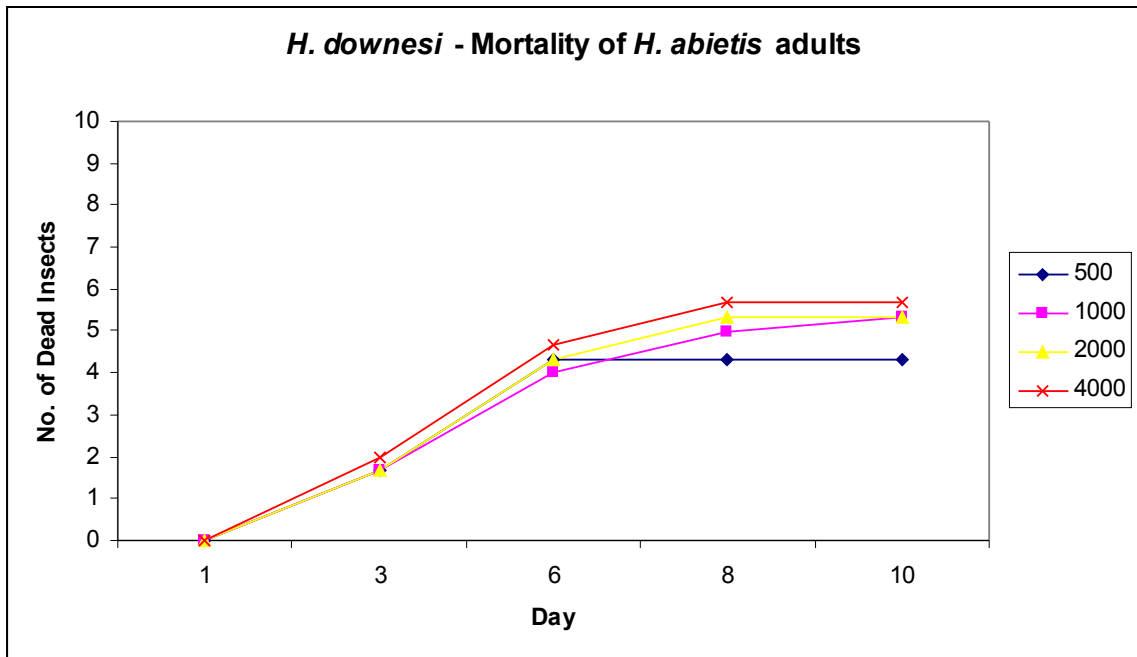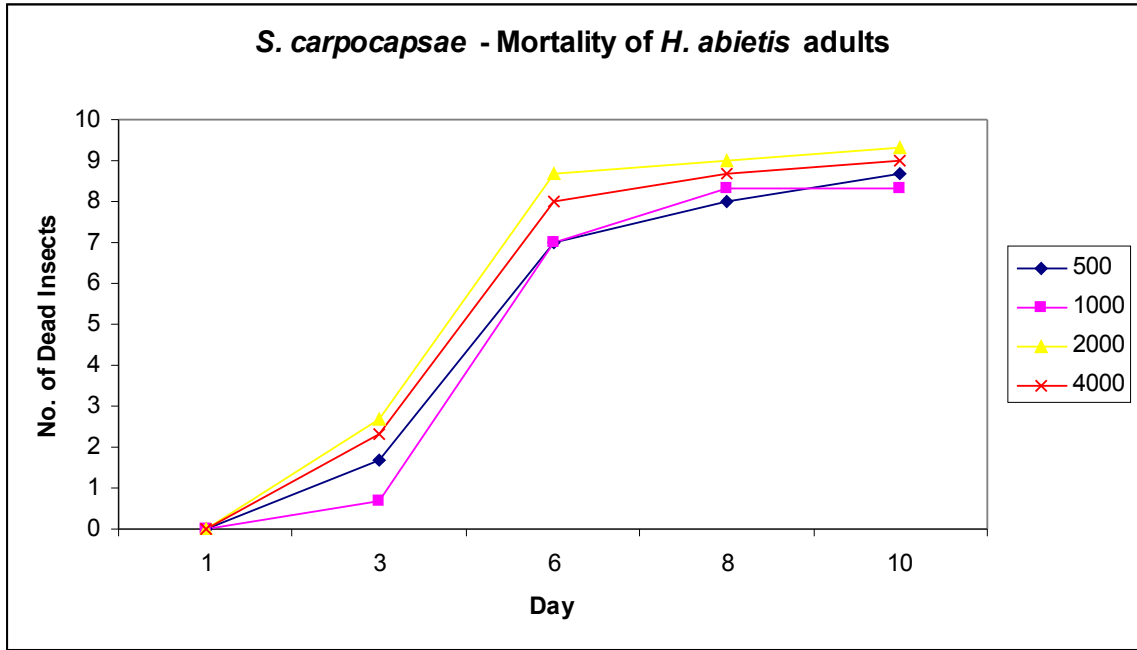
Fig 4.3.4a: Mean number of *H. abietis* adults killed after exposure to various concentrations of *S. carpocapsae* (Upper figure) and *H. downesi* (Lower Figure) in a peat medium for 8 hours. Data shown are a mean of three experiments. For each treatment n = 10 insects.
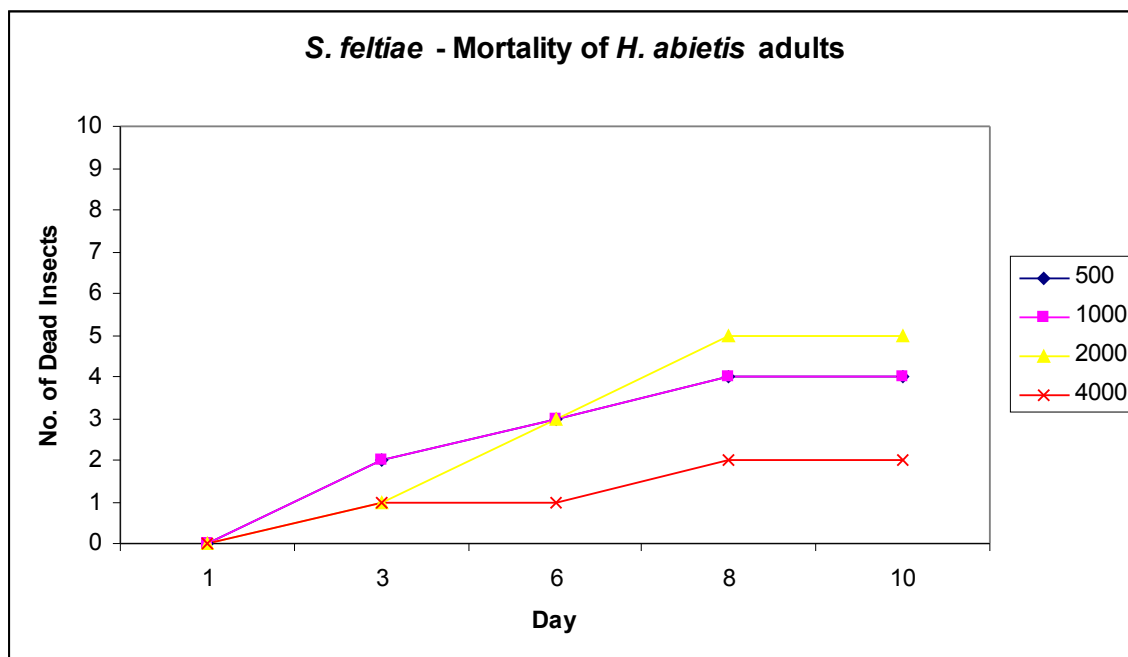
Fig 4.3.4b: Number of *H. abietis* adults killed after exposure to various concentrations of *S. feltiae* for 8 hours in a peat medium. For each treatment n = 10 insects.

Table 4.3.4b: LT_{50} values (in days) and 95% CI values for different concentrations of two EPN species applied to adults *H. abietis* in a peat medium.

Species	EPN Concentration	LT50 +/- SE (days)	Lower F.C. – Upper F.C.
H. downesi	500	8.42 +/- 0.61	7.35 - 9.81
	1000	7.62 +/- 0.47	6.76 – 8.65
	2000	7.37 +/- 0.48	6.50 – 8.41
	4000	7.07 +/- 0.43	6.27 – 8.00
S. carpocapsae	500	4.87 +/- 0.30	4.26 – 5.46
	1000	5.15 +/- 0.29	4.56 – 5.73
	2000	3.54 +/- 0.24	3.04 – 4.01
	4000	4.18 +/- 0.26	3.65 – 4.68

4.3.5 *Route of entry of entomopathogenic nematodes into adult* Hylobius abietis.

Of the five adult *H. abietis* exposed to EPN for one hour two had nematodes in their body tissues after one hour. Both had single nematodes in their bodies that were found in the intestinal tract, in the oesophagous. The insects exposed to EPN for longer periods had nematodes in the haemocoel in some instances and as such could not reliably have their route of entry indicated by their location upon dissection. Due to difficulties in locating single juveniles upon dissection it was not possible to definitively state that EPN are or are not capable of invading the insect through the spiracles.

4.4 Discussion

Many of the studies into entomopathogenic nematode foraging strategies were carried out on flat surfaces such as agar or filter paper (Lewis *et al.*, 1992; Campbell and Kaya, 1999) or in very simple substrates such as sand (Campbell and Kaya, 1999). It is likely that the more complex substrates such as forest soil would affect the movement of EPN differently. While the more simple substrates are more easily reproduced in the lab, they are unrepresentative of Irish forest soils. Much of the coniferous forest plantations in Ireland occur on peaty soils such as those found on cutaway bogland and peaty mountainous soils. Previous studies have shown that *Steinernema carpocapsae* does not actively search for hosts, instead it remains on the surface of the substrate nictating to attach to a passing host (Campbell and Gaugler, 1993). Applications of *S. carpocapsae* to tree stumps in Ireland have shown that the nematodes can invade and kill insects up to 50 cm below the soil level (Dillon *et al.*, 2006) and up to 70 cm along the tree roots (Dillon, NUIM, Unpublished data). It was suggested that the presence of the tree stumps in the soil could increase the likelihood of the nematodes finding and invading their insect hosts (Dillon *et al.*, 2006). The stump and its roots could carry the vibrations of the feeding *H. abietis* larvae into the soil. Such vibrations attract entomopathogenic nematodes (Torr *et al.*, 2004) and would increase the likelihood of parasitism.

When both *H. downesi* and *S. carpocapsae* were tested to investigate the influence of routes and feeding on parasitism of *H. abietis* in sand, the presence of twigs in the substrate did not make a difference with no clear pattern between the treatments. When only *S. carpocapsae* was tested with a longer exposure time in two different substrates the presence of the routes had a more obvious effect with a highly significant difference in the number of killed insects compared to treatments without twigs in the substrate. In both sand and a sand/peat mix the larvae that were able to feed on a twig had a higher mortality rate than the other treatments. In the sand substrate the insects that were beside a twig that they could or could not feed on were more likely to be infected. This suggests that the physical surface of the twig was an important factor in nematode host finding. Other cues such as vibrations and volatiles produced by a feeding insect were not as critical as both the "Food Only" and "None" treatments produced similar results. In the mixed substrate the "Route & Food" treatment again killed the most insects. In this substrate the other three treatments are

more closely grouped. This suggests that a combination of cues and a physical surface caused the higher mortality in the "route & Food" treatment. It is possible that the smaller particle size found in the mixed substrate reduced the space around the surface of the twig thus reducing its efficacy as a travelling surface for the nematodes. It is also possible that the higher organic content of the mixed substrate would reduce the concentration of volatiles reaching the surface of the substrate. In this environment it is possible that only a combination of physical and chemical cues as found in the "Route & Food" treatment would cause a higher invasion rate of insects.

In the first experiment on the effect of routeways, *H. downesi* was killed more insects than *S. carpocapsae*. This data is consistent with the classification of *S. carpocapsae* as an ambush forager and the Heterorhabditids as more active cruise foragers. It is also consistent with field trial results of these two nematodes species against *H. abietis* populations in tree stumps (Dillon *et al.*, 2006).

One of the key factors in a successful biological control program is time of application. It is important that factors such as temperature, time of year and life stage of the pest are taken into account. As *H. abietis* life cycle can take up to 3 years to complete in the UK (Leather *et al.*, 1999) depending on climatic conditions, time of tree felling and the species of host tree (Bakke and Lekander, 1965; Christiansen, 1971; Lekander *et al.*, 1985; Thorpe and Day, 2002) and the life stage at any given time of year in a stump is dependent on all of these factors, it would be important to time application to target the most susceptible stage of the insect. As adult *Hylobuis* are relatively insusceptible to even high concentrations of EPN applied to the substrate (Pye and Burman, 1978; Dillon, NUIM, Pers. Comm.) (Table 4.3.3), and even with continuous exposure (Fig 4.3.2d) only higher concentrations above 500 IJs per weevil were capable of killing all the insects, the immature life stages represent a more likely target for control with entomopathogenic nematodes (Table 4.3.3). It is also likely that even these low levels of adult mortality would not be reflected in the field as a limited exposure in a more complex substrate than filter paper gives lower levels of adult mortality, even at relatively high concentrations (Table 4.3.4). With low concentrations of EPN giving a higher mortality in *H. abietis* when the insects are continuously exposed it is probable that the IJs require a certain time period before

they can invade and establish in the adult weevils (Fig 4.3.2d).The larval stages of *H. abietis* commonly persist in the stumps for more than a year in Ireland. As they actively feed on the phloem layer of the stump it is likely that they would produce more volatiles and vibrations than the more sedentary pupal stage. As both chemical cues (Burman and Pye, 1980; Lewis *et al,* 1992) and vibrations (Torr *et al.*, 2004) are known to attract entomopathogenic nematodes and both species of nematode tested killed fewer pupae than larvae (Figs 4.3.2a and 4.3.2b) any inundative application of EPN should be targeted at larvae. It should be noted that once pupae finish their pupation period and had turned into callow adults they became very susceptible (Fig 4.3.2b) (Table 4.3.3) with 68% of callow adults dying within 3 days of ending pupation after exposure to a relatively low maximum concentration of 200 IJs. As it has been observed that IJs can be found alive under tree bark several months after application (Chris Harvey, NUIM, Pers. Comm.) an application when the majority of the insects are in the late larval stage would be optimal. This would ensure that the nematodes would be able to target both of the most susceptible stages, the larvae and the callow adults, while reducing the likelihood of the adults developing and leaving the stumps before a sufficient reduction in *H. abietis* population can be achieved. It would also ensure that the stumps would be sufficiently degraded that they would no longer be attractive as ovipositing sites to adults migrating onto the site.

Chapter 5.

USE OF THE AMPLIFIED FRAGMENT LENGTH POLYMORPHISM METHOD TO ASSESS STRAIN HYBRIDISATION IN *Steinernema feltiae.*

5.1 <u>Introduction</u>

One of the key concerns of biological control programmes is the effect that the applied organisms will have on indigenous populations. It is usually desirable for any control agent to be native to the region, specific to the target pest and for populations of the agent to fall back to background levels once pest populations are under control. Soil sampling prior to and for several years after application of EPN in Irish forests have shown that after two years the proportion of samples positive for EPN falls back to levels similar to those found before application (Dillon *et al.*, 2006). In a study by Somasekhar *et al.* (2002), the application of *H. bacteriophora* and *H. indica* reduced the number of genera and general abundance of plant-parasitic nematodes. Dillon *et al.* (2008) assessed the EPN population 60 months after an inundative application of *S. feltiae* in a forest soil system. It was found that the isolates found were genetically closer to the indigenous strain than the applied strain.

One of the key problems with assessing the impact of applied EPN is that identification of entomopathogenic nematodes by morphological methods is difficult and time consuming. It usually requires expert knowledge and can often only be carried out on adults as features such as the spicule and labial papillae are key identifying features (Nguyen and Smart, 1996). This can make single worm samples impossible to identify if the individual is female. These difficulties have meant that molecular genetic identification is becoming more common (Reid *et al.*, 1997; Grenier *et al.*, 1996). While interspecific identification can be difficult using non-molecular methods, intraspecific identification of strains would not be possible. Very

few studies have investigated intraspecific populations of EPN (Rolston *et al.*, 2006; Blouin *et al.*, 1999; Dillon *et al.*, 2008) so the impact of applied strains on indigenous populations remains largely unstudied.

It is believed that populations of nematodes are clumped and patchy (Stuart and Gaugler, 1994). Each group is likely to consist of closely related individuals, often having emerged from the same insect. The small size of each nematode coupled with low dispersal abilities means that even strains of the same species in close geographic proximity can have a large molecular variation, particularly in the non-coding DNA segements. Rolston *et al.* (2006) showed that populations of *S. feltiae* isolated from a relatively small (100 m X 800 m) sampling area had a wide variation of the β-tubulin intron. Variation in such regions of the genome is expected between geographically isolated populations. Random mutations in non-coding regions of DNA occur at a steady pace and comparison of non-mating species or strains show that they diverge irreversibly (Kimura, 1991). As mutations in coding regions are less common the non-coding regions evolve faster and thus show higher intraspecific variability (Kimura 1991; Brady *et al*, 1990).

There are several methods that would allow an analysis of the genome of different EPN strains to assess gene flow between applied and indigenous populations. These include sequencing sections of the genome, random amplified polymorphic DNA (RAPD) analysis, microsatellite analysis and amplified fragment length polymorphism (AFLP) analysis. Sequencing sections of the genome was rejected for this project due to the high costs involved. RAPD analysis while simple to carry out has been shown to have a low reproducibility (Jones *et al.*, 1997; Penner *et al.*, 1993). The AFLP method was selected due to its relatively low costs, previous use on nematode populations and the fact that no knowledge of the nucleotide sequence is required in advance. This method has also been found to be highly reproducible (Huys *et al.*, 1996; Janssen *et al.*, 1996). The use of microsatellites is also highly reproducible (Jones *et al.*, 1997) but as the AFLP technique had been used successfully in similar tests of EPN samples (Dillon *et al.*, 2008; Rolston *et al.*, 2006) it was deemed the most suitable technique to use.

The AFLP method of genomic analysis was developed by Vos *et al.* (1995) and involves the selective amplification of restriction fragments generated from a whole genome digest. It analyses the entire genome, is highly reproducible and only a small amount of DNA is required. The method is based on the use of restriction enzymes, one a frequent cutter and one a rare cutter, to digest the entire genome into fragments. The fragments then have adaptors ligated to them. The adaptors ligate specifically an end of a fragment cut by one of the restriction enzymes. The subset that are found via restriction by both enzymes are amplified using primers complementary to the adaptors. The size of this subset is dependent on the specificity of these primers, with less selective primers amplifying a larger proportion of fragments. Amplified fragments are then visualised using a fluorescence-based DNA analyser.
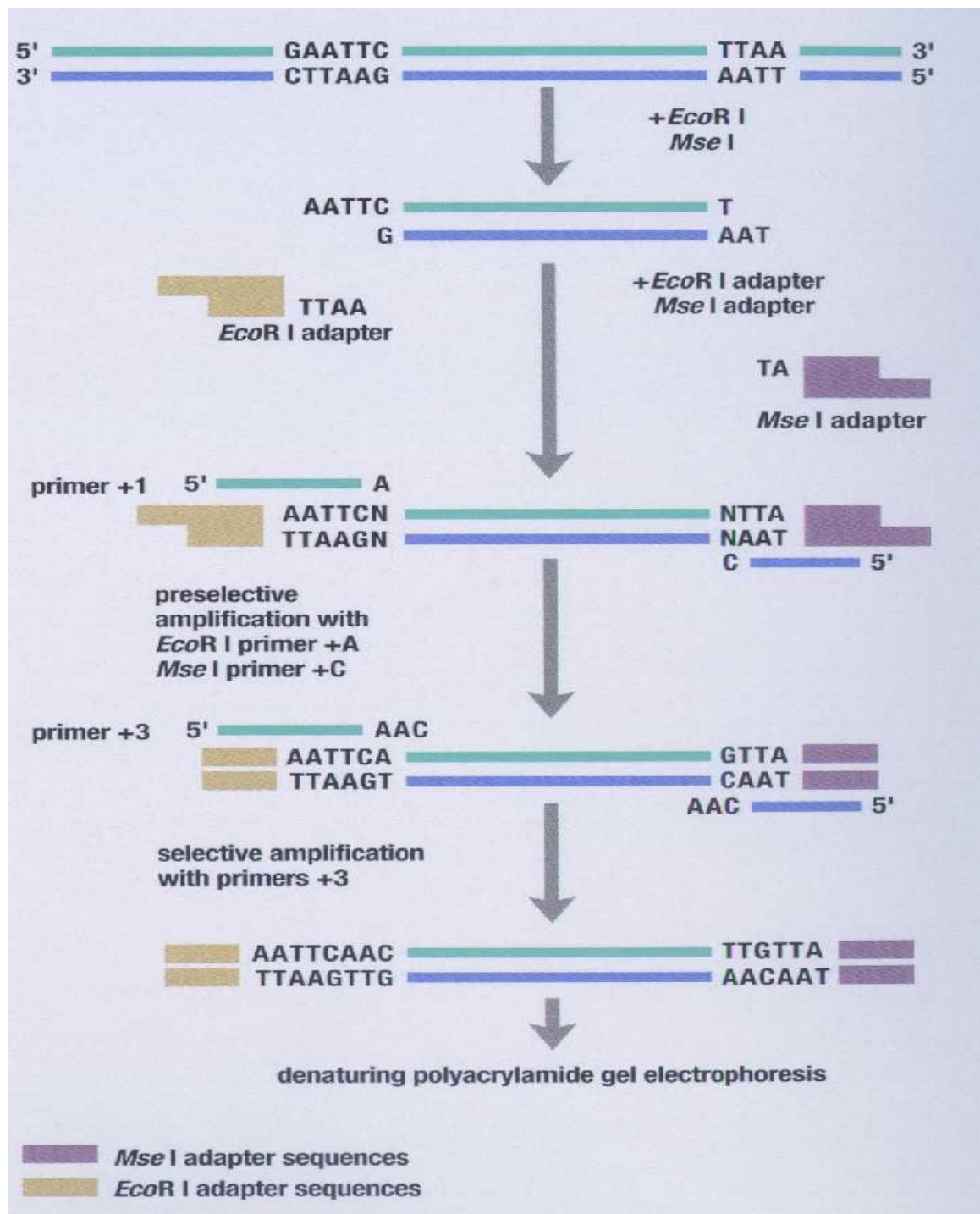
Fig. 5.1.1: Diagrammatic representation of the amplified fragment length polymorphism method of genetic analysis.

(www.msu.edu/course/mmg/835/snapshot.afs/DNAmarkers/aflp.jpg)

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The AFLP method has been used to analyse several nematode species, for example *Haemonchus contortus* (Otsen *et al.*, 2001), *Meloidogyne spp.* (Semblat *et al.*, 2000; Tzortzakakis *et al.*, 1999) and *Globdera spp.* (Picard and Plantard, 2006; Manduric and Andersson, 2003). The method has been less commonly used for EPN species (Hominick *et al.*, 1999; Rolston *et al.*, 2006; Dillon *et al.*, 2008) and these studies were used to assess hybridisation of strains isolated in the field without prior knowledge of the isolates' background. In this project the robustness of the method was tested by crossing three strains of *S. feltiae* and with prior knowledge of the pedigree of each hybridised strain the efficacy of the AFLP method to detect hybridisation of strains of EPN could be examined.

The aims of this chapter were:

(1) To compare different methods of DNA extraction from entomopathogenic nematodes.
(2) To examine the viability of using the AFLP method to detect hybridisation of applied and indigenous strains of *S. feltiae.*
(3) To investigate the accuracy of the results attained from strains of known hybridisation.
(4) To investigate the level of relatedness that can be detected by the AFLP method in EPN.

5.2 Materials and Methods

5.2.1 *Nematodes*

All nematodes used were *Steinernema feltiae.* Three strains were selected for use: 4CFMO, originally isolated in a clearfell forest in Co. Mayo, Ireland; UK76 a commercially produced strain supplied by Becker Underwood Ltd., UK; EN02 a commercially produced strain supplied by Koppert Ltd, The Netherlands. Hybrid strains are denoted as Strain 1 X Strain 2, e.g. EN02 X 4CFMO.

5.2.2 *Hybridisation of nematode strains*

Single infective juveniles of two of the three strains uses were injected into a *Galleria mellonella* larva using a syringe. Each of the three possible combinations of strains was performed in 20 different insects. Insects that died were placed on White traps and emerging juveniles were collected. These juveniles were then backcrossed with the original parent strains using the method above. Killed insects were placed on White traps and the emerging juveniles were collected. After collection all juveniles were washed by sedimentation and stored at 9°C until use.

5.2.3 *DNA Extraction: Single nematode extraction*

Single infective juveniles from each of the parent and hybrid strains from section 5.2.2 were added to 10µl drops of PCR buffer on a glass slide. Each IJ was then crushed using a sterile syringe needle. Each drop was then added to a 0.2 ml PCR tube containing 10 µl of PCR buffer. The tubes were then placed at -70°C for 20 minutes. The tubes were then placed in boiling water for 30 seconds. Two microlitres of proteinase K was added to each tube. The tubes were then incubated at 65°C for 3 hours followed by 95°C for 15 minutes in a thermocycler. They were then placed in a centrifuge and spun at 14,000 rpm for 20 minutes. The upper 15 µl was pipetted off into a sterile 0.2 ml PCR tube and this was used for analysis.

5.2.4 *DNA Extraction: Grouped nematode extraction using a spin column kit*

A pellet of infective juveniles from each strain, approximately 50 µl in volume, was created by centrifuging a suspension of nematodes at 10,000 rpm for 1 minute and removing the supernatant. The tubes containing the pellets were then dipped into liquid nitrogen for 5 seconds and the nematodes were crushed using plastic pestles. The DNA from the nematodes was then extracted using a DNeasy® purification kit (Supplied by Qiagen Ltd.) using the following protocol:

1. Twenty microlitres of proteinase K and180 µl of ATL Buffer was added to each tube. The tubes were then mixed by vortexing and incubated at 55°C for 4 hours.
2. The tubes were vortexed for 15 seconds. Two hundred microlitres of AL Buffer was added to each sample. The samples were then mixed by vortexing and incubated at 70°C for 10 minutes.
3. Two hundred microlitres of 96-100% ethanol was added to each sample.
4. The contents from step 3 were pipetted into a mini-spin column which was then place in a 2 ml collection tube. This was then centrifuged at 8000 rpm for 1 minute. The collection tube and flow-through were discarded.
5. The mini-spin columns were placed in a new 2 ml collection tube and 500 µl of AW1 buffer was added to each tube. This was then centrifuged at 8000 rpm for 1 minute. The collection tube and flow-through were discarded.
6. The mini-spin columns were placed in a new 2 ml collection tube and 500 µl of AW2 buffer was added to each tube. This was then centrifuged at 14,000 rpm for 3 minutes. The flow through and collection tubes were discarded.
7. The spin-columns were placed in a clean 2 ml centrifuge tube and 200 µl of AE buffer was pipetted onto the membrane in each column. This was incubated for 1 minute at room temperature and then centrifuged at 8,000 rpm for 1 minute. The DNA was collected from the flowthrough.
8. Step 7 was repeated using the same 2ml centrifuge tubes.

5.2.5 *DNA Extraction: Grouped nematode extraction using the chilled centrifugation technique*

A pellet of infective juveniles from each strain, approximately 50 µl in volume, was created by centrifuging a suspension of nematodes at 10,000 rpm for 1 minute and removing the supernatant. One hundred microlitres of worm lysis buffer (0.1M Tris-Cl, 0.1M NaCl, 50mM EDTA and 1% SDS) was added to each tube. The tubes were then dipped in liquid nitrogen for 5 seconds and the pellet was then crushed using plastic pestles. The DNA was then extracted using the following method:

1. Five microlitres of Proteinase K (2 mg/ml) was added to each tube. The tubes were then incubated at 56°C for 1 hour.

2. Two and a half times the volume in the tube of Phenol : Chloroform: Isoamyl alcohol (25:24:1) was added to each tube and left for 10 minutes

3. The tubes were spun in a refrigerated centrifuge at 4°C for 15 minutes at 12,000 rpm. The supernatant was removed to new Eppendorf tubes.

4. Steps 2 & 3 were repeated.

5. Two and a half time the volume in the tubes of ethanol (chilled to – 20°C) and 1/30 the volume in the tubes of 3M NaOAc was added to each tube.

6. The tubes were stored at – 20°C overnight.

7. The tubes were spun in a refrigerated centrifuge at 4°C for 15 minutes at 12,000 rpm. The supernatant was removed and 1.5 ml of 75% ethanol was added to each tube.

8. Step 7 was repeated.

9. The supernatant was removed and the pellet was allowed to air dry for 1 hour.

10. The pellet was re-suspended in 100 µl of 10mM Tris-HCl (pH 8.5).

5.2.6 *Gel Electrophoresis*

All electrophoresis was carried out on 1.5% agarose gels prepared in TAE buffer and stained with 5 µl of ethidium bromide. All gels were 60 ml in volume. Gels were placed in a gel rig and submerged in TAE (X1) buffer for electrophoresis. Ten microlitres of each sample was mixed with 2 µl of loading dye and then pipetted into a well of the gel. Five microlitres of 1 kb DNA ladder was used to determine fragment size. Samples were electrophesised for 25 minutes at 120 volts and then visualised by UV illumination.

5.2.7 *Amplified Fragment Length Polymorphism (AFLP)*

The following master mix of reagents was prepared in a sterile 0.5 ml microcentrifuge tube:

10 µl 10X T4 DNA ligase buffer with ATP
10 µl 0.5M NaCl
5 µl 1mg/ml BSA
100 units Mse1
500 units Ecor1
100 Weiss units T4 DNA ligase

Restriction Ligation: 5 µl of each digested DNA sample was placed in a sterile 0.5 ml microcentrifuge tube. To each tube the following reagents were added:

1 µl 10X T4 DNA ligase buffer with ATP
1 µl of 0.5M NaCl
0.5 µl 1 mg/ml BSA
1 µl Mse1 adaptor
1 µl Ecor1 adaptor
1 µl of master mix

Samples were mixed by vortexing and incubated at 37°C for 2 hours on a thermocycler. Each tube then had 179 µl of TE 0.1 buffer added and were then mixed by vortexing. Samples were stored at 4°C until use.

Preselective amplification: In a sterile 0.5 ml microcentrifuge tube for each sample the following was added:

4 µl of diluted Restriction-ligation product from above
1 µl of AFLP preselective primer pairs
15µl of AFLP Core mix

The tubes were mixed gently then place in a thermal cycler. The PCR parameters on Table 5.2.1 were then performed:

Table 5.2.1: PCR parameters used for pre-selective amplification.

HOLD	CYCLE			HOLD	HOLD
	EACH OF 20 CYCLES				
72°C	94°C	56°C	72°C	60°C	4°C
2 mins	20 secs	30 secs	2 mins	30 mins	forever

10 µl of the PCR product for each sample was added to a sterile 0.5ml microcentrifuge tube. To each tube 190 µl of 0.1 TE buffer was added.

Selective amplification: In a sterile 0.5 ml microcentrifuge tube for each sample the following was added:

3 µl of the diluted preselective PCR product from above
1 µl of both selective primers
15 µl of core mix

The tubes were mixed gently then place in a thermal cycler. The PCR parameters detailed in Table 5.2.2 were then performed:

Table 5.2.2: PCR parameters used for selective amplification – "Touchdown PCR"

HOLD	CYCLE			NO. OF CYCLES
94°C for 2 mins	94°C for 20 secs	66°C for 30 secs	72°C for 2 mins	1
-	94°C for 20 secs	65°C for 30 secs	72°C for 2 mins	1
-	94°C for 20 secs	64°C for 30 secs	72°C for 2 mins	1
-	94°C for 20 secs	63°C for 30 secs	72°C for 2 mins	1
-	94°C for 20 secs	62°C for 30 secs	72°C for 2 mins	1
-	94°C for 20 secs	61°C for 30 secs	72°C for 2 mins	1
-	94°C for 20 secs	60°C for 30 secs	72°C for 2 mins	1
-	94°C for 20 secs	59°C for 30 secs	72°C for 2 mins	1
-	94°C for 20 secs	58°C for 30 secs	72°C for 2 mins	1
-	94°C for 20 secs	57°C for 30 secs	72°C for 2 mins	1
-	94°C for 20 secs	56°C for 30 secs	72°C for 2 mins	20
60°C for 30 mins	-			1

Denature: After selective amplification 0.5 µl of each amplification product was placed in a 0.5ml genetic analyser sample tube (Applied Biosystems P/N: 401957). 25 µl of loading buffer (24:1 de-ionised formamide : Rox size standard solution) was added to each tube. A sample tube septa (Applied Biosystems P/N 401956) was placed on top of each tube to seal it. The tubes were mixed gently and then were heated to 95°C for 5 minutes and were then cooled to 4°C for 2 minutes on a thermocycler. The samples were then removed and quick-chilled on ice before analysis on the AB310 genetic analyser.

Table 5.2.3: Selective primer combinations used for selective amplification of fragments.

Mse1 Primer	Ecor1 Primers (Label)
CTG	TG (FAM), AT (NED)
CAA	TG (FAM), AT (NED)
CAT	TG (FAM)
C	TG (FAM)

5.3 <u>Results</u>

The three extraction methods (Sections 5.2.3, 5.2.4 and 5.2.5) used gave variable levels of success.

1. Single nematode DNA extraction (5.2.3) was not successful for this method as the yield of DNA was too low for the reaction and could not be visualised on an electrophoresis gel.

2. Grouped extractions of nematode DNA using a spin-column based kit (5.2.4) was more successful as the extracted DNA could be visualised on a gel but gave very low or no yields when the selective amplification product was analysed.

3. Grouped extractions using the chilled centrifugation technique (5.2.5) was the most successful of the extraction methods. The DNA could be visualised on an ethidium bromide electrophoresis gel prior to amplification, a band of high molecular weight DNA is clearly visible at the top of lane B (Fig 5.3.1). The quantity and quality of DNA yielded from this method as assessed by spectrophotometry (Table 5.3.1) were sufficient for the AFLP technique. The preselective amplification product could also be visualised on an electrophoresis gel indicated by a clear spread of DNA fragments between 500 bp and 4,000 bp (Fig 5.3.2).

The fragment profiles generated were, for the most part, of too low a yield to assess. In those profiles were peaks above the minimum threshold recognised by the Genescan software the majority showed peaks that were unique to individual samples. Only three samples generated in one analysis were informative as they showed a possible inheritance pattern of one allele across hybrid strains (Fig 5.3.3).
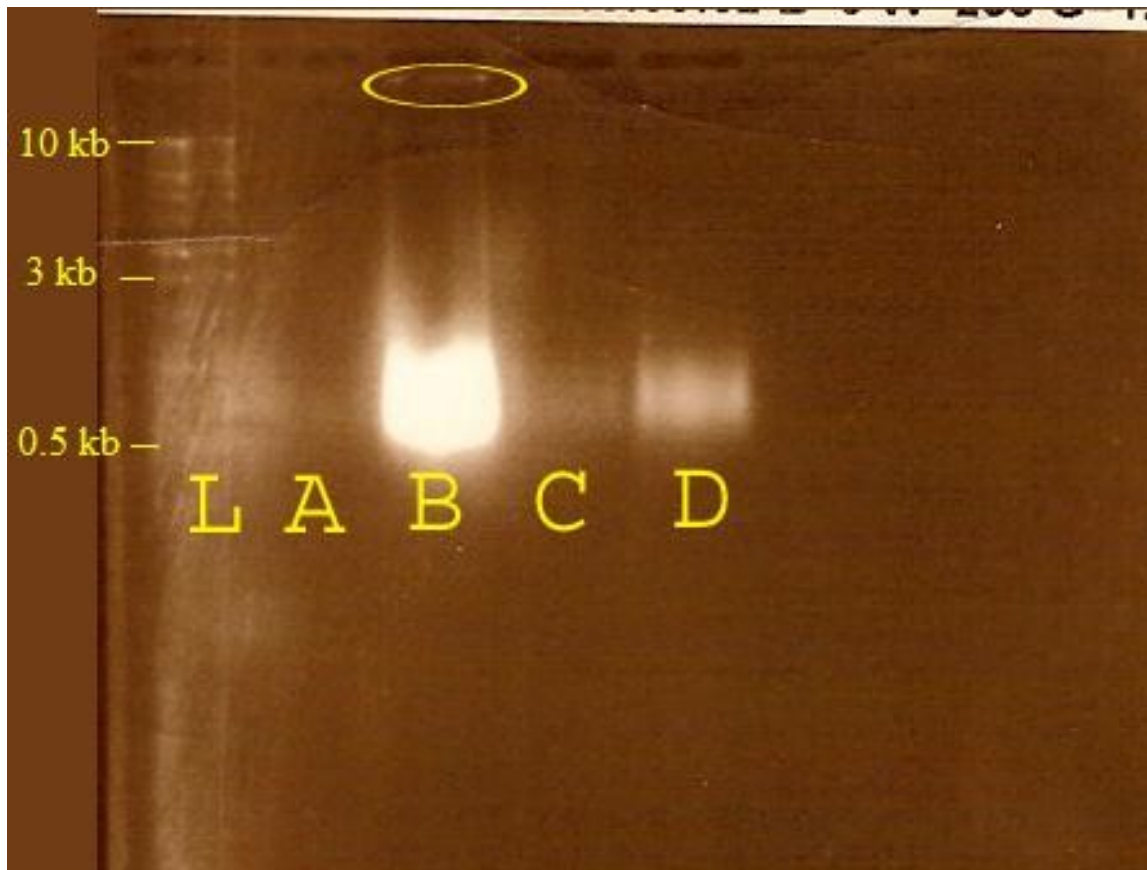
Fig 5.3.1: Electrophoresis gel of DNA after extraction using method described in 5.2.5. L = 1kb ladder; A = UK76 sample; B = 4CFMO sample; C = EN02 sample; D = UK76 X EN02 sample. Circled region = High molecular weight DNA from the 4CFMO sample.
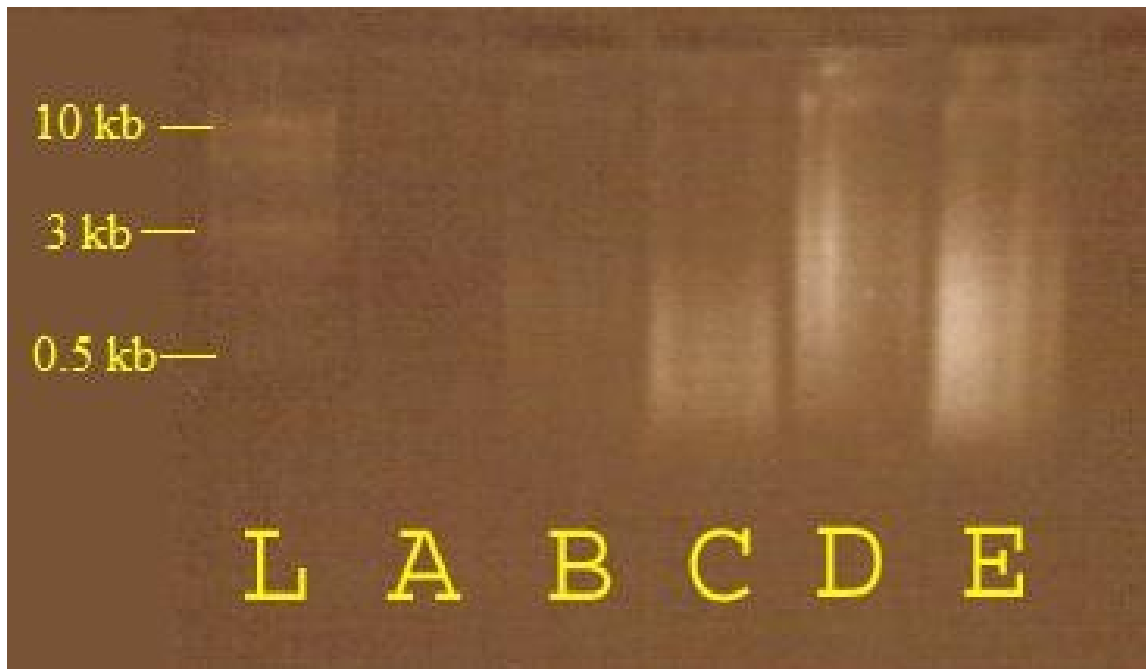
Fig 5.3.2: Electrophoresis gel of preselective amplification product.

L = 1kb ladder; A = UK76 sample; B = 4CFMO sample; C = EN02 sample; D = UK76 X EN02 sample; E = EN02 X 4CFMO sample.

Table 5.3.1: Spectrophotometer readings of DNA samples after extraction using method described in 5.2.5.

Sample	Absorbance @ 260 nm	Ratio 260/280nm	Concentration μg/ml
EN02	0.0601	1.2305	0.15
4CFMO	0.0333	1.2733	0.083
UK76	0.0425	1.0257	0.106
EN02 X 4CFMO	0.0314	1.1746	0.08
4CFMO X UK76	0.0628	1.0473	0.15
UK76 X EN02	0.0698	1.0622	0.175
EN02 X 4CFMO X EN02	0.0204	1.1827	0.05
EN02 X 4CFMO X 4CFMO	0.0668	1.1256	0.17
UK76 X 4CFMO X 4 CFMO	0.0446	0.9727	0.111
Control (Plant) DNA	0.0166	3.0816	0.041
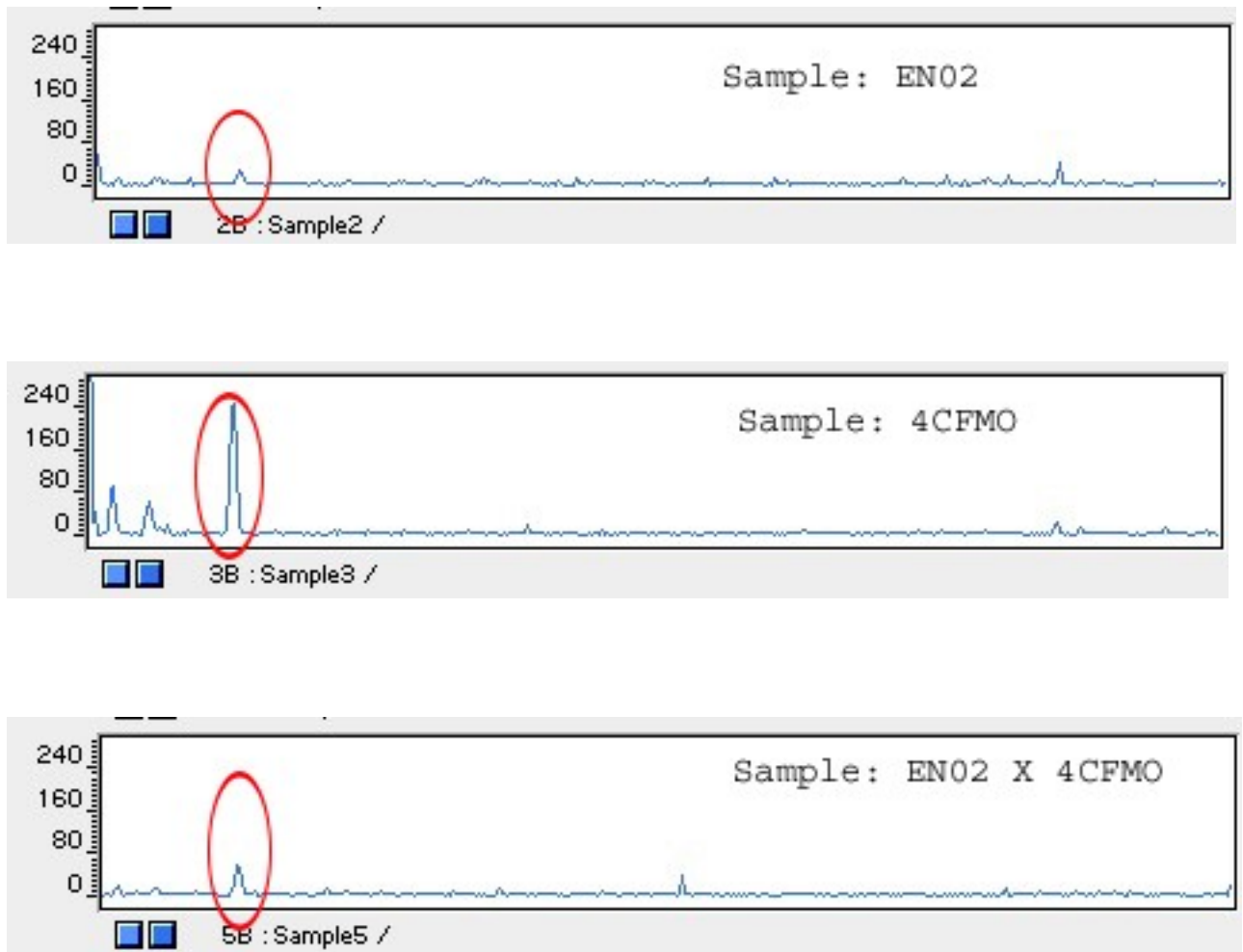
Fig 5.3.3: Peaks representing AFLP fragments of *S. feltiae* strains. The strains represented are two parent strains, EN02 and 4CFMO, and the hybrid of those two strains, EN02 X 4CFMO. The Y-axis represents fluorescence of those fragments. The X-axis represents the size of those fragments when compared to an internal size standard. Circled regions highlight a possible pattern of inheritance at approximately 70 base pairs.

5.4 <u>Discussion</u>

Much of the focus of EPN field trials in the UK has been on the use of *S. carpocapsae* to suppress *H. abietis* populations in the stumps (Brixey *et al.*, 1997; Torr *et al.*, 2005; Brixey *et al.*, 2006). Other nematode species such as *S. kraussei* (Torr *et al.*, 2007) and *H. megidis* (Torr *et al*, 2005) have had less investigation. In Ireland *S. carpocapsae* and *H. downesi* have been widely studied (Dillon *et al.*, 2006; Dillon *et al.*, 2007; Dillon *et al.*, 2008). In these trials *H. downesi* has been most effective but had only been commercially produced on a trial basis in 2008. It may not be possible to continue producing this species on a commercial basis in the long-term. *Steinernema carpocapsae* has been less successful in reducing *H. abietis* populations in Ireland (Dillon *et al.*, 2006; Dillon *et al.*, 2007; Dillon *et al.*, 2008) but as it is non-indigenous to Ireland there are some environmental concerns with mass application of an exotic EPN species.

Steinernema feltiae has also been shown to be successful in parasitising *H. abietis* in stumps (Dillon *et al.*, 2006). It is also the only EPN species to be found in soil samples of Irish coniferous forestry. Dillon *et al.* (1999) found isolates in 4 of 60 forest sites sampled. As no Irish strain of *S. feltiae* is produced commercially it is likely that a strain from the UK or continental Europe that is already being commercially produced would be used if this species was ever to be adopted in large-scale applications of EPN to Irish forests. Dillon *et al.* (2008) assessed hybridisation of two strains of *S. feltiae*, an indigenous strain (4CFMO) and a commercially available strain from continental Europe (EN02), after application in an Irish forest. Recovered isolates were found to more closely resemble the indigenous strain than the commercial isolate. They suggested that this was because the indigenous strain was better adapted to Irish forest conditions. Though the results obtained by Dillon *et al.* (2008) suggest a low risk of exotic applied strains replacing indigenous strains it is

still a concern. The accuracy and level to which hybridisation in EPN can be detected by the AFLP method is also a concern and was assessed in this chapter.

The AFLP fragment profiles obtained from analysis of fluorescently labelled fragments were not sufficient for statistical analysis. A possible inheritance pattern was seen in three of the samples using the primer set Mse1 – CAA and Ecor1 – TG and the FAM label. Three peaks were found of approximately 70 base pairs in size in the EN02, 4CFMO and the hybrid of these two strains, EN02 X 4CFMO. The area of the peak is lowest in the EN02 sample. It is much greater in the 4CFMO sample and is intermediate in the hybrid sample. This pattern is as expected in hybrid strains of the same species. The yield is too low however to perform statistical analysis and the small number of peaks in this small number of samples makes it difficult to assess the AFLP technique as a method of detecting strain hybridisation in *S. feltiae*. As previous studies have successfully used the AFLP technique to analyse relatedness of *S. feltiae* strains (Rolston *et al.*, 2006; Dillon *et al.*, 2008) it is likely that an error in the system has caused the failure of this technique.

In order to locate any error in the system, checks were performed at different stages of the protocol. Three different extraction techniques were used and the quantity and quality of the DNA yielded was assessed by spectrophotometry (Table 5.3.1) and gel electrophoresis (Fig 5.3.1). The preselective amplification product was examined by gel electrophoresis (Fig 5.3.2) and the profile recovered from the gel was as expected with a wide range of fragment sizes found for each sample. Several different primer sets of varying specificity and two different fluorescent dyes were used in the selective amplification process (Table 5.2.3). Some of the combinations of selective primers have been used successfully to analyse EPN populations in previous AFLP studies, e.g. Mse1 – CTG and Ecor1 – TG (FAM), (Rolston *et al.*, 2006). This suggests that it is unlikely that the primer sets are unsuitable for analysis *S. feltiae* genomes. The use of a primer with a single nucleotide selective site (Mse1 – C) would also suggest that the specificity of the selective primers is not the cause of the low yield of results.

In total 12 selective amplification reactions were carried out. They were analysed on 14 different reaction runs on an Applied Biosystems AB310 capillary electrophoresis

genetic analyser. Of the 14 selective amplification reactions analysed 6 generated fragment peaks of sufficient magnitude to be scored by the AB310 Genescan software. In many cases the peaks generated were all below the peak recognition threshold of 50 fluorescence intensity units.

The Mse1-CAA/Ecor1-TG selective primer combination was the most informative, generating 19 fragment peaks across the 56 samples analysed. However, in all but 5 cases, the peaks generated were unique to an individual sample. In just 3 cases peaks were informative and shared across samples (Fig 5.3.3). In these three samples a peak at 70 b.p. was found in both the EN02 and 4CFMO samples at a lesser and greater amplitude respectively. In the hybrid of these two strains the peak was found at intermediate amplitude. This is suggestive of an inheritance pattern for this fragment. However, in the absence of repeated presence of this pattern it is not possible to statistically assess this data. Further work on the methods that are most effective in differentiating populations of *S. feltiae* are required.

Chapter 6

General Discussion

The ongoing large-scale trials of the use of entomopathogenic nematode applications to suppress *H. abietis abietis* populations in tree stumps offers a means to reduce the damage caused by this pest without chemical insecticides (Dillon *et al.*, 2006). How these applications will affect adult weevils on site, those that emerge from the stumps and those that migrate onto the site is also key to the efficiency of the pest control strategy. This study has shown that the various life stages of *H. abietis* are different in their susceptibility to infection by entomopathogenic nematodes (Chapter 4) and that careful timing of the application of EPN could be critical in the success of such applications in population suppression. It was found in assays on filter paper, that larvae and callow adults are the most susceptible life stages of *H. abietis* to EPN infection, with adults and the non-feeding pupal stage less susceptible (Chapter 4). Applications of EPN timed for when the majority of the insects in the stumps are in the final larval instar would be optimal. Stumps would be degraded enough to allow the EPN access to larval galleries (Brixey, 2000) and with previous applications at this time many of the susceptible insects would be infected within 4 weeks (Dillon *et al.*, 2006). Recycling in these infected insects would cause EPN populations in the stumps to remain high over the month following application. This would mean that those larvae that had pupated would form callow adults, another highly susceptible stage, that could become infected also.

A high level of understanding of the foraging strategies of the control agent allows for successful planning of the control program. Many applications of EPN are believed to have failed as this understanding was lacking (Georgis and Gaugler, 1991). As many laboratory based studies that have studied EPN foraging strategies have used simple

surfaces such as agar (Lewis *et al.*, 1992; Grewal *et al.*, 1994; Grewal *et al.*, 1997) it is likely that complex three-dimensional surfaces such as soil will cause different foraging reactions to occur.

In several field trials in Ireland and the UK *S. carpocapsae* was found to infect *H. abietis* larvae in tree stumps (Dillon *et al.*, 2006; Dillon *et al.*, 2007; Torr *et al.*, 2007) up to 50cm from the point of application. This is contrary to the classification of this nematodes species as an ambush forager that does not actively seek out a host (Lewis *et al.*, 1992). It is possible that the soil and stump habitat of *H. abietis* larvae alters the manner in which *S. carpocapsae* juveniles search for hosts. The physical surface of the tree roots could act as a routeway along which the IJs could travel thus avoiding the more complex substrate of the soil as was suggested by Dillon *et al.* (2007). It is also possible that the vibrations of feeding *H. abietis* larvae on the roots could act to attract the EPN (Torr *et al.* 2004).

The presence of twigs in the substrate caused *S. carpocapsae*, classified as a sedentary ambush forager (Lewis *et al.*, 1992), to kill more *H. abietis* larvae than larvae where there were no twigs in the substrate (Chapter 4). Trials of *S. carpocapsae* against pests in cryptic habitats have shown that it is capable of actively foraging for hosts (ref). It is possible that factors such as physical routeways that have not been taken into account in simple assays assessing foraging strategies have led to misleading classifications. As many of the lab-based experiments studying foraging strategies are based over short time periods it is also unlikely that these will reflect how EPN will react to host cues over several days or weeks in the field. Selection of EPN species for biological control programs on the basis of limited behavioural studies in the lab may not be optimal for field applications. Field trials of prospective EPN species such as those carried out on *H. abietis* by Pye and Pye (1985), Dillon *et al.* (2006) and Torr *et al.*, 2007 would give a more accurate portrayal of possible control.

Large scale EPN applications in the UK are reported to cause greatly reduced feeding in adult *H. abietis* on site (Heritage, UK Forestry Commission, Pers. Comm.). It is of interest to know if infection by EPN causes this behaviour. As it is the adult *H. abietis* stages that cause the economically important damage to seedlings, any reduction in feeding would increase the efficacy of EPN as a biological control agent of this pest.

It was thought that the adult *H. abietis* emerging from stumps could already be infected by EPN and this was causing them to feed less. It is possible for insects to be infected by EPN for several days and for them to continue walking and even flying (Downes and Griffin, 1996; Lacey *et al.*, 1995). Adult *H. abietis* rarely die within the first two days after exposure to EPN (chapter ref). Indeed it is possible even under continuous exposure for adult *H. abieits* to survive for up to ref days before dying from EPN infection (Chapter ref). While the effect EPN have on adult *H. abietis* mortality has been studied before (ref) short term effects of EPN infection on adult feeding were unknown. It was found in this study (Chapter 3) that adult *H. abietis* exposed to EPN did not decrease their feeding in the days following exposure to EPN when compared to non-exposed weevils. It was also found that weevils that died during each week-long assay did not significantly differ in the amount of bark eaten than those that survived the assay. Weevils that contained EPN upon dissection were found to have eaten a greater weight of bark than those in which no EPN were found. This would indicate that instead of reducing feeding infected adults may well cause more damage upon emergence from the stumps, at least in the short term. The observation of non-feeding adult weevils on nematode-treated sites therefore cannot be supported by results obtained in experiment 3.2.7.

It is also possible that EPN may influence non-infected adults, either emerging from the stumps or travelling on to the site. In chapter three it was demonstrated that adult weevils could detect the presence of EPN on their bodies. The weevils also displayed a grooming response to this presence that could in part explain their relative insusceptibility to EPN infection. The level of this grooming response was dependent on the species of EPN applied with *S. carpocapsae* eliciting a more sustained response than *H. downesi*. This is likely due to the different behavioural patterns of the IJs observed on the insect. Grooming and other aggressive anti-parasite responses have been shown to decrease infection by EPN (Mankowski *et al.*, 2005; Gaugler *et al.*, 1994) and it could be partially responsible to the relatively low susceptibility of adult *H. abietis*. In assays on filter paper (Chapter 4) adults were shown to be rarely infected by low concentrations of EPN, even with continuous exposure. When the weevils were placed in vials of compost as an exposure medium however, infection rates were high with only an 8 hour exposure (Chapter 4). As adult weevils regularly overnight in the top layers of soil and leaf litter it is likely that this is a more realistic

exposure method than filter paper assays. Thus in the areas surrounding stumps that have EPN applied to them or at the base of EPN-treated seedlings, a reduction in adult populations is possible.

Adults migrating on to clearfell sites from surrounding sites can cause damage to transplanted seedlings, as is evident from increased damage to seedlings on the edges of felled compartments (Wilson *et al.*, 1996). Seedlings dipped in high concentrations of EPN had reduced damage by *H. abietis* (Pye and Pye, 1985). The impact of the repellent effects of EPN on the substrate was investigated in chapter 3. While there was a reduction in feeding on EPN treated substrates compared to non-treated controls, this reduction was only temporary and was not seen two days post-application. It was also found that the presence of high concentrations of nematodes on the substrate did not alter the amount of time the insect spent on that substrate. These results indicate it is unlikely that EPN in the substrate will prevent weevils from approaching or feeding on seedlings in the long term. Conord *et al.* (2006) showed that *H. abietis* populations in Ireland are distinguishably different using molecular methods indicating a low level of gene flow between populations less than 200 km apart. This would indicate that area wide applications of EPN to stumps should reduce the number of weevils in a region that would not be replenished quickly from populations in other areas of the country. This could reduce the numbers of weevils migrating on to clearfell sites and help further reduce local *H. abietis* populations in the short to medium term, though increased temperatures associated with climate change may alter this.

The downstream effect of applied EPN on indigenous nematode populations has been the subject of some studies (Millar and Barbercheck, 2001; Somasekhar *et al.*, 2002; Duncan *et al.*, 2003; Dillon *et al.*, 2008). Due to difficulties in morphological identification (Nguyen and Smart, 1996), especially of different strains, genetic methods offer the most likely method of assessing the impact of applied strains. When mass produced strains are applied in massive concentrations, especially if they are of exotic origin, it is a concern that the indigenous strains will be replaced. Molecular methods to assess gene flow have been used on EPN samples gathered from the field (Dillon *et al.*, 2008; Rolston *et al.*, 2006). In chapter 5 one of these methods, the amplified fragment length polymorphism (AFLP) method was assessed. This method

was used by Dillon *et al.* (2008) in an Irish forest ecosystem after application of several strains of *S. feltiae* to assess gene flow and hybridisation. In this study the efficiency and accuracy of the AFLP method to assess gene flow and strain hybridisation was tested. Hybrids of known crosses were tested using the AFLP method to see if the results accurately reflected the hybridisations performed in the laboratory. Ultimately the results obtained were not robust enough to make accurate assessments of strain hybridisation in *S. feltiae*, in the most part due to difficulties with the method. Similar optimisation difficulties with the AFLP method have been found in several laboratories across Europe (Jones *et al.*, 1997) and further investigations could increase the level of results obtained with this method. It is possible that other methods such as microsatellite analysis may offer a more straightforward method of assessing gene flow and hybridisation in EPN populations.

From this study several issues that merit further research have arisen. The influence of substrate type and physical routeways on infection of *H. abietis* larvae by EPN has shown that classification of EPN species into behavioural types may be misleading. Further work using experimental setups that more accurately reflect field conditions could demonstrate variability in host finding strategies of EPN not found in simpler laboratory assays. The influence of insect feeding on infection by EPN also merits further work to investigate the mechanisms behind this increase in mortality. The high susceptibility of the callow adult stage of *H. abietis* to EPN infection would suggest that this life stage of this insect pest may be the most successful stage for field applications to target. A larger, possibly field-based, study into this susceptibility would help promote greater understanding of the varying success of EPN against the different *H. abietis* life-stages. A more reliable method of molecular testing of gene flow between native and applied EPN populations needs to be developed. It is possible that other methods, such as microsatellite analysis, could be reliably used to assess the impact that inundative applications of EPN have on background populations.

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Appendix

Table 3.3.6a: Numbers of live, dead and encapsulated nematodes or nematodes under the elytra found upon dissection of weevils 6 days after exposure to a low dose of *Steinernema carpocapsae*.

S. carpocapsae Low Dose - Week 1

	Live	Dead	Encapsulated	Elytra	Weevil Alive?
1	0	0	1	0	Y
2	0	0	0	0	Y
3	0	0	0	0	Y
4	12	0	0	38	N
5	8	0	15	74	Y
6	0	0	0	0	Y
7	0	0	0	0	Y
8	1	0	1	0	Y
9	2	0	0	0	Y
10	0	0	0	0	N
11	0	0	0	0	Y
12	0	0	0	0	Y
13	2	0	0	0	Y
14	1	0	0	0	N
15	6	0	0	18	Y
16	2	0	0	0	Y
17	3	0	9	68	Y
18	2	0	0	0	N
19	21	5	0	80	N
20	1	0	2	0	Y
Mean	3.05	0.25	1.4	13.9	
Std dev	5.26632799	1.118033989	3.789181275	27.53352024	
Std Error	1.17758674	0.25	0.847286691	6.156682292	

Table 3.3.6b: Numbers of live, dead and encapsulated nematodes or nematodes under the elytra found upon dissection of weevils 6 days after exposure to a high dose of *Steinernema carpocapsae*.

S. carpocapsae **High Dose – Week 1**

	Live	Dead	Encapsulated	Elytra	Weevil Alive?
1	0	0	0	0	Y
2	5	0	2	0	Y
3	0	0	0	0	Y
4	0	2	0	0	N
5	1	0	0	0	Y
6	0	1	0	0	Y
7	5	0	3	19	Y
8	0	0	7	0	Y
9	7	2	4	0	Y
10	0	0	0	0	Y
11	0	0	0	0	Y
12	0	0	0	0	Y
13	9	0	0	0	N
14	0	0	0	0	Y
15	2	0	0	25	N
16	4	0	0	0	Y
17	1	0	7	0	Y
18	0	0	0	0	Y
19	0	0	8	0	Y
20	0	0	15	0	Y
Mean	1.7	0.25	2.3	2.2	
Std Dev	2.754899941	0.638666374	4.040583595	6.841052551	
Std Error	0.616014354	0.142810143	0.903501959	1.529705854	

Table 3.3.7c: Numbers of live, dead and encapsulated nematodes or nematodes under the elytra found upon dissection of weevils 6 days after exposure to a low dose of *Heterorhabditis downesi.*

H. downesi Low Dose – Week 1

	Live	Dead	Encapsulated	Elytra	Weevil Alive?
1	0	0	0	0	Y
2	0	0	0	0	Y
3	6	0	0	0	Y
4	2	0	0	0	Y
5	0	0	0	0	N
6	3	0	0	0	Y
7	0	0	2	0	Y
8	0	0	0	0	Y
9	2	0	0	0	N
10	1	0	0	29	N
11	0	0	3	44	Y
12	10	0	0	0	Y
13	0	0	0	0	N
14	3	0	0	0	Y
15	1	0	0	0	N
16	0	0	0	0	Y
17	0	0	0	0	Y
18	0	0	2	0	Y
19	0	0	0	12	Y
20	3	0	0	0	Y
Mean	1.55	0	0.35	4.25	
Std dev	2.56443284	0	0.87509398	11.60705321	
Std Error	0.57342462	0	0.195676963	2.595416	

Table 3.3.6d: Numbers of live, dead and encapsulated nematodes or nematodes under the elytra found upon dissection of weevils 6 days after exposure to a high dose of *Heterorhabditis downesi*.

H. downesi High Dose – Week 1

	Live	Dead	Encapsulated	Elytra	Weevil Alive?
1	0	0	0	0	Y
2	0	0	0	0	N
3	0	0	2	0	Y
4	0	4	0	0	N
5	3	0	0	0	N
6	0	0	0	0	N
7	0	0	1	117	Y
8	2	0	2	0	Y
9	19	0	0	0	N
10	0	0	0	0	Y
11	0	0	15	0	Y
12	0	0	0	0	N
13	5	0	1	0	N
14	9	0	0	26	Y
15	0	0	0	0	Y
16	1	0	0	0	Y
17	0	0	1	0	Y
18	1	0	0	0	N
19	0	0	0	0	N
20	0	0	0	0	Y
Mean	2	0.2	1.1	7.15	
Std dev	4.599771161	0.894427191	3.3387675	26.49980139	
Std Error	1.0285401	0.2	0.746571109	5.92553573	

Table 3.3.6e: Numbers of live, dead and encapsulated nematodes or nematodes under the elytra found upon dissection of weevils 2 weeks after exposure to a low dose of *Steinernema carpocapsae*.

	Live	Dead	Encapsulated	Elytra		Weevil Alive?
1	0	0	0		0	Y
2	0	0	0		9	N
3	0	0	0		0	Y
4	0	0	0		0	N
5	0	2	0		0	N
6	0	0	0		0	Y
7	0	0	0		0	Y
8	0	0	0		0	Y
9	0	0	0		0	N
10	0	0	0		0	N
11	0	0	0		4	Y
12	0	0	0		0	Y
13	0	0	0		0	Y
14	0	0	0		0	Y
15	0	0	0		0	Y
16	0	0	0		0	Y
17	0	0	0		0	Y
18	0	0	0		0	Y
19	0	5	0		0	Y
20	0	0	0		0	Y
Mean	0	0.35	0		0.65	
Std dev	0	1.18210339	0		2.158825217	
Std Error	0	0.26432635	0		0.482727994	

S. carpocapsae Low Dose – Week 2

Table 3.3.6f: Numbers of live, dead and encapsulated nematodes or nematodes under the elytra found upon dissection of weevils 2 weeks after exposure to a high dose of *Steinernema carpocapsae*.

S. carpocapsae **High Dose – Week 2**

	Live	Dead	Encapsulated	Elytra	Weevil Alive?
1	0	0	0	0	Y
2	0	0	0	0	Y
3	0	0	0	3	Y
4	0	0	0	0	Y
5	0	0	0	0	Y
6	0	0	0	0	Y
7	0	0	0	0	Y
8	0	0	0	0	Y
9	0	0	0	0	Y
10	0	1	0	0	Y
11	0	0	0	0	Y
12	0	0	0	0	Y
13	0	0	0	0	N
14	0	0	0	0	Y
15	0	2	0	0	Y
16	0	0	0	1	N
17	0	0	0	0	Y
18	0	0	0	0	Y
19	0	0	0	0	Y
20	0	0	0	0	Y
Mean	0	0.15	0	0.2	
Std dev	0	0.489360485	0	0.695852374	
Std Error	0	0.109424331	0	0.155597321	

Table 3.3.6g: Numbers of live, dead and encapsulated nematodes or nematodes under the elytra found upon dissection of weevils 2 weeks after exposure to a low dose of *Heterorhabditis downesi.*

H. downesi Low Dose – Week 2

	Live	Dead	Encapsulated	Elytra	Weevil Alive?
1	0	0	0	2	N
2	0	0	0	0	N
3	0	1	0	0	N
4	0	0	0	0	N
5	0	0	0	0	Y
6	0	0	0	0	N
7	0	0	0	0	Y
8	0	0	0	0	Y
9	0	0	0	0	N
10	0	0	0	0	N
11	0	0	0	0	N
12	0	0	0	0	N
13	0	0	0	0	Y
14	0	0	0	0	N
15	0	0	0	0	Y
16	0	0	0	0	N
17	0	0	0	0	N
18	0	1	0	0	Y
19	0	0	0	0	Y
20	0	0	0	7	Y
Mean	0	0.1	0	0.45	
Std dev	0	0.30779351	0	1.605090586	
Std Error	0	0.06882472	0	0.358909166	

Table 3.3.6h: Numbers of live, dead and encapsulated nematodes or nematodes under the elytra found upon dissection of weevils 2 weeks after exposure to a high dose of *Heterorhabditis downesi*.

H. downesi High Dose – Week 2

	Live	Dead	Encapsulated	Elytra	Weevil Alive?
1	0	0	0	0	Y
2	0	0	0	0	Y
3	0	0	0	0	N
4	0	0	0	0	N
5	0	0	0	0	Y
6	0	0	0	0	Y
7	0	0	0	0	Y
8	0	0	0	0	N
9	0	0	0	0	Y
10	0	1	0	0	N
11	0	0	0	0	N
12	0	0	0	0	N
13	0	0	0	0	N
14	0	1	0	6	N
15	0	0	0	0	Y
16	0	0	0	0	N
17	0	0	0	0	N
18	0	0	0	0	Y
19	0	0	0	0	Y
20	0	0	0	0	Y
Mean	0	0.1	0	0.3	
Std dev	0	0.307793506	0	1.341640786	
Std Error	0	0.06882472	0	0.3	

Table 4.3.1: Number of dead weevils after exposure to entomopathogenic nematodes in a peat substrate for 8 hours. For each treatment n = 10. Each section of the table represents a different experiment using nematodes reared on different dates and insects collected on different dates. Sf = *Steinernema feltiae*, Sc = *Steinernema carpocapsae*, Hd = *Heterorhabditis downesi*.

Day	No. of Dead Weevils				
	3 Days	4 Days	6 Days	8 Days	10 Days
Sf 500	2	2	3	4	4
Sf 1000	2	3	3	4	4
Sf 2000	1	1	3	5	5
SF 4000	1	1	1	2	2
Hd 500	2	2	2	1	2
Hd 1000	3	3	3	3	3
Hd 2000	1	1	1	1	1
Hd 4000	1	1	1	1	1
Sc 500	2	3	8	9	9
Sc 1000	0	2	7	8	8
Sc 2000	5	7	9	10	10
Sc 4000	2	3	6	8	9
Control	0	0	0	0	1

Day	No. of Dead Weevils							
	1	2	3	4	5	6	7	10
Hd 500	0	1	3	3	6	6	6	6
Hd 1000	0	2	2	4	5	5	6	6
Hd 2000	0	2	4	5	6	6	7	7
Hd 4000	0	1	3	4	5	6	6	6
Sc 500	0	2	3	6	9	9	9	9
Sc 1000	0	2	2	4	8	8	8	8
Sc 2000	0	2	4	7	9	9	9	9
Sc 4000	0	2	3	5	7	8	8	8
Control	0	0	0	0	0	0	0	0

Day	No. of Dead Weevils							
	3	4	5	6	7	8	9	10
Hd 500	0	5	5	5	5	5	5	5
Hd 1000	0	4	4	4	5	6	7	7
Hd 2000	0	6	6	6	7	8	8	8
Hd 4000	2	6	6	7	10	10	10	10

Sc 500	3	4	4	4	5	5	6	8
Sc 1000	2	5	6	6	7	7	9	9
Sc 2000	3	8	8	8	8	8	8	9
Sc 4000	5	9	10	10	10	10	10	10
Control	0	1	1	1	1	2	2	4