Ecological impact of entomopathogenic nematodes used to control the large pine weevil, *Hylobius abietis* (Coleoptera: Curculionidae)

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Declaration

This thesis has not been submitted in whole or in part to this or any other university for any other degree and is, except where otherwise stated, the original work of the author.

Signed

Christopher Harvey

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You seekers of the truth accepting that reason will relive and

breathe and hope and chase

and love for you

and you and

you

Jon Anderson, 1974

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List of Abbreviations

%	percent
<	less than
=	equals
>	greater than
°C	Degrees Celsius
µl	microlitre
AFLP	Amplified Fragment Length Polymorphism
ANOVA	Analysis of variance
cm	centimetre
e.g.	For example
EPN	Entomopathogenic Nematode(s)
et al.	and others
Fig.	Figure
g	gravity force
h	hours
ha	hectare
i.e.	that is
IJ	Infective Juvenile
K.-W.	Kruskal-Wallis
L	Litre
LAB	Laboratory Exposure Experiment
M	Molar
M.-W.	Mann-Whitney
m^3	Cubic metres
ml	millilitre
mm	millimetre
N	Number
RFLP	Restriction Fragment Length Polymorphism
SE	Standard Error
sec	seconds
sp(p).	Species
StDev.	Standard deviation
U	Unit
vs.	against

ABSTRACT

The large pine weevil (*Hylobius abietis*; Coleoptera: Curculionidae) is one of the most economically damaging pests in forestry across Northern Europe. Its larvae develop in coniferous tree stumps on clearfell sites and adult weevils feed on seedlings that are replanted on these sites, causing substantial mortality. As the drive towards achieving the sustainable management of natural resources increases, biological control agents are being considered as an alternative to chemical pesticides. Though such agents have great potential for contributing towards a reduction in the ecological impact of pest insect control, they must also be investigated for any potentially damaging effects they may have in whichever context they are being employed.

Entomopathogenic nematodes (EPN; *Steinernema carpocapsae* and *Heterorhabditis downesi*) are currently being inundatively applied to tree stumps on coniferous clearfell sites in the UK and Ireland as biological control agents against the large pine weevil. The aim of the laboratory and field-based experiments and field sampling of clearfell sites that were carried out for this thesis was to investigate the risks associated with this use of EPN in this setting. To this end, the persistence of EPN in soil and bark after application to clearfell sites was recorded and attempts were made to investigate the possibility of hybridisation occurring between an exotic and a native *Steinernema* strain. Effects of EPN on some important non-target insects on clearfell sites, the service-providing wood decomposer *Rhagium bifasciatum* and the pine weevil parasitoid *Bracon hylobii* were also assessed in laboratory and field experiments.

EPN presence in soil samples collected around tree stumps on clearfell sites five months, one year and two years after application of EPN decreased significantly from year one to year two. EPN presence on all sites sampled two years post EPN application was relatively low (less than 7 % positive soil samples). A similar trend was observed for EPN persistence under the bark of stumps. No EPN at all were detected two years after application on those sites treated on a small scale by manually applying nematodes. EPN persistence appeared to be better predicted by tree stump species (which in turn is indicative of the number of pine weevils developing within them) compared to soil type. Spread of nematodes was limited mostly to a 40 cm radius around stumps and little evidence of EPN presence in untreated areas was found on any of the investigated sites. While some spread of EPN to areas adjoining two of the sites occurred, there was no evidence of establishment off-site. (Chapter III).

Attempts to distinguish between an indigenous and an exotic strain of *S. feltiae* and their hybrid using two molecular methods (Amplified Fragment Length Polymorphism or AFLP and Restriction Fragment Length Polymorphism or RFLP) were confounded by methodological obstacles. However, RFLP analysis of the strains did indicate a high rate of intraspecific and even intrastrain variation for *S. feltiae*. Using this method it was also possible to tentatively identify a field-isolated *Steinernema* sp. as *Steinernema kraussei*, though two other unidentified isolates could not be assigned to a particular species (Chapter IV).

The saproxylic beetle community in deadwood other than tree stumps on Irish clearfell sites appears to be dominated by the longhorn beetle *Rhagium bifasciatum* (Coleoptera: Cerambycidae; Fabricius 1775). Both *S. carpocapsae* and *H. downesi* invaded decomposing logs and infected all stages of this species within logs when applied directly to them. However, infection only occurred to a large extent (> 50 % of insects) if the applied dose of EPN was half that applied to tree stumps (1.8 million IJs) to control the pine weevil and infection was also significantly lower when such logs were incubated in the field rather than the laboratory. *R. bifasciatum* individuals were infected in all regions of logs and baiting of wood samples revealed that IJs were penetrating logs to their very centre, indicating high mobility and infectivity of IJs within this substrate. *S. carpocapsae* emerged in numbers of 1,000 IJs or more per week for up to eight weeks after first emergence while *H. downesi* emergence had effectively ceased by the fifth week. A substantial number of live *S. carpocapsae* IJs remained in host cadavers even after eight weeks of emergence. Infection of *R. bifasciatum* was also recorded on three clearfell sites to which EPN had been applied around tree stumps previously (only a single infected larva found on one of the sites). Wood samples from some of these logs contained high numbers of EPN IJs (> 6,000), suggesting that EPN were recycling within logs. However, since infection of *R. bifasciatum* decreased significantly as the distance between a log and treated tree stumps increased it appears that the risk to this service-providing non-target insect can be minimised by being as accurate as possible when applying nematodes. (Chapter V).

The ectoparasitoid wasp *Bracon hylobii* Ratz. (Hymenoptera: Braconidae) is closely associated with the large pine weevil and may therefore be at risk if EPN are applied against the latter (eggs laid on EPN-infected host larvae do not develop to adulthood). Female wasps did not parasitise dead hosts in standard trials (small bark patch [2.25 cm^2]; host either freeze-killed or killed by EPN). However, naive wasps parasitised hosts that were moribund (i.e. those that died of EPN-infection

within the 24 h period they were offered to wasps) significantly less frequently than either healthy control hosts or infected hosts that survived the 24 h trial period. Experienced wasps (one egg laying experience) parasitised all of these host types with similar frequency.

Behavioural observations provided no evidence of wasps locating heavily infected or dead hosts and then rejecting them. Heavily infected hosts that died within the 24 h trial period during which they were offered to wasps tended to move less than their surviving counterparts and wasp oviposition was significantly correlated with host movement and host feeding during trials. Naïve wasps were significantly less likely to parasitise hosts that had their mandibles glued shut than those that did not (this had no effect on parasitism by experienced wasps). Thus, it is suggested that vibrations caused by these host activities were the most important cue driving host location and/or host acceptance in *B. hylobii*.

In continuous 30 minute observation of trials in which wasps were offered a host at the centre of a large bark patch (20.25 cm^2), experienced wasps spent more time close to the host when it was moving and were also significantly more likely to approach the host when it was moving, indicating a vibrotaxic response. Moreover, they only were attracted to dead hosts (freeze-killed or killed by EPN) when the bark covering them was being scratched manually from below to simulate host movement. Some experienced wasps parasitised freeze-killed and EPN-killed hosts when an artificial vibrational stimulus was added in this way and the dead host was moved about inside its chamber. *B. hylobii* also parasitised hosts the species is not known to be associated with, including larvae of longhorn beetle *R. bifasciatum* and waxmoth larvae (*Galleria mellonella*). These results suggest that wasp foraging behaviour, host acceptance and oviposition success is most affected by vibrational cues and that naïve wasps are less likely to locate moribund hosts that move and feed less due to infection with EPN. The risk is expected to be further mediated in a field situation where wasps are likely to encounter several hosts in one host patch and more likely to be attracted to those hosts that are healthy and producing more or stronger vibrational and volatile cues compared to moribund hosts (Chapter VI).

In summary, persistence and spread of EPN after application to tree stumps on clearfell sites was low and there was no evidence of a substantial impact on non-target insects. It can therefore be concluded that the overall risks associated with the use of EPN as an inundative biological control agent against the large pine weevil are low.

CHAPTER I

General Introduction

1.1 Biological control

Biological control (or 'biocontrol') is defined as the *'artificial control of pests and parasites by use of organisms or their products'* by the Penguin dictionary of Biology (Thain & Hickman, 2004). It presents an alternative to conventional control of pest organisms which since the middle of the 20th century has mostly consisted of the use of chemical pesticides. Biological control methods have been used on varying scales to control a range of pests around the globe, including invasive plants and mammals as well as invertebrate and microbial pathogens (Jaksic & Yáñez 1983; Weller 1988; McEvoy & Coombs 1999). Many of the pests that are targeted with biological control are invasive species that lack natural enemies in the environments they have been introduced into (Messing & Wright 2006). A large proportion of pests causing problems around the world are insects. One of the earliest examples of classical biological control targeting an insect pest in an agricultural setting is the use of the vedalia beetle, *Rodolia cardinalis*, which was introduced to citrus groves in California from Australia in the late 19th century to counteract the cottony cushion scale, *Icerya purchasi* (Caltagirone & Doutt 1989). Since then, biological control organisms such as fungi, bacteria and entomopathogenic nematodes have been used against various insect pests (Ferron 1978; Priest 1992; Gaugler et al. 1997a; Georgis et al. 2006). A comprehensive review of the prospects of biological control against insect pests using entomopathogens was published by Lacey et al. in 2001. The authors expect the importance of this branch of biological control to increase in coming years:

'*We envision a broader appreciation for the attributes of entomopathogens in the near to distant future and expect to see synergistic combinations of microbial control agents with other technologies.*' (Lacey et al. 2001)

Three distinct types of biological control efforts can be distinguished: (a) the *importation* of exotic species to combat a particular pest, (b) the *augmentation* of the numbers or quality of the present indigenous enemy or enemies of the targeted pest and (c) the *conservation* of such indigenous enemies of pests (DeBach & Rosen, 1991).

A further distinction in biological control methods is made between *inundative* and *inoculative* or *classical* biological control. Inundative biological control usually entails the release of large numbers of the biocontrol agent at a single time point or for a short period of time. Examples of this would be the application of large numbers entomopathogenic nematodes or microbial pathogens or the release of large numbers of parasitoid wasps against pest insects (Waage & Hassel 1982; Lacey et al. 2001; Georgis et al. 2006).

1.1.1 Classical biological control

As the name implies, classical biological control was the initial mode of biological control employed in most parts of the world. In contrast to inundative control, it is an attempt to establish a biocontrol agent in the target ecosystem by encouraging it to reproduce and maintain a population alongside the pest, which is why it is also referred to as inoculative biological control. The general intention is to create a stable system in which the biocontrol agent keeps the pest at low levels indefinitely and thus exerts a continuous suppressive effect (Murdoch et al. 1985). This eliminates the need for repeated release of biocontrol agents and thus reduces the cost and effort involved. Data on the success rate of classical biocontrol and the interpretation thereof varies considerably among studies and researchers in this field (Hall et al. 1980; Hokkanen & Sailer 1985; Stiling 1993; Lacey et al. 2001). Factors such as climate, predation by the local fauna and the lack of alternative food sources once the target host is depleted have been suggested as possible explanations for the failure of some classical biocontrol efforts (Stiling 1993; Snyder & Ives 2001). A summary of some of the more successful cases has been collected by Caltagirone (1981).

1.1.2 Inundative biological control

The intention of inundative biological control is generally to create a significant, but short-term impact on the target pest population by releasing or applying a large number of the antagonising biocontrol agent. In this context, long-term establishment or dispersal of the biocontrol agent is generally not regarded as desirable (Bigler et al. 2006). In many cases, exotic species are used for inundative biological control. Ideal candidates are invertebrate organisms that are easy to produce in large numbers at affordable costs (Ehlers 2001; Thangavelu et al. 2004). Mainly insects, fungi, nematodes and other small pathogens, parasites or predators fall into this category. Genetic modifications of microbial pathogens such as *Bacillus thuringiensis,* for example, may lead to more efficient and precise applications of inundative biocontrol in the near future (Lacey & Goettel 1995; Lacey et al. 2001). The more host-

specific a biocontrol agent is the better, as this reduces the likelihood of undesirable non-target effects (van Lenteren et al. 2003). Examples of promising trials using inundative biological control include the release of stink bugs to control the potato beetle (Houghgoldstein & Whalen 1993) and the use of entomopathogenic nematodes against corn pests in the USA (Feaster & Steinkraus 1996).

1.1.3 Biological control used against forestry pests

Up until the past two decades, non-chemical control against forestry pests has mainly been focused on direct efforts to reduce the numbers and breeding success of adult individuals by using pheromone-baited traps (Duelli et al. 1997; Wermelinger 2004) or to manipulate the local environment to make it less hospitable to the pest by physical means, for example by scarifying the soil (von Sydow 1997; Örlander & Nordlander 2003) or by removing breeding opportunities for it. Both inundative and classical biological control has been considered as a viable option and pursued with some success in forestry management for the past decades, both in North America (Miller et al. 1987) and in Europe (Pschorn-Walcher 1977). The scientific literature on pathogens and parasitoids associated specifically with bark beetles that is available has been reviewed by Kenis et al. (2004).

One group of pathogens that has been tested for its potential as biocontrol agents extensively are entomopathogenic fungi such as *Beauveria bassiana.* This species in particular has been shown to be effective against bark beetle pests and wood boring pests in laboratory assays (Wegensteiner & Fuhrer 1988; Kreutz et al. 2004) and is a potential candidate for inundative biocontrol in forestry. The possibility of using parasitoids as biological control agents has been examined in a variety of forestry settings, for example to control the emerald ash borer (*Agrilus planipennis*) in the USA (Duan et al. 2009), the argentine stem weevil (*Listronotus bonariensis*) in New Zealand (Goldson et al. 1993) or the large pine weevil (*Hylobius abietis*) (Henry & Day, 2000). Since most parasitoids will only oviposit and develop successfully on their target host, the culturing of large numbers of parasitoid females for inundative biological control is prohibitively labour-intensive and expensive in most cases. Protecting local and/or indigenous parasitoid populations (conservation) or introducing parasitoids for classical biological control with the intention of establishing the parasitoid in the target ecosystem may be more promising approaches for this group of biocontrol organisms. Entomopathogenic nematodes were first considered as inundative control agents in forestry in the 1970s (Burman & Pye 1979). In recent years this group of insect pathogens has emerged as a promising candidate for biological control of forestry pests such as

the Asian longhorn beetle or the large pine weevil (Leather et al. 1999; Fallon et al. 2004; Långström & Day 2004; Dillon et al. 2006).

1.1.4 Risk assessment of biological control

While biological control holds great promise for replacing chemical pesticides or at least reducing the quantities they are used in, they also pose their own set of risks. Introducing exotic insects or pathogens to combat a local pest population can lead to unforeseen and unwanted changes in ecosystem balance, including the collapse of the target population to the point of extinction (Thomas & Willis 1998; van Lenteren et al. 2003). Since most biological control organisms have a host range that goes beyond the specific target they are intended to control, they may have an adverse effect on other organisms in the respective habitat, so-called 'non-target' organisms (Bathon 1996; Follett & Duan 1999; van Lenteren et al. 2003). There are cases in which the successful biological control of a particular pest causes another pest that had previously been of little importance to fill the now vacated niche and present a new problem. Ehler (2000) describes the case of the olive scale (*Parlatoria oleae*) in California, which was combated by importing a parasitoid wasp in the 1960s. In this case, biocontrol was so successful that the use of insecticides was discontinued. This allowed another olive tree pest, black scale (*Saissetia oleae*) to increase in numbers. Additional parasitoids were subsequently introduced to control this pest and establish a stable biocontrol system (Shoemaker et al. 1979; Ehler 2000). Adverse effects on non-target organisms are particularly problematic if the introduced organism has the ability to self-perpetuate once released as is the case with organisms used for classical biological control (Simberloff & Stiling 1996).

Up until recent decades, little information was gathered to follow up on the environmental impacts of both classical and inundative biological control efforts. Consequently, there is an ongoing debate about the risks posed by both of these biological control approaches and the purported cases of adverse effects each has had on non-target organisms or entire ecosystems (Bathon, 1996; Simberloff & Stiling 1996; Thomas & Willis 1998; Louda 2003; van Lenteren et al. 2003). As a result of this debate and with the intensified use of biological control in recent times, efforts have been under way to assess the risks biological control agents may pose and to investigate how best to maximise their efficacy and safety so as to have a solid evidentiary basis upon which to base decision about regulation and licensing (van Lenteren et al. 2005; Bigler et al. 2006). Van Lenteren et al. (2003) have proposed a five-pronged approach to estimating the risks associated with inundative biological control organisms. They suggest that five key factors associated with the respective organism are assessed:

(a) Establishment

(b) Dispersal

(c) Host range

(d) Direct effects on non-target organisms

(e) Indirect effects on non-target organisms

These parameters can be tested for in the laboratory to some extent, but field trials are generally necessary to gain an insight into their relative contributions to the risk in under natural conditions.

Administrative initiatives with the goal of increasing the available information on biological control organisms and their risks include the EU-funded projects REBECA (‚Regulation of Biological Control Agents') and ERBIC (Evaluating Environmental Risks of Biological Control Introductions into Europe) (van Lenteren et al. 2003). The Biological Pesticide Registration Program of the OECD only recently published the document ‚Guidance for Information Requirements for Regulation of Invertebrates as Biological Control Agents (IBCAs)' to aid member states in making regulatory decisions about biocontrol organisms (OECD, 2004). One outcome of these programs is expected to be a harmonisation of the regulation of the import and use of biological control agents across Europe (Babendreier et al. 2005).

1.2 Irish forestry and current forestry policies

Forestry in the Republic of Ireland falls under the supervision of the Department of Agriculture, Fisheries and Food. According to the National Forest Inventory published by the Department in 2007, approximately 10 % of the Irish land area is occupied by forests, with spruce being the dominant species (Anon. 2007a). Public ownership accounts for 57 % of forests and is represented by the State forestry company Coillte Teoranta, which maintains, harvests and restocks them. In the Strategic Plan for Irish forestry that was adopted as part of the Operational Programme for Agriculture, Rural Development and Forestry (OPARDF) in 1999, a target of sustainable forestry was laid out for the Irish forestry industry (Bacon et al. 2003). This initiative was part of a Europe-wide emphasis on promoting sustainable management methods in agriculture and forestry as discussed by the United Nations Conference on Environment and Development and the Ministerial Conference of Forests in Europe in Helsinki in 1993 (Anon. 1999). Coillte adopted the sustainability target and formulated its 'Sustainable Forest Management Initiative' based on the Helsinki Criteria for sustainable management in a framework

document published in 1999 (Anon.). Sustainable Management was adopted not only to ensure continued productivity of forestry operations by Coillte, but also to protect resources and the benefits to environment, biodiversity and socio-economic benefits provided by forest ecosystems in Ireland:

'To us, this means recognising all the social, environmental and economic values that forestry brings, and translating them into management objectives, so that we can provide a natural resource that is both renewable and sustainable in the long-term.'

('Commitment', Framework Document for Sustainable Management, p. 9; Anon. 1999)

In 2007, Coillte reported € 106 million of revenue from forestry operations. The National Forestry Inventory results for 2007 label 13,620 ha of forest area in Ireland as clear-cut and Coillte reports reforestation efforts covering 7,157 ha for that year (Anon. 2007a & 2007b). The rate of reforestation by Coillte has remained consistent at around 80 - 90 % in the years 2004 to 2007, with the total area of clearfelled forest owned by Coillte being reported as 8,106 ha in 2007. With regards to the species that were used for reforestation, the emphasis was on non-native conifer species (including spruce), which accounted for 89.64 % of the seedlings planted by the company. Sitka spruce made up the largest proportion of standing trees in 2007 (51.87 %), with lodgepole pine being the second most common species in Coillte forests (11.90 %). Broadleaf trees comprised 13.08 % of standing forest in 2007 (Anon. 2007c). According to Coillte projections published in 2000, the forest areas estimated to be clearfelled in 2008, 2009 and 2010 were 7,144 ha, 6,365 ha and 6,176 ha, respectively (Anon. 2000b).

The large pine weevil (*Hylobius abietis*) is a beetle common to forest ecosystems across Northern Europe (Scott & King 1974). Larvae of *H. abietis* develop in coniferous tree stumps. It represents a significant threat to reforestation efforts as the adults of this beetle will feed on the bark of young seedlings which are planted to restock clearfell sites (Wilson & Day 1994; Leather et al. 1999). The beetles will attack both coniferous and broadleaf trees, but have been shown to favour coniferous species (i.e. pine, spruce) in laboratory choice trials (Leather et al. 1994; Toivonen et al. 2006). It is the only pest insect against which Coillte takes routine action via pesticides, or, since 2007, attempts to control using entomopathogenic nematodes as biological control agents (Anon. 2007a).

1.2.1 The large pine weevil as a forestry pest

The large pine weevil (*H. abietis*) is distributed across Europe and Asia. It is classified as a major forestry pest in 15 countries across Europe, including Ireland, the UK, Sweden and Germany, threatening an

estimated 3.4 million hectares of forests (Långström & Day 2004). Records of the species being recognized as a forestry pest date back to the 19th century (Germany: Ratzeburg 1839; England: Olmerod 1890). Where it is practised, forestry based on coniferous monoculture has greatly facilitated the spread and establishment of large pine weevil populations. Current control efforts based around the use of chemical pesticides are costing countries affected by the pine weevil millions of Euros *per annum* (e.g. £ 2 million a year in the UK and up to € 30 million annually in Sweden) (Weslien et al. 1998; Leather et al. 1999; Långström & Day 2004). If no chemical control measures were fielded against the pine weevil, the most recent estimate for the resulting economic damage across Europe is € 140 million (Långström & Day 2004).

Female *H. abietis* lay eggs on or beside tree stumps within which larvae then feed and develop until a new generation of adult beetles emerges. As a consequence, densities of weevil adults and larvae can be very high on forestry clearfell sites (Leather et al. 1999). Since adult pine weevils feed on the bark of young seedlings (among other food sources such as the tops of mature or growing trees and brash mats on the site), the main damage they do is to the seedlings that are replanted on recently felled coniferous sites (von Sydow & Örlander 1994; Örlander & Nilsson 1999; Petersson et al. 2005; Leather et al. 1999). On Irish clearfell sites, mild to severe feeding damage was recorded on up to 95 % of spruce seedlings on three clearfell sites (Aoife Dillon, unpublished data). Mortality rates of damaged seedlings on clearfell sites in the UK have been reported to range from 30 % to 100 % (Heritage et al. 1989). Adult weevils may emerge from stumps for several years after felling. While adults do not necessarily remain on the sites they have emerged on to feed on seedlings planted there, their ability to migrate large distances (possibly up to 80 km) to seek out other clearfell sites means that seedlings on any site within range of emerging pine weevil populations are at risk (Solbreck 1980; Wilson & Day 1994). Thorsen et al. (2001) found that seedlings damaged by pine weevils tended to grow slower than those not fed upon. Bark damage on seedlings can provide avenues of infection for pathogenic fungi and bacteria. The ultimate result of severe feeding damage in most cases is seedling death (Eidmann et al. 1996; Von Sydow 1997; Hannerz et al. 2002). This means that replanting of seedlings on the affected site(s) is often necessary at additional cost to the forestry operator. Plate 1.1 shows a photograph of a young pine seedling replanted on a clearfell site in Ireland with feeding damage caused by an adult pine weevil.

Plate 1.1: Pine seedling on Irish clearfell site with clear feeding damage on bark caused by adult pine weevils. Orange patches on the seedling stem represent feeding scars. Photograph by Aoife Dillon.

Due to the nature of monoculture plantation forestry with clearfelling of individual plots as practised by Coillte on the majority of its sites, the pine weevil is provided with optimal breeding conditions. Clearfell sites that have been harvested within one to five years of each other are often separated by distances easily traversed by adult weevils and in some cases are adjacent to each other (author's observation). This means that adult pine weevils emerging from stumps on site will find suitable food resources in the form of seedlings as well as breeding sites with freshly cut tree stumps within close vicinity of each other. Studies in Swedish forests indicate that forestry practice can have a significant effect on the damage caused to seedlings. For instance, delaying the planting of seedlings on a clearfell site (i.e. planting them four instead of two years after felling) decreased the amount of feeding damage recorded (von Sydow 1997; Örlander & Nilsson 1999). Other factors found to influence the damage caused to seedlings include amount of vegetation on clearfell sites (Örlander & Nordlander 2003) as well as the soil characteristics on the site (Petersson et al. 2005). Leather et al. (1999) have published a comprehensive review of the biology of the large pine weevil and the factors that will affect the risk of seedling damage.

Due to the significant amount of economic damage that is caused to the forestry industry by *H. abietis*, it is ranked as one of the most important pest insects across Northern and Eastern Europe. Control of this pest insect has been a main focus of forestry industry in Europe over the past century (Långström & Day 2004). While the large pine weevil is a considerable pest in and of itself, its ability to disperse over

great distances makes it a potential vector for associated plant pathogens. These include the butt rot fungus *Heterobasidion annosum*, also a major concern for forestry across Europe, and the blue stain fungus *Leptographium procerum* (Kadlec et al. 1992; Levieux et al. 1997). More recently, a new species of pine wood nematode, *Bursaphelenchus antoniae*, was found to be associated with *Hylobius* sp. in Portugal (Penas et al. 2006a). This genus also contains the pine wilt nematode *Bursaphelenchus xylophilus,* which has also been reported in Portugal, though it is not known to whether *B. antoniae* is similarly damaging or if *B. xylophilus* is also associated with *Hylobius sp.* (Penas et al. 2006b). While these associations may be of importance in parts of Europe, there is currently no evidence that Irish pine weevils carry these pathogens.

1.3 The large pine weevil *Hylobius abietis* (Coleoptera: Curculionidae)

H. abietis (Coleoptera: Curculionidae) was first described by Linnaeus in 1758. Adult *H. abietis* are approximately 6 to 14 mm in length, dark brown in colour and feature three irregular bands of yellow to orange hairs on their elytra (Plate 1.2). Adult pine weevils mainly feed on the bark of young tree seedlings and the treetops of mature trees (Örlander et al. 2000; Hannerz et al. 2002). On clearfell sites, the density and seasonal development of pine weevil populations is influenced by the felling date of the previous crop. The earlier trees are cut within the time period when pine weevil are active, the greater the proportion of adults emerging early in the summer when emergence commences (Moore et al. 2004). The species of the previous crop also has an effect on pine weevil population density, as pine weevil larvae develop more successfully and with greater speed while reaching a higher end weight in pine stumps compared to spruce or fir (Leather et al. 1999; Thorpe & Day 2002).

Following hibernation in the soil or litter or after emerging from a tree stump, adult pine weevils are attracted to clearfell sites by volatiles released from cut trees (Nordenhem & Eidmann 1991; Tunset et al. 1993; Leather et al. 1999). It has generally been thought that female pine weevils lay their eggs directly into crevices and cracks or chewed niches in the bark of stumps of freshly felled trees. However, experiments by Nordlander et al. (1997) have demonstrated that females may also lay their eggs in the soil close to tree stumps. The hatching larvae then migrate to the tree stump, most likely following the gradient of volatiles released by it (Nordenhem & Nordlander 1994).

Plate 1.2: Adult pine weevil (*H. abietis*), dorsal view (left) and anterior view (above)

Females are capable of depositing up to 120 eggs in the first year of maturity (Novak, 1976) and may continue to oviposit in the following season after having overwintered on the site. In Sweden, the end of the reproductive period usually falls within the middle of summer in the month of August (Örlander et al. 1997). Once hatched, first instar larvae will begin to feed on the phloem layer under the bark of tree stumps. Larval morphology of *H. abietis* is similar to that of most xylophagous Coleopterans. The body is pale, ranging from a yellowish pallor to a clean white colour. The larval cuticle may be slightly transparent. The head capsule is reddish brown and features strong and prominent mandibles that are oriented ventrally when the larva is in the resting position (Plate 1.3).

The number of larval instars pine weevil pass through before pupation can range from four to six, depending on food availability, quality and other environmental factors (Bejer-Petersen et al. 1962; Christiansen & Bakke 1971; Thorpe & Day 2002). Fig 1.2 shows the life cycle of *H. abietis* in relation to the time of year. As larval development may take up to two years, a range of pine weevil stages is

Plate 1.3: Final instar pine weevil larva (*H. abietis*)

usually encountered on colonized sites one to two years after felling. Larval stages are usually found distributed throughout the bark of the stump under the soil horizon, whereas adults and pupating individuals are found around the soil horizon and close to the bole (Henry 1995, Brixey et al. 2006). Most of the studies on the development time and emergence patterns of pine weevil have been conducted in Southern Scandinavia (Bakke & Lekander 1965; Havukkala 1976; Lekander et al. 1985). In these regions, pine weevil development in non-optimal host stumps such as spruce may take up to four years. Data from studies in Britain, in particular Scotland and Northern England, suggest that in their more temperate climate, development is accelerated compared with Scandinavia and may take as little as two years in spruce stumps (Henry 1995; Moore et al. 2004). Stump sampling in Ireland has indicated that in lodgepole pine tree stumps, the first generation of adults is usually found in the first summer season after felling, with a total development time of roughly 14 to 16 months (Dillon et al. 2007), whereas larval development and emergence of adults may be delayed by several months in spruce, causing the first adults to emerge in the second summer after felling on sites stocked with this stump species (Dillon et al. 2008a; Aoife Dillon, personal communication).

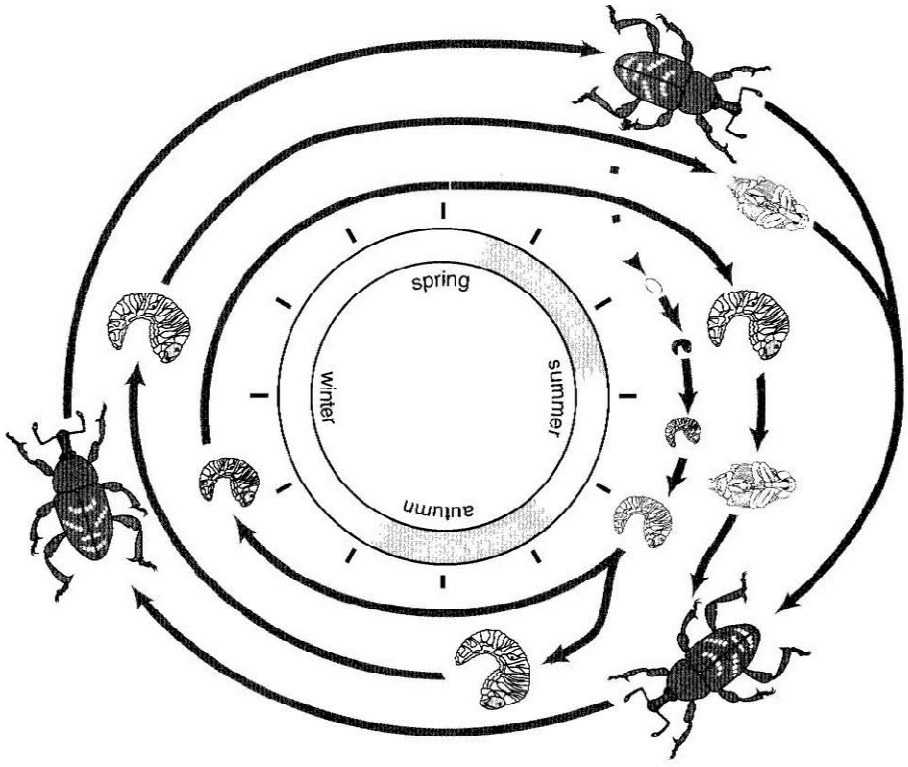


Fig 1.2: Life cycle of the large pine weevil (*H. abietis*) (from Heritage, 1996).

In stumps, *H. abietis* larvae generally feed in areas of the bark situated below the soil horizon and most will stay close to the bole of the stump (Henry 1995). Pupation and emergence of adults in the first year after felling in the UK and Ireland occurs in summer (July to September), peaking late in August and early September (Heritage cited in Torr et al. 2007; Aoife Dillon, unpublished data). Not all adults will emerge before the autumn, with a portion overwintering in their pupation chamber within the tree stump (Nordenhem 1989; Leather et al. 1999). As mentioned, the length of the developmental period is influenced by the host species, but may also be prolonged if eggs were laid late in the previous season or temperature conditions are unfavourable for larval feeding and development (Christiansen 1971; Lekander et al. 1985). Adults may continue to emerge for several years after first emergence. Örlander et al. (1997) report that pine weevils were abundant on clearfell sites in Southern Sweden five years after felling. No long-term population studies on clearfell sites have been conducted in Ireland, but the available data indicates that pine weevil populations in stumps peak one and two years after clearfelling and fall off thereafter (Dillon et al. 2007; Griffin et al. 2008; author's observation).

1.4 Entomopathogenic nematodes (EPN)

Two families of roundworms (Phylum Nematoda; Diesing 1861) are known to act as entomopathogens: the Steinernematidae and Heterorhabditidae (Nematoda: Rhabditida). Their lethality toward many insects is the result of the symbiotic bacteria they carry. *Steinernema* spp. carry symbiotic bacteria of the genus Xenorhabdus, while *Heterorhabditis* spp. carry bacteria of the genus Photorhabdus (Adams & Nguyen 1993, Boemare 1996). Photorhabdus bacteria are luminescent and host insects infected by them can be identified by luminescence of the cadaver. Due to their ability to infect most insects, their short generation time, their propensity for being produced in large numbers in artificial media as well as their convenient non-pathogenicity against vertebrates they have become popular biological control organisms for use against insect pests (Kaya & Gaugler 1993; Boemare et al. 1996; Gaugler et al. 1997; Ehlers 2001).

1.4.1 Distribution and occurrence of EPN

EPN are globally distributed (with the exception of Antarctica) and are found in virtually every terrestrial ecosystem (Hominick 2002). Their predominant natural habitat is the soil, and EPN can be isolated from soil samples either directly or by baiting the samples with susceptible insects (Jian et al. 1997; Spiridonov & Moens 1999; Sturhan & Ruess 1999; Rosa et al. 2000). Bedding & Akhurst (1975) suggested a very simple baiting technique using the larvae of the waxmoth (*Galleria mellonella*) that has proved to

be very successful and has been used in many subsequent studies. EPN distribution can be patchy, but high or repeated recovery from sites has been reported in a number of instances (Hominick & Briscoe 1990; Griffin et al. 1991; Haukeland 1993; Campbell et al. 1995). Hominick (2002) has compiled an exhaustive review of EPN biogeography.

Steinernema carpocapsae (Weiser, 1955) is not native to Ireland, but is found across Europe (e.g. Austria, France, Sweden, and a disputed report from Britain) (Hominick 2002; Christine Griffin, personal communication). As one of the most successful and widely available EPN biocontrol agents, it has been introduced for inundative control in several countries, including Ireland (Dillon et al. 2008a).

Plate 1.4: Infective juvenile of *S. carpocapsae*

Several species of EPN have been isolated in the UK and Ireland. These include *S. affine, S feltiae, S. kraussei* (UK only), *S. carpocapsae* (UK only, disputed) and *H. downesi* (Blackshaw 1988; Griffin et al. 1994; Gwynn & Richardson 1996). In the Republic of Ireland, the only nematode species to have been recovered from woodland thus far is *S. feltiae* (Griffin et al. 1991; Dillon, 2003). *Steinernema affine* has only been found in tillage and grassland in Ireland, while *H. downesi* appears to favour sandy coastal soils and dunes. The latter species seems to favour sandy soils in general, as it was also predominantly isolated from sandy, coastal localities in Estonia and Denmark (Griffin et al. 1999; Stock et al. 2002; Rolston et al. 2005).

The free-living stage of EPN is a specialised third stage juvenile (J3), which is often referred to as the infective juvenile (IJ). Another common name for this stage is the 'dauer' stage (from the German word 'dauerhaft' or 'durable'), as this stage is protected by an additional cuticular layer (the cuticle of the second moult is retained) and can survive the comparatively unfavourable conditions the IJ faces when emerging from a host cadaver in search of a new host (Poinar & Leutenegger 1968; Glazer 2002; Grewal et al. 2002). Since the infective juvenile stage does not feed while outside of the host, it relies on stored reserves, mainly lipids. IJs can be stored for several months in the laboratory (Selvan et al. 1993; Patel & Wright 1997; Grewal 2000; Chen & Glazer 2005). IJs produced on an industrial scale can be concentrated and stored in a dessicated state (anhydrobiosis) in special storage formulations at very high densities (Grewal 2000; Ehlers 2001).

Depending on the foraging strategy, infective juveniles seek out a new host or simply wait for one to pass by. They may enter the host via its natural orifices (spiracles, anus or mouth), or, in the case of heterorhabditid species, they may cut their way directly through the cuticle using an oral tooth (Bedding & Molyneux 1982; Wang & Gaugler 1998; Dowds & Peters 2002). Some steinernematids may enter directly via the cuticle as well (Peters & Ehlers 1994). It is therefore not surprising that penetration rates of IJs vary considerably among different host types, depending on factors associated with host cuticle thickness, mandibular activity, spiracle size and defecation frequency (Caroli et al. 1996; Dowds & Peters 2002). Upon invading the host insect, the IJ sheds its protective sheath and releases the symbiotic bacteria it has carried in its gut from the previous host (in the case of some *Steinernema* spp., the bacteria are carried in a specialized vesicle). The bacteria produce toxins which kill the insect within 24 to 48 h and create an amenable environment for the now feeding juveniles (Forst et al. 1997; Johnigk & Ehlers 1999; Dowds & Peters 2002). Nematodes feed on the bacteria colonising the host and the bacteria also produce antimicrobial compounds that prevent secondary infection of the host cadaver with saprophytic bacteria and fungi (Forst et al. 1997; Hu & Webster 2000; Forst & Clarke 2002). Some bacterial products have also been shown to deter insect scavengers (Zhou et al. 2002). Due to the delay between nematode penetration and mortality caused by bacteria, infected hosts may sometimes serve as dispersal vehicles for the nematodes, a process termed 'phoresis' (Timper et al. 1988; Downes & Griffin 1996).

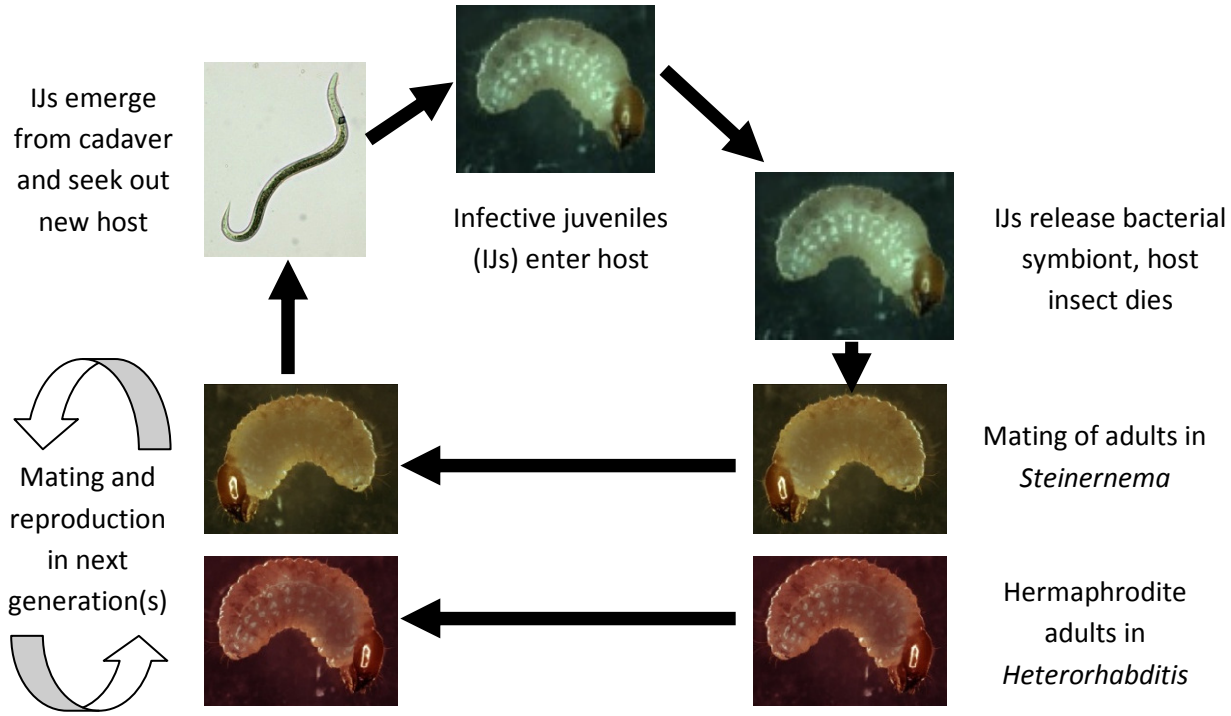


Fig 1.3: Basic life cycle of *Steinernema* and *Heterorhabditis*.

The IJ feeds in the dead host and passes through five larval stages. After two to four days, depending on host and EPN species, the J5 stage becomes the adult that then sexually matures. Adults are dioeceous in *Steinernema* spp. and hermaphroditic females in *Heterorhabditis* spp. (Poinar 1993). One female is capable of producing thousands of eggs that she releases into the haemocoel of the host (Zioni et al. 1992, Nguyen & Smart, 1992; Boemare et al. 1996). The juveniles that emerge pass through the four juvenile stages as they feed and develop (J1 – J2 – J3 – J4) and may mature to a second generation of adults. In *Heterorhabditis*, dioeceous as well as hermaphroditic adults develop from the second generation onward. The number of reproductive generations produced by a population of EPN in a host insect depends on the number of nematodes invading and the available resources. Evidence from in vitro experiments suggests that depletion of resources either directly or indirectly (in some species via the release of a pheromone), leads to arrestment of juvenile development in the J3 stage that in turn leads to the accumulation of infective juveniles in the host cadaver (Popiel et al. 1989; Johnigk & Ehlers 1999; Ehlers 2001; Johnigk et al. 2004; San-Blas et al. 2008). Once IJs have developed, crowding effects and the accumulation of waste products such as ammonia in the host cadaver may trigger subsequent IJ emergence (San-Blas et al. 2008). Depending on the host, one infected insect can produce hundreds of thousands of IJs.

1.4.2 Nematode movement and host location

Based on their host-finding strategies, EPN have traditionally been classified as either 'cruisers' (i.e., species whose IJs actively move through a substrate to locate a host) and 'ambushers' (i.e. species that employ a 'sit and wait' strategy that involves little displacement and active searching). Species that do not clearly fall in either category are classified as 'intermediates' (Lewis et al. 2006). Lewis (2002) has also provided a classification in this respect for steinernematids. *Steinernema carpocapsae* is listed as an ambusher, *S. feltiae* as an intermediate. *H. downesi* – like almost all heterorhabitids - is considered a cruiser (Dillon 2003). It has been argued that the strict distinction between ambushers and cruisers may only be applicable in some environments. For example, Kruitbos et al. (2010) have shown that *S. carpocapsae* can readily move through peat substrate to infect hosts.

The most basic mode of locomotion observed in EPN is sinusoidal movement over a flat surface as is typical of nematodes (Wallace 1968; Croll 1975). EPN may follow gradients when performing such movement, for example gradients of temperature or host cues (Burman & Pye 1980; Wang & Gaugler 1998). EPN may also display other behaviour or movement types under certain circumstances. Especially ambushers have been observed to stand on the substrate. When standing, IJs raise a portion of the anterior section of their body off the substrate, sometimes waving it back and forth (also referred to as 'nictation'). Plate 1.5 shows *S. carpocapsae* IJs standing and nictating on the surface of wood pieces to which they had been applied in the laboratory. This behaviour is thought to facilitate the attachment of the IJ to a passing host insect, as standing and/or nictating species have been shown to be many times more successful at attacking highly mobile hosts than species that do not show this behaviour are (Campbell & Gaugler 1993; Lewis et al. 2006). Standing and nictation is most commonly observed in *Steinernema* spp. and is of varying duration, with *S. carpocapsae* being one of only three steinernematid species in which standing occurs over protracted periods of time (Campbell & Kaya 2002).

In some *Steinernema* spp., IJs have been observed to jump (Reed & Wallace 1965). This mode of locomotion allows an IJ to traverse a distance of several times its own body length, which would facilitate dispersal. It could also function as an attack mechanism by which nematodes launch themselves at passing hosts (Campbell & Kaya, 1999a & b; Lewis et al. 2006). It has also been suggested that jumping and/or standing behaviour may allow some EPN species to bridge large pores found in some substrates (e.g. loose, porous soils or organic litter) with greater ease than cruiser species that do not nictate and instead move cross the surface of soil particles (Reed & Wallace 1965; Kruitbos et al. 2010).

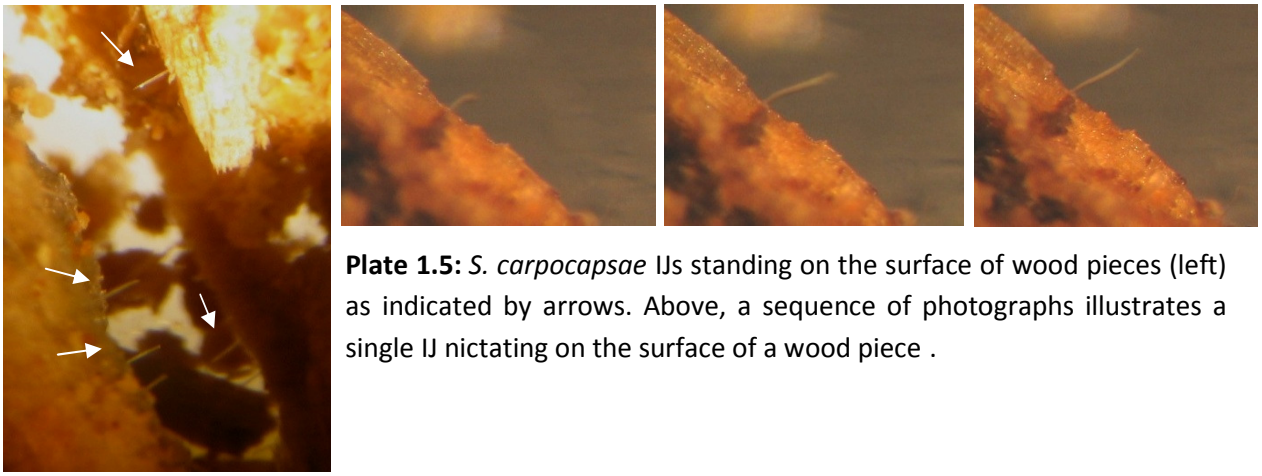


Plate 1.5: *S. carpocapsae* IJs standing on the surface of wood pieces (left) as indicated by arrows. Above, a sequence of photographs illustrates a single IJ nictating on the surface of a wood piece .

Cruiser species are thought to be more attracted by host volatiles and other host cues, since they are capable of moving towards the source of such cues from a distance (Grewal et al. 1994a). This has been borne out by experimental evidence. Lewis et al. (1992, 1995) have found that *S. carpocapsae* does not change behaviour in response to host cues, while cruiser species *S. glaseri* does. *S. carpocapsae* does however aggregate around host compounds once it has come in contact with the host cuticle. Other cues that might attract cruising IJs are CO_2 gradients, fecal matter and other waste generated by the host and vibrations created by host activity (Grewal 1993; Lewis et al. 1993; Torr et al. 2004). Once a host has been invaded by nematodes and bacterial infection has taken hold, the volatiles released may make hosts more or less attractive to IJs of the same or other species. Experimental results on *Steinernema* spp. have shown that *S. carpocapsae* is repelled by hosts infected with most heterospecifics and also conspecifics, but that attraction and deterrence effects differed among *Steinernema* spp. (Grewal et al. 1997). Some *Steinernema* spp. will co-infect hosts together with heterospecifics and in some cases are able to develop in one host simultaneously (Lewis et al. 2006).

In recent years, the more or less dichotomous distinction between ambushers and cruisers has been called into question by researchers observing nematode behaviour in substrates more reflective of their natural habitat than those typically used in the laboratory assays (i.e. agar plates, filter paper and sand). For instance, Kruitbos et al. (2010) recently demonstrated that IJ foraging behaviour can be significantly affected by the substrate texture. In their trials, *S. carpocapsae* (ambusher) showed greater mobility and host taxis than *H. megidis* (cruiser) in peat, while the reverse was true in sand (Kruitbos et al. 2010). In field trials, *S. carpocapsae* outperformed the intermediate *S. feltiae* when either species was applied to tree stumps to control for the large pine weevil (Dillon et al. 2006).

1.4.3 Use of EPN for biological control

The use of EPN as biocontrol agents has a long history. Early uses going back to the 1930s were geared towards classical biological control, as in the case of the introduction of *S. glaseri* to control the Japanese beetle *Popilla japonica* in the USA. The advent of chemical pesticides shortly thereafter, however, stifled research in this area early on (Smart 1995). EPN re-emerged as potential biocontrol agents in the 1960s and 70s, with research mainly focussing on *S. carpocapsae* (then classified as *Neoaplectana carpocapsae*) (Jackson & Moore 1969; Pye & Burman 1977). By the 80s, large scale production of EPN in bioreactors was being actively researched (Bedding 1981; Gaugler 1981). Several EPN species (including *S. carpocapsae*, *S. feltiae* and *H. megidis*) are now being produced commercially and are available to the public in a formulation suitable for short-term storage (Ehlers 2001; Dillon 2003). Only recently, trial production of *H. downesi* was commenced by Koppert in the Netherlands. Since IJs can now be produced relatively cheaply in large numbers, the preferred method of application is inundative, i.e. short-term application of large numbers of nematodes to create a direct impact on the pest population (Feaster & Steinkraus 1996; Shapiro-Ilan et al. 2006; Dillon et al. 2007). For a review of some of the current biocontrol applications of EPN, see Georgis et al. (2006).

EPN have several features that make them ideal candidates for biological control of pest insects: They are relatively easy and cheap to produce, they are not damaging to vertebrates, have a wide host range, can actively seek out target insects, kill quickly (24 – 48 h) and are able to reproduce in the field and may thus have a limited inoculative effect and finally, they can be used in combination with other biocontrol agents (e.g. microbes, parasitoids etc.) (Gaugler 1981; Kaya & Gaugler 1993; Smart 1995; Ehlers 2001). Since some EPN are resistant to commonly used pesticides, they can also be used in tandem for an additive effect (Rovesti et al. 1990). In addition, nematodes can easily be applied by mechanical and automated means, for example via the irrigation system on plantations or in greenhouses, thus reducing the labour costs associated with their application (Shapiro-Ilan et al. 2006).

1.4.4 Survival and efficacy of EPN

Several abiotic factors are known to adversely affect EPN pathogenicity in a variety of ways. While most EPN species can tolerate and remain active and infective in a range of temperatures spanning up to 30°C, there is a temperature optimum for these parameters that appears to correlate with the geographic distribution of each EPN species, but is consistent among populations of species established at different localities (Grewal et al. 1994b). Some species such as *S. kraussei* are favoured by low

temperatures and can infect insects at temperatures below 10°C (Mráček et al. 1998; Willmott et al. 2002; Torr et al. 2007), while others like *H. megidis, S. carpocapsae* and *S. feltiae* are adapted to moderate temperatures and are most infective in a range between 20 and 25°C, values similar to those reported for most *Heterorhabditis* spp. (Dunphy & Webster 1986; Saunders & Webster 1999). Though IJs are sheltered from abiotic stress in the host, non-optimal temperatures or low humidity can also delay or prevent development of nematodes in host cadavers and their subsequent emergence (Brown & Gaugler 1997).

IJs are very susceptible to UV irradiation and die quickly when exposed to light at a frequency of around 300 nm for more than a few minutes. Direct sunlight is tolerable for up to half an hour before adverse effects on infectivity and survival of nematodes are observed. *Steinernema carpocapsae* has been shown to be susceptible to short UV (254 nm), but is more resistant to longer UV (366 nm) compared to *H. bacteriophora* and may therefore be more effective in biocontrol applications involving exposed surfaces (Gaugler & Boush 1978; Gaugler et al. 1992).

Soil moisture and air humidity has also been shown to affect EPN survival and activity in the laboratory and the field. Most species prefer low to intermediate soil moisture and perform poorly when the soil is saturated with water, either due to reduced mobility or oxygen deprivation (Koppenhöfer et al. 1995; Shapiro-Ilan et al. 2006). The lack of a sufficiently thick water film which facilitates sinusoidal nematode movement on soil particles may also contribute to poor EPN movement in dry soils (Gaugler 1988). However, these experimental results do not necessarily apply to all members of a species, especially since inter-strain variation in response to abiotic factors has been reported (Dunphy & Webster 1986; Molyneux 1986; Grewal et al. 2002). Addition of antidessicants to the nematode suspension prior to application may increase efficacy of nematodes by increasing their survival time in exposed and dry environments (Glazer 1992). Soil pH can also have an effect on nematode survival and infectivity (Kung et al. 1990b; Koppenhöfer & Fuzy 2006).

Pore size of soils has been identified as one of the major factors influencing nematode movement in soils. Pore size in soils decreases as clay content increases. Dispersal and infection success of several EPN species, including cruiser species *S. glaseri* and *Heterorhabditis* spp. but also *S. carpocapsae* has been shown to be greatest in soils with high sand (large pore size) and low clay content (small pore size), an observation that may be due to small pore sizes restricting the movement of IJs, particularly that of large species (Georgis & Poinar 1983; Molyneux & Bedding 1984; Koppenhöfer & Fuzy 2006). In substrates of more heterogenous and on average greater pore size, for example peat, EPN species

conventionally categorised as ambushers may perform as well as or better than cruiser species (Molyneux & Bedding 1984; Gaugler 1988; Kruitbos et al. 2010). It is therefore advisable to not only take factors like temperature and soil moisture into account when selecting EPN species for biological control, but to consider the target substrate texture and properties as a whole (Gaugler 1988; Gaugler et al. 1997a; Koppenhöfer & Fuzy 2006).

1.4.5 Production and application

In the laboratory, IJs can be produced in vivo, usually using a low-resistance host such as the final instar larvae of waxmoth *Galleria mellonella* L. (Lepidoptera: Pyralidae). One waxmoth larvae can yield up to 3.8×10^5 infective juveniles (author's observation). Rearing of nematodes in this way is not economically feasible when the goal is large scale application requiring many millions of nematodes. In this case, commercially produced nematodes are reared in vitro and stored in a desiccated (anhydrous) state in a formulation (usually a gel, a powder or of granular texture) that allows storage and suspension in water (Ehlers 2001; Gaugler & Han 2002). Production on a medium to large scale can include a solid substrate, such as a spongy material, or it can be carried out in a liquid medium contained in a fermenter (Bedding 1981; Ehlers 2001; Johnigk et al. 2004). Growth media for the nematodes are basic (for example, EPN have been reared on kidney extract medium), requiring a source of nitrogen, carbohydrates and lipids along with some mineral salts (Ehlers 2001; Gaugler & Han 2002). In large-scale rearing of EPN, the timing of the inoculation of the medium with the symbiotic bacteria and the nematodes as well as the final harvest is of great importance, as poor development of the nematode population (e.g. due to population dynamics) and phase-shifts in bacterial populations may significantly decrease the overall yield (Ehlers 2001; Johnigk et al. 2004).

Since health risks to humans posed by EPN are very low, application of EPN on a large scale can be carried out by having workers spray the suspension using common equipment such as pressurized sprayers that can also be fitted to helicopters or other vehicles (Georgis 1993) (Plate 1.6). The infective juveniles can also be delivered via automated systems, for example the irrigation systems of plantations and greenhouses (Georgis 1992; Wright et al. 1993; Shapiro-Ilan et al. 2006). Such automated application techniques not only reduce application costs, but may also allow precisely timed and synchronized application of IJs to all parts of a target area.

Plate 1.6: Infective juveniles of *S. carpocapsae* being applied to tree stumps on an Irish clearfell site using spray nozzles connected to a tank mounted on a motorized forwarder (photograph by Aoife Dillon).

Biotic factors like predation or competition with other insect pathogens or parasites may reduce the efficacy of EPN in a field situation (Kaya & Koppenhöfer 1996). Nematode-trapping or pathogenic fungi, protozoans and mites can prey on IJs in soil (Kaya 2002). Generalist predators may scavenge infected host cadavers, but the symbiotic bacteria of EPN have been shown to produce compounds that can deter some members of this group (Zhou et al. 2002). Estimating the potential reduction in biocontrol efficacy of applied EPN due to predation is not practical as these factors are poorly understood at this time and their estimation would require preparatory research in advance of application on an unfeasible scale.

Antagonism between EPN and other naturally present organisms or concomitantly applied biocontrol agents may also influence the efficacy of the control effort, though experimental results in this area are not always in agreement. Barbercheck et al. (1990, 1991) have reported that application of EPN together with insect pathogenic fungi *Beauveria bassiana* can increase the overall mortality and shorten the time to death of target insects, but that the two agents antagonize each other when invading hosts, with EPN preventing *B. bassiana* reproduction and vice versa, depending on which agent established in the host first. Additive and even synergistic effects were observed by Ansari et al. (2004) when applying *S. glaseri* and *H. megidis* in combination with fungal pathogen *Metarhizium anisopliae* against the turf grass pest *Hoplia pilanthus.* The IJs of EPN also seem not to be adversely affected by a commonly used microbial

inundative biological control agent, *Bacillus thuringiensis*. This allows application of the two agents in one suspension to control multiple pests (Kaya et al. 1995). Synergistic effects of EPN and *B. thuringiensis* have been demonstrated in laboratory trials (Koppenhöfer & Kaya 1997). EPN are resistant to many commonly available and widely employed pesticides and may compliment their efficacy if applied together with them. For instance, Koppenhöfer et al. (1998 & 2000a) were able to demonstrate that imidacloprid and the species *H. bacteriophora* and *S. glaseri* interacted synergistically in controlling the Japanese beetle *Popilla japonica* in greenhouse and field trials. While EPN can be combined with most other biocontrol agents used against insects and even with pesticides, there is often interspecific competition between EPN when applied together or when an applied species has to compete with a local population, in some cases even leading to extinction of said population (Kaya & Koppenhöfer 1996).

EPN of the two different genera (*Steinernema* and *Heterorahbditis*) cannot coexist and reproduce in the same host, and steinernematid species tend to gain the upper hand and displace the heterorhabditid competitor (Alatorre-Rosas & Kaya 1991; Kaya & Koppenhöfer 1996). In *Steinernema* spp., infected host insects have been shown to be unattractive to heterospecific IJs in some cases. IJs of *S. carpocapsae* and *S. glaseri* were also shown to be deterred or at least less attracted to host insects infected by conspecifics once infection had progressed (Grewal et al. 1997).

1.4.6 Risks associated with EPN as biological control agents

EPN have not been shown to pose any significant health risks to vertebrate species, including humans (Boemare et al. 1996; Ehlers & Hokkanen 1996; Gaugler 2002). Since EPN also do not pose any other significant environmental risks, such as pollution or release of toxins, there is currently no restriction on their use on the European level, though some member states have internal licensing procedures for EPN. In Ireland, the Pesticide Control Service which is part of the Department of Agriculture, Fisheries and Food, gives out permits for large scale pest control with EPN, while additional permits from the National Parks and Wildlife service are required if EPN are to be used in areas (i.e. nature reserves etc.) overseen by this body or when an exotic species is being applied.

Due to the frequent use of exotic EPN species to control pest insects, their potentially wide host range and the ability of these nematodes to reproduce and disperse in the environment, they may adversely affect non-target species, thus posing a potential risk (Bathon 1996; van Lenteren et al. 2003). Studies on non-target effects of EPN are few, but research in the area has intensified in recent years. EPN effects

on soil microbial community and nutrient cycling were examined by De Nardo et al. (2006), who found that most parameters measured were not affected by EPN presence. When both EPN and a host (*Galleria mellonella*) were introduced into the system, however, ammonia accumulated in the soil, indicating an indirect effect of the nematodes. In one study, EPN not only had no adverse affect on soil nematode community, they had the positive side effect of selectively reducing the abundance of plant pathogenic nematodes (Somasekhar et al. 2002).

Bathon (1996) has reviewed the literature pertaining to non-target effects of EPN on vertebtrates and molluscs. They are only susceptible to EPN if exposed to IJs at high densities in the laboratory. For example, IJs of *H. bacteriophora* killed frog tadpoles in Petri dish assays but failed to do so in aquaria more representative of the field situation (Bathon 1996). Arthropods are at a greater risk of infection. Poinar and Paff (1985) found that isopods of the genus *Porcellio* were infected by *S. carpocpasae* in the laboratory and that the nematodes were able to develop in the host hemocoel.

Based on the results of both laboratory assays in which EPN have infected insects from a large number of species across several orders and the diversity of insects from which some species have been recovered in nature, EPN are widely considered as extreme generalists (Bathon, 1996; Peters 1996). Such laboratory assays may, however, only give a poor approximation of EPN infection in the field. In addition, knowledge of the natural host ranges of EPN isolates is often lacking due to the widespread use of *G. mellonella* as a bait insect for field samples in place of recording emergence from insects within the samples themselves (Griffin et al. 2005). Bioassays or mesocosm experiments in which EPN are applied to soil containing larval or pupal stages of insects (e.g. Diptera, Lepidoptera or Coleoptera) may help address some of these issues as they give an indication of how successful EPN are at infecting and reproducing in hosts encountered in a complex substrate similar to that present in a field situation (Bathon 1996). Depending on the EPN species and target insect used and the experimental conditions, infection rates can vary considerably (e.g. 90 % of larvae of *Mamestra brassicae* [Lepidoptera: Noctuidae] killed by *S. feltiae* and *H. bacteriophora* but only 30 % infection of pupae of the hover fly *Eupeodes corollae* killed by *S. feltiae*) (Jung cited in Bathon 1996).

Few studies include follow-up investigations of the fate of EPN and their infection of non-target insects in the field after inundative application (Bathon 1996). Where data has been published, however, it seems to indicate that the non-target impact of EPN is low. Georgis et al. (1991) found that both *Steinernema* and *Heterorhabditis* species applied as biocontrol agents in a variety of settings (e.g. against the Japanese beetle and the black vine weevil) did not have a significant impact on soil

arthropod communities (e.g. crickets and carabid beetles), while commonly used chemical pesticides did. In a trial conducted by Koch and Bathon (1993) in Germany, insects were collected from plots in four ecosystems, including the edge of a pine forest, after these had been treated with *S. feltiae* and *Heterorhabditis* spp. (approximately 1 million IJs/m^2). The authors reported no extinctions and impacts on only a small set of the insect species were recorded, while the abundance of some species increased after EPN application.

Several parasitoid wasps that are associated with pest insects have been found to be susceptible to EPN infection in laboratory assays, especially during the larval stages of development (Kaya 1978; Kaya & Hotchkin 1981; Georgis & Hague 1982). Similar effects have been reported after field application of EPN, for example *S. feltiae* application to control the web-spinning sawfly *Cephalcia arvensis* reduced the eclosion of ichneumonid parasitoid *Xenoschesis fulvipes* by over 66 % (Battisti 1994). Reduction of parasitoid populations associated with the targeted pest are not only undesireable because they affect a non-target insect, but especially because they reduce the naturally occurring biological control by the parasitoid. Such negative effects on parasitoids are not always observed, however. Larvae of the parasitoid *Habrobracon hebetor* are susceptible to infection with *H. indica*, but when nematode and wasp were used together against Indian meal moth *Plodia interpunctella* in laboratory assays, no antagonistic effect was observed (Mbata & Shapiro-Ilan 2010).

In Ireland, application of EPN to tree stumps did not appear to significantly affect the beetle community associated with them, as trapping data has revealed (Griffin et al. 2008). In the laboratory, however, *S. carpocapsae* and *H. downesi* were shown to cause infection in a parasitoid wasp (*B. hylobii*) associated with the target pine weevil *H. abietis* against which these EPN were being applied in the field (Everard et al. 2009, author's observation). Larvae, cocoons and adults were susceptible to nematodes in the laboratory and ovipositing females did not reject infected hosts in most cases (Everard et al. 2009). Application of EPN to tree stumps in the field did not adversely affect parasitism rates of *H. abiteis* by *B. hylobii*, however, indicating that the nematodes were not affecting the parasitoid population (Dillon 2008b).

Establishment and long-term persistence of inundative biological control agents is not intended and may have damaging consequences. However, establishment of EPN after application has not been found in many cases (Smits 1996). The nematode *S. scapterisci*, which was introduced into Florida to control mole crickets in 1985 was still found in field populations eleven years later (Parkman & Smart 1996). *S. glaseri*, which was introduced into New Jersey in 1939 to control the Japanese beetle *Popila japonica*

was present at low levels some 50 years later (Gaugler 1992). Persistence rates and duration of persistence after inundative application varies with the applied species, field conditions and target hosts. Though EPN persistence may be high in the short term (weeks to months), in most studies, persistence of significant numbers of EPN on site does not exceed periods of two to three years, with the majority of IJs disappearing within the first few weeks after application (Klein & Georgisi 1992; Wright 1993; Smits, 1996; Susurluk & Ehlers 2008), though in some cases, high rates of persistence (e.g. EPN presence in up to 44 % of samples) have been reported two years and more after original application (Shields et al. 1999; Dillon et al. 2008a). Due to the small size of nematodes and consequently their limited mobility, their potential for long-range dispersal is low. However, phoresis (transport by a host) may allow EPN to travel great distances in some instances and establish a population in a new environment (Downes & Griffin 1996).

Transgenic or exotic EPN strains or species that are released as control agents may pose a risk as they could displace or hybridize with indigenous EPN populations. Examples for this have not been published yet, however. Gaugler and Wilson (1997b) found no difference in the persistence of a transgenic strain of *H. bacteriophora* and the wild type strain. Likewise, Dillon et al. (2008a) found no evidence that an exotic strain of *S. feltiae* had displaced or hybridized with a local strain on Irish clearfell sites.

Though results so far regarding the safety of EPN are promising, further research with regards to non-target effects, spread and persistence of EPN is necessary to ensure the continued safety and viability of the use of this group of insect pathogens as biological control agents against insect pests.

1.5 Control of the large pine weevil

Efforts to control the large pine weevil have been undertaken for the greater part of the past two centuries in most regions in which the beetle is considered a pest. The early efforts focussed mainly on debarking and removing tree stumps from clearfell sites to reduce breeding sites available to *H. abietis*. This is no longer practiced as a control method due to the excessive costs and ecological damage incurred (Långström & Day 2004), though Finnish forestry has recently begun removing tree stumps from clearfell sites as a fuel source on a limited scale. Early results, however, do not indicate that this is having the side-effect of controlling pine weevil damage to seedlings on these sites (Heli Viiri, personal communication). Subsequent attempts to control the pine weevil on clearfell sites have been mainly centred on chemical control via pesticides, physical protection of seedlings, sylvicultural management and – later on in the 20th century - biological control. With the increasing restrictions placed on the use

of chemical pesticides, it is expected that research efforts in this area will be increased in the coming years (Leather et al. 1999; Kenis et al. 2004).

1.5.1 Physical barriers and management of clearfell sites

As the main damage caused by pine weevils is to the seedlings in replanted clearfell sites, one of the first ideas to reduce this damage was to create a physical barrier on or around seedlings that would prevent access to pine weevil adults. Sheltering seedlings physically, for example by applying a coat of latex to their bark or by placing a protective plastic collar around their base has been shown to significantly reduce feeding damage in Sweden (Hagner & Jonsson 1995; Petersson et al. 2004). For instance, Eidmann et al. demonstrated that plastic sheaths around seedlings can reduce seedling mortality by up to 90 % (Eidmann et al. 1996). Plastic collars with a lip were shown to confer the greatest protective effect in field studies compared to latex coating and lipless collars (Petersson et al. 2004). This method, however, is cost- and labour-intensive and – depending on what type of protection is used – usually is not as effective as applying chemical pesticides (Petersson et al. 2003 & 2004).

Other physical alterations to entire clearfell sites that have proved to be somewhat effective in reducing seedling damage have been scarification of the soil (i.e. removing all vegetation to expose the soil surface) (Örlander & Nordlander 2003) and the addition of alternative food sources for pine weevils on replanted sites (Örlander et al. 2001). Retaining some shelter wood (i.e. standing stems) has also been shown to reduce the damage to seedlings caused by weevils (von Sydow & Örlander 1994; Petersson et al. 2003). All of these methods, however, have potential drawbacks that must be taken into account. Increasing the amount of shelter wood on a site to protect seedlings increases the risk of injury to forestry workers and makes their work more cumbersome, increasing the cost of planting seedlings. Retaining small stands of trees on clearfell sites and leaving behind deadwood may help to maintain the local diversity of carabid beetles and other invertebrates, some of which may be beneficial in controlling pine weevil populations (Siitonen 2001; Grove 2002; Koivula & Niemelä 2002; Jonsell et al. 2007; Niemela & Koivulä 2007). One example for this is provided by Salisbury and Leather (1998), who report that pine weevil larvae migrating in the soil are prone to predation by carabid beetles.

Delaying the restocking of clearfell sites by five to six years so as to reduce the feeding damage caused by adults emerging on-site has not emerged as a viable control method either, as migrating weevils cannot be prevented from invading these sites from other locations. In addition, economic pressures force forestry operators to keep fallow period as short as possible and current forestry practice is to

replant as soon as possible and allow establishment of the seedlings to pre-empt the emergence of large numbers of weevils on-site (Schaible, 1999). In Ireland, coniferous clearfell sites operated by Coillte are by law required to be replanted within two years of cutting (Aoife Dillon, personal communication).

1.5.2 Chemical control of the large pine weevil with pesticides, antifeedants and antiattractants

Two methods of chemical control can be distinguished: the use of chemical pesticides to kill or reduce the feeding of adult pine weevils (these have included DDT and permethrin compounds, among others) (Hagner & Jonsson 1995; Leather et al. 1999; Torstensson et al. 1999) and the use of naturally occuring organic compounds such as plant- or insect-associated volatiles or their derivatives as antifeedants (Unelius et al. 2006; Bohman et al. 2008; Eriksson et al. 2008).

The first chemical pesticide that was wildly used against the pine weevil was the very popular and effective organochlorine DDT (dichlorodiphenyltrichloroethane). Beginning in the 1950s, seedlings were routinely dipped in a DDT solution before being planted and would often receive follow-up treatment with DDT once planted (Stoakley 1968; Långström & Day 2004). Due to its well-publicized detrimental ecological effects, including human health concerns, DDT was banned in forestry across most of Europe in the 1970s (Turusov et al. 2002; Långström & Day 2004; Beard 2006). Once it was banned, other chemical pesticides took its place in most European countries. Lindane (*gamma*-hexachlorocyclohexane, also an organochlorine) was used as a replacement for DDT in some countries (Annila 1982, Stoakley & Heritage, 1989). Lindane presented its own set of environmental problems and its use was discontinued in most European countries during the 1980s and 1990s to be gradually replaced by the most commonly used forestry pesticide in current use, the synthetic pyrethroid permethrin (and the related pyrethroid cypermethrin).

As with DDT and lindane, the primary application method for permethrin is the dipping of seedlings prior to planting. Since permethrin is relatively unstable when exposed to light and field conditions, especially in soil, repeated spraying of seedlings is necessary to maintain its protective effect (Kaufman et al. 1977; Heritage et al. 1997). Permethrin provides an effective means of reducing pine weevil damage to seedlings at a comparatively low financial cost (Hagner & Jonsson 1995; Von Sydow 1997; Örlander & Nilsson 1999). In the cited studies by Örlander & Nilsson and von Sydow, survival of seedlings on clearfell sites that were dipped and sprayed with permethrin during the course of trials was increased by 20 % to more than 60 % compared to untreated controls. Laboratory studies, however, have shown that pine weevil adults may avoid seedlings treated with pyrethroid pesticides and instead

choose to feed on untreated seedlings. Moreover, depending on the dosage and subsequent degradation of pyrethroid compounds, toxic effects on feeding pine weevils may be delayed by several weeks, allowing them to feed extensively before they die (Rose et al. 2005). While permethrin and other pyrethroid compounds break down readily in soil (Lord et al. 1982; Sakata et al. 1992), they can make their way into waterways and other aquatic environments where they can have toxic or endocrine disruptive effects on aquatic organisms (McLeesc et al. 1980; Anderson 1982; Siegfried 1993; Xue et al. 2005). Permethrins can also have adverse effects on the health of forestry personnel exposed to them (Miyamoto et al. 1995).

Due to the environmental effects associated with pyrethroid pesticides and permethrin/cypermethrin specifically, European countries are now restricting their use in forestry or banning them altogether. In Sweden, permits for the use of cypermethrin expired in 2003, but were renewed until 2005 due to the lack of effective biological control methods at that point in time (Långström & Day 2004).

In Irish forestry, cyperpermethrin is the only chemical pesticide that is used on a large scale. It is used to dip and spray conifer seedlings to protect them against feeding damage by the pine weevil. In the most recent year for which data is available, Coillte applied 0.1 kg of active pesticide per hectare of forest (Anon., 2007b). Coillte is only authorized to use pesticides that have been licensed by the Irish Department of Agriculture, Food and Rural Development (Anon., Coillte website retrieved 2010). The license that is granted by the Forestry Stewardship Council (FSC) upon which any national permits depend will lapse in 2014 as a consequence of the European Union deciding to cease the use of permethrins (Aoife Dillon, personal communication, Anon. 2000a). This is in agreement with the 'Integrated Pest Management Strategy' adopted by Coillte as part of the 'Sustainable Forest Management Initiative' which has the express target of reducing the use of pesticides and focusing on the sustainable management of forests (Anon. 1999, Anon. 2007a, Anon. on Coillte website [retrieved 13.9.2010]). However, it also means that viable alternatives, including biological control mechanisms to combat the pine weevil are in urgent need (Dillon et al. 2006; Dillon et al. 2007).

In addition to the general insect pesticides, a number of organic compounds with a more specific action against pine weevils have been the subject of active research, some of which occur naturally in forest habitats. The advantage of using naturally occurring organic compounds for control is clear: they are usually less likely to cause a significant ecological impact than general pesticides and since they may already be present in the respective ecosystem, biological degradation processes are more likely to be in

place to break down these compounds. In many cases, for example in the case of mating pheromones, they are generally far more specific in their effect on the target pest than conventional chemical control methods such as pesticides.

Biological control of pine weevils using biogenic chemical compounds has been approached from several angles. Tapping adult beetles by using feeding attractants and pheromones has been attempted (this is known to be an effective method for controlling some bark beetle pests such as *Ips typographus*) (Raty et al. 1995; Jakuš 1998; Schlyter et al. 2001; Långström & Day 2004). These efforts have been confounded by the fact that pine weevils apparently do not produce an aggregation or sex pheromone that is attractive over long distances and general host attractants are too ubiquitous on clearfell sites to be effective bait for mass trapping of pine weevils (Selander 1978; Nordlander et al. 1986; Nordlander 1987). Consequently, this method of control has proved to be impracticable. Traps baited with volatiles attractive to pine weevils are used to predict seedling damage and estimate weevil populations, but are not effective enough to significantly reduce populations on clearfell sites (Nordlander 1987; Zumr & Starý 1992; Wilson & Day 1995).

Conifer volatiles that are known to attract pine weevils over long distances as well as help adults to locate hosts at a close range include terpenes and pinenes (Nordlander et al. 1986; Tilles et al. 1986a; Wibe et al. 1997). Volatiles released as a consequence of the feeding activity of pine weevils have been shown to stimulate the aggregation of adults (Tilles et al. 1986b). Ethanol is often used in *H. abietis* traps as it greatly augments the efficacy of pinenes and terpenes in attracting adult individuals, but is also a suitable lure on its own (Tilles et al. 1986a). *H. abietis* has been shown to be more attracted to ethanol-baited pitfall traps than other *Hylobius* spp. as well as other bark-feeding beetles (Lindelöw et al. 1993). Ethanol is a basic breakdown product of organic compounds and has been found to be present in higher concentrations in pine stumps compared to spruce stumps, which may be a contributing factor in the beetle's preference for this host (von Sydow & Birgersson 1997).

A number of volatiles can be used as 'antifeedants' to either deter pine weevils from feeding or to mask the attractant volatiles and thus make it harder for the beetles to locate seedlings. These antifeedants have been shown to successfully reduce the feeding damage to cuttings and seedlings they have been applied to in the laboratory and field (Klepzig & Schlyter 1999; Bratt et al. 2001; Eriksson et al. 2008). Limonene has been shown to effectively inhibit the attractant effects α-pinene has on pine weevils (Nordlander 1990). Field trials have demonstrated that this volatile is highly effective in reducing the number of pine weevils attracted to traps baited with pine as well as reducing feeding damage to

seedlings supplemented limonene (Nordlander 1991). Most research in this area is still focussed on the identification of potential antifeedants (Månsson & Schlyter 2004) and the evaluation of their projected efficacy in laboratory experiments on pine weevils (Klepzig & Schlyter 1999). As of yet, no literature on extensive field trials or commercial application of the discussed compounds is available, though some limited field trials have shown a significant reduction in seedling damage due to antifeedants (Bratt et al. 2001). Thacker et al. (2003) have suggested the application of neem oil to seedlings as an antifeedant in the UK. Antifeedants are currently not being used in Irish forestry.

1.5.3 Biological control of the pine weevil using natural predators, parasitoids and pathogens

The pathogens and predators that are known to be associated with *H. abietis* and other bark beetle pests have been reviewed by Kenis et al. (2004). For the purpose of this introduction, entomopathogenic nematodes (EPN) will be classified as pathogens, since they generally kill the host if they are able to establish within it insect and do so using a bacterial symbiont that itself is an insect pathogen (see 1.4.1).

a) Predators

The larvae of the large pine weevil are sheltered inside the bark of tree stumps and are thus considered to be at a very low risk of predation by generalist predators. Adults may be at a greater risk during their migration from one site to another as well as during feeding. Not much information on predators of the pine weevil is available and virtually nothing is known about the quantitative impact predators may have on pine weevil populations (Kenis et al. 2004). Several studies list predators that have been found in or around tree stumps harbouring pine weevil larvae, but only a few of these are actually known to feed on pine weevil larvae (Leather et al. 1999). Salisbury and Leather (1998) did observe carabid beetles predate pine weevil larvae migrating through soil in the laboratory, but to what extent this occurs in the field is not known. The author has observed birds feeding on pine weevil larvae offered to them during field sampling of tree stumps, but never saw birds actively foraging for pine weevil at or around tree stumps. Due to the lack of information on predators of the adult stage of *H. abietis* and the sheltered location of the larvae, predators have not been used as biological control agents against the pine weevil thus far.

b) Parasitoids

Kenis et al. (2004) found mention of only eight to ten parasitoids that have been connected to *H. abietis* in the literature (some of which are most likely false records). These parasitoids are mainly larval

parasitoids belonging to the Braconidae and Ichneumonidae (Kenis et al. 2004). Wasp species parasitizing the adult stage of the pine weevil have also been reported, for instance the braconid species *Perilitus areolaris*, which has so far only been found on *H. abietis* (Gerdin & Hedquist, 1985). This species has been reported to parasitise up to 16 % of pine weevil beetles in field-collected samples in the Czech Republic (Schindler, 1964; Novák, 1965). Extensive studies on the distribution and impact of these parasitoids on pine weevil have not been conducted.

Known larval parasitoids of *H. abietis* include the generalist ichneumonid species *Dolichomitus tuberculatus* and the braconid *Bracon hylobii*. While the former has been reared from field-collected larvae (Munro 1928; Hanson, 1943) and is known to parasitise a range of bark and wood-dwelling beetle larvae (Kenis et al. 2004; author's observation), it is *B. hylobii* that is the most widely distributed and important larval parasitoid of the large pine weevil. It is also the parasitoid on which the majority of research has been conducted in this context (Henry, 1995; Henry & Day 2001; Kenis et al. 2004; Everard et al. 2009). *B. hylobii* can be reared in the laboratory and released in the field (Henry, 1995). Henry and Day (2000) have done so and while they did record an increase in the *H. abietis* parasitism rate on some sites on which wasps were released compared to control sites, these effects only manifested themselves after one to two years post-release of wasps and had little to no effect in reducing weevil numbers in the year of release. This and other factors (poor synchronization of host and parasitoid life cycle, labour and material costs involved in producing high numbers of females) limits their potential as a biocontrol agent that may reduce pine weevil populations below an economically acceptable threshold (Henry & Day, 2001). Due to its reproductive potential and its ability to parasitise a high proportion of hosts in optimal conditions it may, however, be a valuable component in an integrated biological control approach against the large pone weevil (Henry & Day 2001; Everard et al. 2009).

c) Pathogens: Microbes

The range of pathogens naturally occurring in *H. abietis* is very poorly known. Gregarines and microsporidia that have been found in the gut or malphigian tubules of field-collected *H. abietis* specimens include *Nosema hylobii*, *Gregarina hylobii* and *Ophryocystis hylobii.* These have been observed to inhabit the midgut cells, midgut lumen and Malpighian tubules, respectively, of the large pine weevil (Purrini 1981; Purrini & Ormieres 1982). It is not known to what extent these parasites may affect the vitality or feeding extent of their host, but Yaman (2007) has reported that the gregarine species *Gregarina typographi* found in the midgut lumen of Turkish populations of the bark beetle *Ips*

sexdentatus damaged the host integument and affected its health adversely. However, to date no information has been published on similar effects of gregarines or other protozoans on *H. abietis*.

Fungal pathogens have been examined in more detail, in particular regarding their use against pine weevil. Generalist insect pathogen fungi such as *Beauveria bassiana* and *Metarhizium anisopliae* that infect insects across several systematic orders have been scrutinized as potential biological control agents. This is mainly due to their efficacy in a wide range of settings and the relative ease with which their infective propagules (spores) can be produced in numbers sufficiently large for inundative biological control (Zimmermann 1993; Feng et al. 1994). *Beauveria bassiana* infects host insects via asexually produced conidia that adhere to the insect cuticle and germinate in humid conditions. The fungus is capable of penetrating the host cuticle and fungal hyphae grow in the haemocoel of the infected insect (Feng et al. 1994). The fungus has been found in pine weevil adults from the field (Gerdin, 1977) and a related species - most likely *B. caledonica* - has also been found to infect pine weevil larvae in tree stumps on Irish clearfell sites (Glare et al. 2008). In the laboratory, *B. bassiana* caused considerable mortality in pine weevil adults when spores were directly applied to the insects, but also when adults were offered food treated with spore suspension. Infection of adults was particularly fatal when they were exposed to fungus-overgrown beetles (Wegensteiner & Fuhrer1988; Wegensteiner cited in Kenis et al. 2004).

Though the fungus can kill pine weevils, it may take a long time to do so (survival times of up to 80 days are reported in the previously cited studies, indicating that the fungus may primarily act as an opportunist rather than an aggressive pathogen). If used as a biological control agent against adults or late larval stages, this long delay may allow adults to emerge and cuase feeding damage before succumbing to fungal infection. Larvae that were collected on a clearfell site in Ireland with *Beauveria* sp. presence in tree stumps and then stored at 9°C in the laboratory showed high infection rates after four to six weeks of storage (approximately 30 % to 50 % infection). It is not clear whether infection of the larvae was aided by starvation or other factors associated with storage (author's observation).

Plate 1.G: Adult pine weevil infected with *Beauveria* sp. White hyphae can be seen at the leg joints and around the edges of the elytra.

d) Pathogens: Nematodes

Most of the nematodes that have been found associated with *H. abietis* have not been reported to cause considerable mortality or adverse effects in the pest. *Hylobius* spp. infected with EPN (*Steinernema* spp.) have however been found in the Czech Republic and the USA (Kenis et al. 2004). Despite EPN of the species *Steinernema feltiae* being present on Irish clearfell sites, no infection of *H. abietis* with this nematode has been reported to date (Dillon, 2003). As described above (1.4.1), EPN are capable of reproducing in large numbers inside an insect host and therefore can also potentially persist and establish in the environment they are applied to. This may increase their efficacy against the target pest, but is not desirable in inundative biological control (Kaya & Gaugler 1993; van Lenteren et al. 2003; Dillon et al. 2008a).

The first proposal for the use of EPN to control the large pine weevil was made by Pye & Burman in 1977. They tested various microorganisms found on and in pine weevil in the field as well as EPN species *S. carpocapsae* as potential biocontrol agents against *H. abietis* and found the EPN to cause 100 % mortality in larvae at low external doses and found the LD_{50} for late instar larvae was 9 IJs for this EPN species. They also found that the IJs were capable of penetrating 20 cm of packed sawdust to reach the host, indicating considerable mobility of the infective stage in this environment (Pye & Burman 1977; Pye & Burman 1978). Adult *H. abietis* seem to be at a much lower risk of infection with EPN, even after prolonged exposure to high doses of IJs (> 500) and are capable of protecting themselves from infection by encapsulating invading nematodes (Pye & Burman 1978; Girling et al. 2008; Everard et al. 2009). Studies following on from these early investigations tested the efficacy of EPN against the pine weevil in field situations. A review of the use of EPN against *H. abietis* has been compiled by Torr et al. (2005).

The British Forestry Commission commenced limited field trials with EPN (species *S. carpocapsae, S. feltiae* and *Heterorhabditis megidis*) as biocontrol agents against the pine weevil in 1988. Timing of EPN application was the most important factor influencing efficacy, with almost no infection occurring if EPN were applied at or shortly after felling, but infection rates in mature weevil populations reaching 50 to 96 % (Brixey 1997). An application rate of 3.5 million infective juveniles per stump was recommended (Collins, 1993; Brixey, 1997). Brixey at al. (2006) found callow adults to be at particular risk of EPN infection in field trials with *S. carpocapsae*, but Dillon at al. (2006 & 2007) found the opposite in field trials in Ireland, with infection rates among late instar larvae of 60 %, but only 36 % in pupae and 18 % in adults. Though early instar larvae of the pine weevil are more susceptible to nematode infection in the laboratory, it is thought that the advanced degradation of stumps and the bark make it easier for

nematodes to locate host insects in mature populations. Though there is some evidence to suggest that nematodes of the species *S. carpocapsae* and *H. downesi* move along roots or bark in the soil to locate a host (Ennis et al. 2010), it is unknown at this time to what extent nematodes may use host galleries in the bark as direct pathways to them.

Extensive field trials investigating the feasibility of EPN use against *H. abietis* on a large scale commenced in 1999 in the UK and in 2007 in Ireland (Evans et al. 2004; Anon., 2007b). In the UK, nematode application has been successful in reducing weevil populations in tree stumps, an effect that will carry over into the following year as nematodes persist (Evans et al. 2004). *Steinernema carpocapsae* is the main species being used in these trials. Torr et al. (2007) suggested *S. kraussei* as an alternative, as it has several advantages over *S. caprocapsae*: It is indigenous to Northern England, is adapted to cooler temperatures and is thought to adopt an active host searching strategy (cruiser) as opposed to the ambushing strategy attributed to *S. carpocapsae*. Despite these contrasting properties they found no significant difference in the efficacy of the two species in infecting *H. abietis* larvae in stumps or reducing adult emergence. In Ireland, the focus has been on *S. carpocapsae*, *S. feltiae*, *H. megidis* and the Irish species *H. downesi*, the latter two species being classified as cruisers. Field trials investigating the efficacy of these species both in small-scale manual application experiments and large-scale trials (*S. carpocapsae* only) have shown that all of them are able to significantly reduce the number of live *H. abietis* individuals in tree stumps and also the number of emerging adults. The nematodes were able to find hosts at considerable distances (up to 50 cm) from the bole of the stump (Dillon et al. 2006; Dillon et al. 2007; Dillon et al. 2008a).

Overall, *H. downesi* was found to have the greatest potential for control of the large pine weevil in small scale trials (Dillon et al. 2006 & 2007). Timing of application has been identified as a key factor in applying EPN, with mature populations that are usually found in pine stumps at least one year after felling in Ireland and the UK being the optimal target (Brixey et al. 2006). Dillon et al. (2007) demonstrated that nematodes can be applied as early as April in Ireland at no reduction in efficacy compared to application in late spring or summer. In Poland, where the climate is much warmer than in Ireland in spring and summer (presumably leading to greater IJ mortality), application of nematodes in June was shown to be ineffective in causing significant weevil mortality in stumps. Delaying application to September under these circumstances was highly effective, however, causing up to 90 % of weevil infection when using *S. feltiae* and *H. downesi* (Iwona Skrzecz, personal communication). Other factors regarding application technique (i.e. IJs in suspension or within infected cadavers) and application

volume (500 ml, 250 ml or 125 ml of suspension) have been shown to have little impact on the infection rate (Dillon et al. 2007). Dillon et al. (2007) also found that halving the applied number of *H. downesi* IJs to 1.8 million IJs per stump still gave a significant reduction in *H. abietis* emergence compared to the control.

Trials using EPN against the pine weevil are ongoing in Ireland. 70 ha of clearfell sites were treated in 2008, and the projected treated area for 2009 was 150 ha (Anon 2007b). Despite the success of EPN infecting *H. abietis* in tree stumps and reducing their emergence in Ireland, damage to seedlings on these sites has not been reduced below a level that would justify the additional economic cost of producing them (Aoife Dillon, personal communication). Especially on sites with pine stumps, where emerging weevil numbers are generally up to ten times higher than in spruce stumps in Ireland, control efforts may not be sufficient to suppress pine weevil numbers below an economically viable threshold (Griffin et al. 2008). An integrated approach that takes into account site management and EPN application, possibly in combination with pathogenic fungi or other microbes while at the same time protecting local parasitoid communities and minimizing potential non-target effects seems to be necessary to create a sufficiently strong biological control impact on the large pine weevil (Leather et al. 1999; Brixey et al. 2006; Dillon et al. 2007; Everard et al. 2009).

1.6 *Bracon hylobii* – a parasitoid of *H. abietis*

The parasitoid wasp *Bracon hylobii* (Hymenoptera: Braconidae; Ratzeburg 1848) is a gregarious, ectoparasitoid wasp that targets the large pine weevil as one of its primary hosts. It was first discovered on *Pissodes pini*, however. Other than *Pissodes* spp., no other natural hosts for *B. hylobii* have been reported to date (Kenis et al. 2004). Munro described the life cycle of the species and was the first to identify *H. abietis* as a host for this parasitoid (Munro 1914 & Munro 1917 cited in Henry 1995).

1.6.1 Life cycle of *B. hylobii*

Adult wasps are approximately 4 to 8 mm in length. There is no pronounced sexual dimorphism, though males tend to be slightly smaller than females. The latter are easily identified by the ovipositor, which is housed in a dark sheath that protrudes in a straight line ventrally from the abdomen. Exsheathed, the ovipositor can be up to 4 mm in length.

The complete life cycle of *B. hylobii* is presented in Fig 1.4. Wasps of both sexes eclose from the cocoon fully mature and can mate immediately. As is common among braconid wasps, unmated females will

oviposit, but unfertilized eggs will produce male offspring exclusively (Wharton 1993; Henry 1995). Emerging females may already carry eggs in their ovaries, though it is not known if these eggs are immediately viable and whether this is the case with all females (author's observation). The mechanisms involved in female *B. hylobii* locating and ovipositing on a host have so far not been investigated extensively, though there is some evidence that females are attracted by volatiles produced by feeding hosts (Faccoli & Henry 2003). Wasps have also been shown to spend less time handling smaller hosts and do not parasitise hosts below a certain size (< 100 mg) (Henry & Day 2001). Once a host is located, a wasp positions itself on the bark directly above it, raise its abdomen and angle the ovipositor downward so that it touches the bark at a right angle and begin penetrating the bark.

A number of ectoparasitic braconids are known to paralyze the host by injecting it with venom, and the same is true for *B. hylobii* (Wharton 1993; Henry 1995; Henry & Day 2001). A paralyzed host will generally cease to feed and move of its own accord, but it is still responsive to agitation (Henry 1995, author's observation). Oviposition may begin before the host is fully paralyzed and can last for several hours (author's observation). A clutch of eggs is laid by the wasp, usually directly on, or sometimes beside it. The wasp then retracts its ovipositor and, usually after resting for several minutes, moves on to seek out a new host (Henry 1995, author's observation).

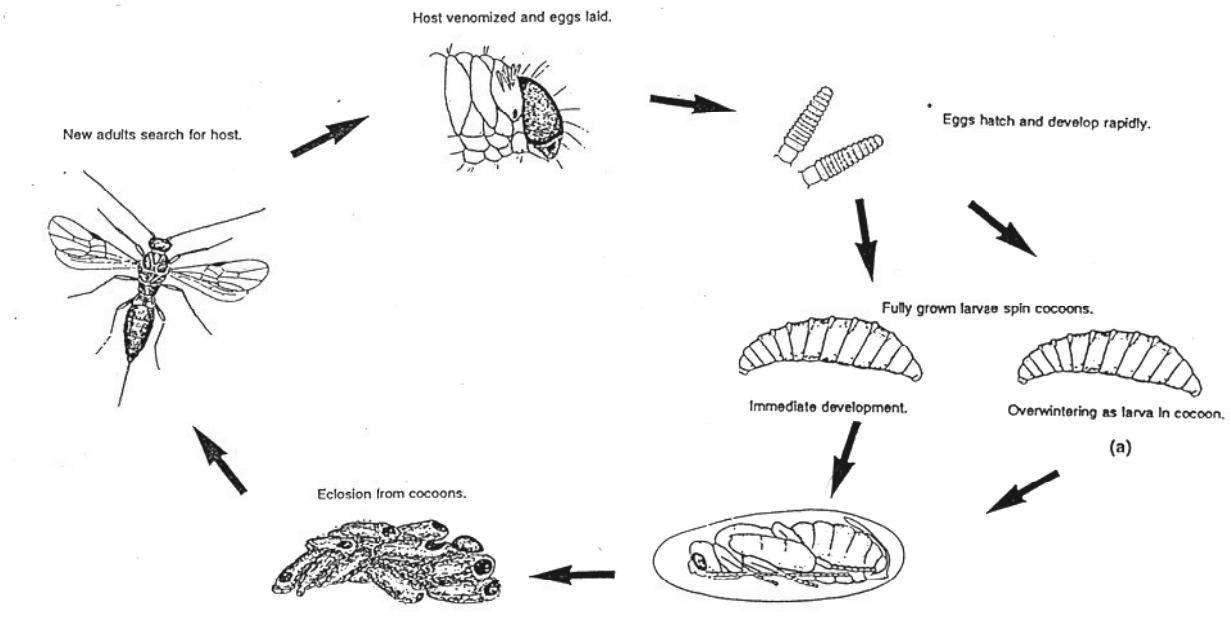


Fig 1.4: Life cycle of *B. hylobii* (from Munro, 1917).

Female wasps can parasitise several hosts in a day, suggesting that they do not lay the full complement of eggs stored in the ovaries at any one time (Henry & Day, 2001). The first egg clutch laid usually contains between 20 and 30 eggs and clutch size decreases in a roughly linear fashion with each new host (author's observation). In the laboratory, wasps have been observed to oviposit up to 17 times over the course of their lifetime and lay up to 93 eggs (Henry & Day 2001). Henry & Day report that at the optimal temperature for wasp activity and reproductive success (20°C), female wasps supplied with food survive for around 24 days on average (Henry & Day 2001). Larvae of *B. hylobii* usually hatch after two days and begin feeding on the host by burrowing through its cuticle with their head. They remain on the outside of the host for the duration of development. The host remains paralyzed, but alive until it succumbs to the feeding by the wasp larvae. After passing through five larval instars, the larvae pupate in a tightly packed mass in the space previously occupied by the now consumed host (Henry & Day 2001; Everard et al. 2009). At 20 °C, mean generation time is 25 day, with males emerging two days earlier than females on average (Henry & Day 2001; author's observation). Plate 1.8 includes photographs of a wasp probing the bark over a host larva and several cocoon batches collected in the field.

When faced with unfavourable temperature or when overwintering, *B. hylobii* pupae within cocoons can enter diapause. At low temperatures (around 4°C), cocoons can be kept in this state for at least six months. When returned to optimal temperature (20 °C), pupae complete development and emergence occurs within one to two weeks (Henry & Day 2001; author's observation).

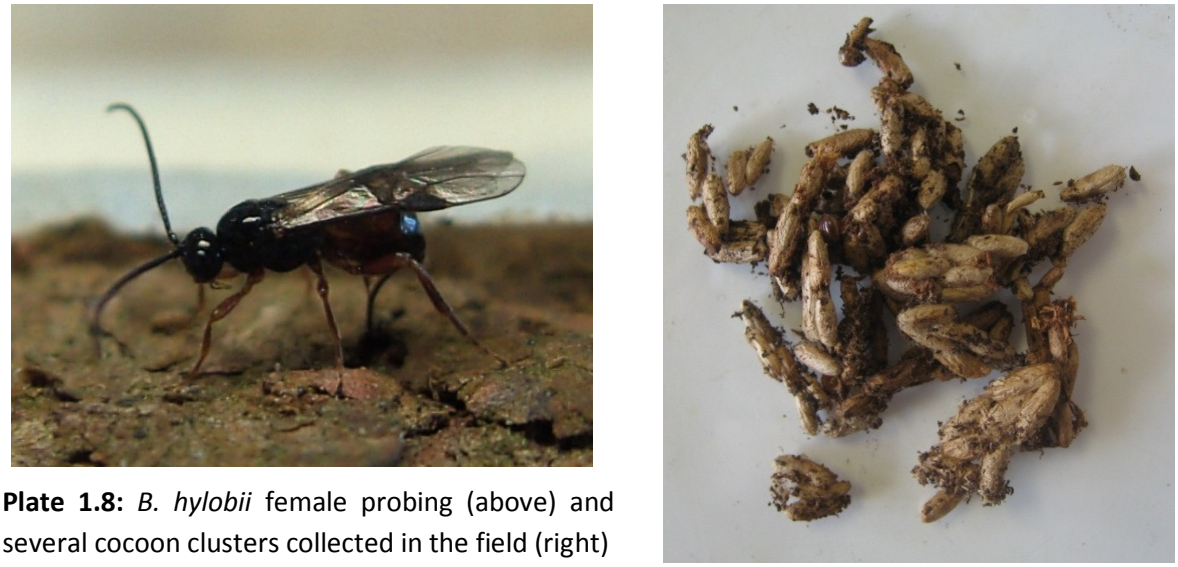


Plate 1.8: *B. hylobii* female probing (above) and several cocoon clusters collected in the field (right)

1.6.2 Distribution and host associations

Bracon hylobii populations associated with the large pine weevil have been reported across much of Northern Europe, including Scandinavia, Germany, the UK and Ireland (Munro 1914; Hedqvist 1958; Von Waldenfels 1975; Gerdin 1977; Henry 1995; Moore 2001; Kenis et al. 2004; Hilszczanski et al. 2005; Dillon et al. 2008b). Their distribution appears to follow that of *H. abietis* quite closely, though populations are patchy, both within clearfell sites and also on a landscape scale (Henry 1995; Kenis et al. 2004; Iwona Skrzecz, personal communication). Where they are present, they are, however, capable of infecting a large proportion of the pine weevil population on site. In the UK and Ireland, infection rates ranging from 20 to 30 % on most sites have been reported, though parasitism rates may be as high as 90 % in pine stumps, which tend to harbour more pine weevil (Henry 1995; Dillon et al. 2008; author's observation). The history and efficacy of *B. hylobii* as a biological control agent against the large pine weevil is reviewed by Kenis et al. (2004).

1.7 Aims of the project

The main objective of the work presented in this thesis was to evaluate potential risks associated with the application of entomopathogenic nematodes to control the large pine weevil, *H. abietis*. Results of the experiments and the field sampling conducted would allow general predictions about the non-target effects and risks of establishment and spread of nematodes once they have been applied to tree stumps on coniferous clearfell sites. Each chapter in this thesis presents the results of experiments designed to examine one potential risk factor associated with EPN biocontrol of the large pine weevil:

Chapter III: Assessing the persistence and spread of EPN in relation to site characteristics such as stump species and soil type. For this purpose, soil samples were collected up to two years after EPN application on and around the edges of clearfell sites to which nematodes (S*teinernema carpocapsae* and *Heterorhabditis downesi*) had been applied on a large scale (i.e. 3.5 million IJs to all stumps on a site) and were baited in the laboratory.

Chapter IV: Distinguishing between the genotype of parent strains and potential hybrids of *Steinernema feltiae*. This would permit samples collected on clearfell sites to which exotic and indigenous strains of this EPN species had been applied to be classified as either one of the parent strains or a hybrid of the two, providing information about the fate of applied nematodes.

Chapter V: Assessing the risk to non-target insects on clearfell sites via laboratory experiments and field experiments in which saproxylic insects (which represent service-providers) are exposed to EPN (S. *carpocapsae* and *H. downesi)*. Also, collection of woody debris from sites treated with EPN and assessment of infection of xylophagous insects within it.

Chapter VI: Assessing the risk to the parasitoid wasp *Bracon hylobii* associated with the large pine weevil by examining its response to infected host larvae. This also included investigations on the wasp behaviour to gain a better understanding of short range host location in this species, thus allowing predictions about the risk posed by EPN via intraguild predation.

CHAPTER II

General Material and Methods

2.1 Source and storage of insects

a) Waxmoth larvae (*Galleria mellonella*)

Waxmoth larvae were obtained from the Mealworm Company (Sheffield, UK) and stored in the containers provided by the supplier at 15°C. Larvae were stored for no longer than four weeks.

b) Pine weevil larvae (*Hylobius abietis*)

Pine weevil larvae were collected in the field from pine tree stumps tree on four separate clearfell sites (see Tables 2.3 and 2.4) and stored at 9°C in 24 well plates to prevent pupation and dessication, one larva per well (Cornel Inc; Cornel, USA). The lid of each plate was lined with five layers of tissue paper soaked with tap water. The tissue paper was replaced every week and larvae were used in experiments within four weeks of collection.

c) Longhorn beetle larvae (*Rhagium bifasciatum* and *Asemum striatum*)

Longhorn beetle larvae were collected from the field by destructively sampling decomposing logs from nine different clearfell sites (see 2.5). Larvae were stored as described for pine weevil larvae, though tissue paper was only replaced every two weeks and larvae were stored for up to three months. At least two weeks before being used in experiments, larvae were transferred to a 5 cm diameter Petri dish filled with decomposing wood. Dishes were wrapped in Parafilm® (Pechiney Plastic Packaging; Menasha, USA) and larvae were allowed to feed for at least two weeks before being used.

d) Parasitoid wasps (*Bracon hylobii*)

Wasps were collected in larval and cocoon stages from three separate clearfell sites (see 2.5). Additionally, a laboratory culture was maintained, the wasps for which were originally supplied by Dr Paddy Walsh at the Galway-Mayo Institute of Technology. Wasp populations reared in the laboratory and brought in from field sites were merged to introduce genetic variation and reduce the effects of

inbreeding. Cocoons collected in the field that were not required immediately were placed in 50 ml plastic tubes (Sarstaedt; Nürnbrecht Germany) with a hole in the lid covered by a layer of gauze and stored at 4°C for at least two months and up to six months. Once emerged, wasps were stored in 50 ml plastic tubes, with no more than 50 wasps per tube. A piece of tissue paper soaked with a 50 % honey/tap water solution was inserted in each tube. A hole was cut in the lid of each tube and covered with a layer of gauze to allow ventilation. Wasps were stored at 20°C and 16 h light/8 h dark cycle until used in experiments.

2.2 Source of nematodes and nematode culturing

Four nematode species were used in this study (Table 2.1). Cultures were carried on from those maintained at NUI Maynooth that had been reared through *G. mellonella* larvae since first being isolated or after being obtained from colleagues. Table 2.1 lists the species cultured and provides information on where they were isolated and their natural habitat.

To avoid cross-contamination, only one species or strain of nematode was handled in the workspace at any given time and all surfaces in contact with nematode containers, White traps etc. were wiped down thoroughly with 70% ethanol and allowed to dry before beginning work on the next species/strain. Nematodes were cultured in final instar waxmoth larvae. Small scale rearing of infective juveniles (IJs) was carried out in 9 cm diameter Petri dishes lined with a single layer of filter paper (Whatman No 1) placed in both the lid and the bottom of the dish. Nematode suspension was added (3 ml containing a total of 3000 IJs) and 10 to 20 waxmoth larvae were placed in each dish. The dishes were incubated at 20°C (23°C for *S. bicornutum*) for 10 days after all waxmoth larvae had died from EPN infection, at which point the cadavers were transferred to White traps prepared with Whatman no. 1 filter paper and tap water according to the protocol described by Kaya and Stock (1997). White traps were incubated at 20°C (23°C for *S. bicornutum*). Once IJs emerged from the cadavers, they were removed from the White traps every two days and washed by allowing them to settle to the bottom of a 2 L glass beaker, pouring off the supernatant and resuspending them in fresh tap water three times. Suspensions were then adjusted to a concentration of 1000 IJs/ml and split into 25 ml aliquots for storage.

Mass rearing to generate large numbers of nematodes (> 10 million) for field and laboratory inundative application was carried out in the plastic containers in which waxmoth larvae were supplied (20 cm long, 10 cm wide, 8 cm high). Waxmoth larvae were first separated from the wood shavings in which they were delivered, before being placed back in the container. The lid of the container was lined with five to

ten layers of tissue paper and approximately 15 to 25 ml of nematode suspension (1000 IJs/ml) were poured into the container. The lid was closed and the container inverted several times to allow the tissue paper to soak up the nematode suspension. The container was then incubated lid-down at 20°C for two weeks. Insect cadavers were transferred to White traps and collection and washing of IJs was carried out as described above for small-scale culturing. Mass reared nematodes were stored at 9°C in 2 L beakers, each containing a 1.5 L aliquot at a concentration of 2000 IJs/ml. Each beaker was aerated and the suspension was continuously agitated by a single air diffuser connected to an Airvolution 4 aquarium air pump (Interpet; Dorking, UK). Nematodes were stored in beakers for no longer than one week before use.

Table 2.1: Strain designation and source of nematode species used.

Species	*strain*	*Source*	*Isolated by*
Steinernema carpocapsae	*US-S-25*	Koppert Ltd., Berkel en Rodenrijs, The Netherlands	N/A
Steinernema feltiae	*4cfmo*	Clearfell site Co. Mayo, Ireland	Dr Aoife Dillon
	EN02	E-nema GmbH, Raisdorf, Germany	N/A
Steinernema bicornutum	*IRA7*	East Azarbaijan, Iran	Eivazian Kary
Heterorhabditis downesi	*K122 (previously Irish type)*	Grassland Co Wexford, Ireland	Dr Christine Griffin

2.3 Storage of nematodes

Nematodes were routinely stored at 9°C in 25 ml aliquots of tap water at a concentration of 1000 IJs/ml. Aliquots were stored in 125 ml sealed plastic food tubs with a diameter of 8.5 cm ('Econo' type by Huhtamaki; Espoo, Finland). Nematodes were reared through waxmoth larvae as described at least every 3 months.

2.4 Counting of nematodes and adjusting suspension concentrations

To increase the concentration of nematode suspension from the storage concentration of 1,000 IJs/ml, nematodes were allowed to settle in a graduated cylinder and excess water was poured off. When counting nematodes to check nematode concentration in preparation of a given experiment, all counts were carried out by transferring a sample (100 µL, 1 ml or 1 ml from a 1:10 tap water dilution of the

stock, depending on the concentration of the stock suspension) to a 9 cm diameter Petri dish marked with a square grid. Tap water was added until the bottom of the dish was covered and live IJs were counted with the aid of a dissecting microscope by moving from one grid square to the next. At least five separate samples were counted for each desired concentration. IJ concentration was adjusted until the concentration estimated using the mean number of nematodes per sample fell in a range ± 5% of the target value.

2.4.1 Counting of nematodes in infected cadavers

Cadavers containing nematodes were placed in a Petri dish with tap water. Using a scalpel, the head capsule was separated from the cadaver and crushed. The contents of the cuticle were then squeezed out of the cadaver by running the blunt side of the scalpel along the length of the cadaver from the posterior to the anterior end while holding the posterior end with a pair of forceps. The empty cuticle was cut into small pieces and the water with the cuticle and its contents was then carefully poured into a 50 ml plastic tube. The tube was shaken vigorously for approximately 15 seconds until the contents had been homogenised. The homogenate was then poured into a 9 cm diameter Petri dish lid marked with a grid and the number of adult nematodes and/or juveniles was counted by moving from one grid square to the next. The homogenate was split into several portions and diluted with tap water if visibility was low. If the number of juveniles in a given sample was too high to be counted by this method, three samples of the homogenate (100 µl or 1 ml, depending on juvenile concentration) were pipetted into a 9 cm diameter Petri dish in a line of small droplets (see Fig 2.1). Nematodes in all of the droplets per sample were counted and the total number of juveniles in the sample was calculated from the mean number of nematodes per sample.

2.4.2 Counting of nematodes emerging from individual White traps

This method was used to count IJs emerging from a single cadaver in an individual White trap. The water from the White trap was carefully poured off into a 50 ml tube and the White trap was rinsed with tap water that was also poured off into the 50 ml tube to remove any IJs remaining in the trap. The tube was inverted three times to homogenize the suspension and depending on the concentration of IJs in the suspension, counts were performed using one of four methods:

a) Topping up 50 ml tube, dilution followed by 100 µl sample count: The 50 ml tube was topped up to 50 ml with tap water. A 1 ml sample was taken from the 50 ml tube and transferred to a 10 ml graduated cylinder. The cylinder was topped up to 10 ml with tap water and inverted

three times. Three 100 µl samples were taken from this dilution and each was pipetted into a Petri dish in a line of droplets (Plate 2.1). The number of IJs per 100 µl sample was determined and the total number of IJs in the White trap was calculated.

b) Sample count (100 µl): The volume of suspension in the 50 ml tube was recorded. Three 100 µl subsamples were taken and pipetted into a Petri dish in a line of droplets each. The number of IJs per 100 µl subsample was determined and the total number of IJs in the White trap was calculated.

c) Sample count (1 ml): Same as b), but IJs in 1 ml samples taken directly from the 50 ml tube were counted.

d) Total count: The entire contents of the 50 ml tube were poured off into a 9 cm diameter Petri dish marked with a grid and the total number of IJs was determined.

Methods a) and b) were used when the IJ concentration was high, usually in the first three to four weeks of emergence. Methods c) and d) were used when IJ concentrations were low.

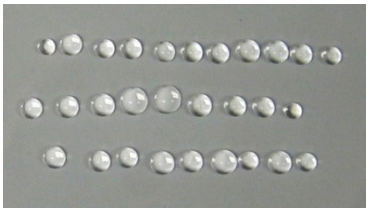


Plate 2.1: Lines of droplets pipetted out in a Petri dish to count IJs within a 100 µl subsample. Each line of droplets represents one subsample.

2.5 Field sites

All field sites visited for this study were forestry sites owned and managed by Coillte Teoranta (main offices in Newtownmountkennedy, Co. Wicklow). Some sites were visited exclusively to monitor persistence of EPN by collecting soil and sometimes bark samples (see Chapter III), others were used only to gather decomposing deadwood for laboratory and/or field experiments on saproxylic beetles (see Chapter V) and some were visited to collect *H. abietis* larvae and cocoons of *B. hylobii* (see Chapter VI). The soil type given for each site is based on the visual examination and evaluation of soil sample characteristics collected at each site or, on sites where only logs or insects were collected, on Coillte records (peat soils with a layer of raw humus or mineral soils with low humus content). Most of the tree stumps on the sampled sites were either Sitka spruce (*Picea sitchensis*) or lodgepole pine (*Pinus contorta*).

a) Clearfell sites treated by manual application of nematodes to stumps (Glendalough and Oakwood)

On these sites, EPN IJs had been manually applied to a limited number of stumps arranged in a randomised block design. Treated stumps on these sites had been labelled at the time of nematode application (Dillon et al. 2008b). A dose of 3.5 million IJs had been applied per stump in 500 ml of tap water. Controls had received 500 ml of tap water only. Applied IJs of *H. downesi* had been cultured for application using waxmoth larvae in the laboratory while *S. carpocapsae* IJs had been produced in bio-reactors and supplied by Koppert (Dillon et al. 2008b). Site characteristics including stump species, felling date and application date of EPN as well as the number of sampled stumps are presented in Table 2.4.

Table 2.2: Characteristics and sampling details for sites at Glendalough and Oakwood, to which EPN had been manually applied. SS = Sitka spruce. S.c. = *Steinernema carpocapsae*, H.d. = *Heterorhabditis downesi*. Soil classification based on Coillte records. N = number of stumps treated. Site location given with latitude, longitude and elevation.

Site location (lat. + long., elevation)	*Felled*	*EPN applied*	*Soil type*	*Stump species*	*Applied EPN species*	*N*
Glendalough 53°03'N 006°28'W 300 m	July 2004	June 2006	Mineral	SS	*S.c.*	15
					H.d.	15
					Control (no EPN)	14
Oakwood 53°03'N 006°26'W 4 m	Dec 2003	June 2005	Peat	SS	*S.c.*	20
					H.d.	20
					Control (no EPN)	20

b) Sites with all stumps treated using a spray rig (Lackenrea, Glendine, Ballymacshaneboy, Knockeen, Kilworth)

As part of a large-scale field trial conducted by Coillte to test the efficacy of EPN at controlling the large pine weevil (*Hylobius abietis*), IJs produced in a bio-reactor by Becker Underwood (Littlehampton, England) had been applied to these sites using a spray rig. *Heterorhabditis downesi* was applied to tree stumps in a section of the site at Kilworth using IJs produced in bio-reactors on an experimental basis by Koppert (Berkel en Rodenrijs, The Netherlands). The spray rig was seated on a forwarder and moved along the rows of stumps on the site. Forestry workers moving alongside the forwarder wielding spray nozzles attached to the rig applied approximately 500 ml of nematode suspension around the bole of

each stump, resulting in each stump receiving an approximate dose of 3.5 million IJs (Plate 2.2). Site characteristics including stump species, felling date and application date of EPN as well as the number of sampled stumps for each site are presented in Table 2.4. This table also indicates whether a site was used only to sample soil for EPN persistence or also to collect or apply nematodes to decomposing logs to be used for risk assessment experiments. Some sites were used for collection of logs, longhorn beetle larvae and/or pine weevil larvae only. Stumps on these sites were not treated with EPN and they were visited only to sample or collect logs or weevil larvae for risk assessment experiments (Table 2.3).

Plate 2.2: Spray rig and forwarder used to apply EPN on trial sites (left) and forestry worker spraying EPN suspension around tree stump (right). (Photographs taken by Dr Aoife Dillon)

Table 2.3: Characteristics and sampling details for sites at which only pine weevil larvae or decomposing logs were collected (Chapters V and VI). FEE II = Field Exposure Experiment II conducted on this site (Chapter V). SS = Sitka spruce, LP = Lodgepole Pine.

Site	*Site location (county)*	*Felling date*	*Soil type*	*Stump species*	*Sampling*
Kildalkey	Meath	Jan 2008	Mineral	LP	Logs/weevils/FEEII
Clonava	Westmeath	May 2009	Peat	LP	Weevils/wasps
Clonkeen	Offaly	2007	Peat	LP	Logs/weevils

Table 2.4: Characteristics and sampling details for sites to which EPN had been applied with a spray rig as part of Coillte trials. SS = Sitka spruce, LP = Lodgepole pine. *Sc* = *Steinernema carpocapsae*, *Hd* = *Heterorhabditis downesi*. *Logs at Featherbed were from a plot untreated with nematodes and weevils collected at Glendine were from an untreated plot also. FEE I = Field Exposure Experiment I conducted on this site (Chapter V). Site location with latitude, longitude and elevation for all sites on which soil samples were collected (Chapter III) or Field Exposure Experiments on *R. bifasciatum* were carried out (Chapter V).

Site	*Site location*	*Felled*	*EPN applied*	*Soil type*	*Stump species*	*Applied EPN species*	*Used for*
Lackenrea	52°08'N 007°48'W 53 m	Dec 2005	2007	Mineral	SS + LP	*Sc*	Soil/logs
Glendine*	53°05'N 007°34'W 458 m	Dec 2005	2007	Peat	LP	*Sc*	Soil/weevils
Ballymacshaneboy	52°18'N 008°36'W 311 m	Dec 2005	2007	Mineral	LP	*Sc*	Soil/logs
Knockeen	52°12'N 007°10'W 79 m	Dec 2005	2007	Peat	SS	*Sc*	Soil
Featherbed*	52°12'N 007°10'W 79 m	2007	2008	Mineral	SS + LP	*Sc*	Logs/ FEE I
Deerpark	Wicklow	Dec 2005	2007	Mineral	SS + Larch	*Sc*	Logs
Raheenkyle	Carlow	2006	2008	Mineral	SS	*Sc*	Logs
Kilworth	Cork	2008	2009	Mineral	SS + LP	*Hd* *Sc*	Soil/logs

2.6 Statistics

All statistical tests were carried out using the MiniTab 15 Statistical Software package (Minitab Ltd.; Coventry, UK). Binomial data were compared using χ^2 – tests. Where $N \leq 10$ or the χ^2 – test yielded cells with a predicted value < 5, Fisher's exact test was used. When comparing the observed outcome of binomial data against chance outcome, a χ^2 –Goodness-of-Fit test was used.

Continuous data sets were tested for normality using the Anderson-Darling method and where data sets were significantly different from a normal distribution ($\alpha = 0.05$), data were transformed by taking the square root or the base 10 logarithm of each datum point. In cases where data sets contained a large number of zeros, base ten logarithm transformation was not used. In instances in which only a small portion (< 5 %) of a data set was represented by zero values, log base ten transformation was carried

out after adding one to each datum point. Whenever this was done it is indicated in the text. All percentage data was transformed by taking the arcsine prior to analysis.

Data sets that conformed to a normal distribution were first tested for equal variances using Levene's test ($\alpha = 0.05$), and if no significant difference among the variances was found, the data sets were compared using either a t-test for two samples or a One-way ANOVA for multiple samples with Tukey's post-hoc test ($\alpha = 0.05$). General Linear Models were used to test for significant effects of multiple fixed variables and wherever possible, interactions of the fixed variables were included in the model. Tukey's test was used to test for significant differences due to individual fixed variables ($\alpha = 0.05$). Residuals of the General Linear Model were visually compared against a normal distribution to confirm the reliability of results. Where residuals did not satisfactorily match a normal distribution, the results of the model were not used unless otherwise stated.

Data sets for which transformation was not successful were compared using a Mann-Whitney U-Test (abbreviated as M.-W. U-test), or when three or more such data sets were being compared a Kruskal-Wallis-test (abbreviated as K.-W.-test) was used. Wherever multiple pairwise comparisons were made between data sets using the M.W. U-test, the χ^2 – test or Fischer's exact test, the level of significance was adjusted according to Bonferroni (P/n where n = number of comparisons).

When performing regression analysis, residuals were visually compared against a normal distribution to confirm validity of the analysis. Wherever data was transformed prior to regression analysis it is indicated in the test. Whenever Binary Logistic Regression was carried out on a binary response variable, Pearson residuals were visually compared against a normal distribution to confirm the reliability of the regression model and suitability of the link function used. Wherever the residuals did not satisfactorily match a normal distribution, the results of the model were deemed not to be valid and were not used.

Random numbers used in experimental designs (e.g. for assigning log segments to climate rooms in Chapter V [5.2.3] or host types to scratching arenas in Chapter VI [6.2.11]) were obtained from the random integer generator at www.random.org.

CHAPTER III

Assessing persistence and spread of entomopathogenic nematodes used for pine weevil control on clearfell sites

3.1 Introduction

Trials in which entomopathogenic nematodes (EPN) are applied to tree stumps on clearfell sites have been carried out in the UK since 1988. Brixey (2000) compared *Steinernema* spp. and *Heterorhabditis* spp. with regards to their efficacy at infecting pine weevils within tree stumps in the field at a dose of 3.5 million IJs per treated stump. *S. carpocapsae* and *S. feltiae* infected 53 % to 56 % of weevils, while *H. megidis* was not as effective (roughly 40 % infection per stump). Differences in infection rate among EPN species were not significant. The results were interesting in that they did not match expectations based on the foraging strategies of these EPN species. *Steinernema carpocapsae*, which performed best, is considered an 'ambusher' species, i.e. it adopts a foraging strategy that involves little movement and active searching behaviour, but rather has the IJs standing or sitting on the surface of the substrate waiting for a host to pass by (Lewis 2002). *H. megidis*, like all heterorhabditids, is considered a 'cruiser' with IJs that actively move through the substrate in search of a host. Based on the efficacy of *S. carpocapsae* reported by Brixey that was confirmed by Heritage (cited in Torr et al. 2005), but also due to the commercial availability of this species, it was chosen as the control agent against the pine weevil in the UK (Brixey 2000, Torr et al. 2005).

Torr et al. (2007) suggested the use of *S. kraussei* as an alternative to *S. carpocapsae*, as the former is native to and widely distributed in the UK and therefore possibly more adapted to local conditions. They found that there was no significant difference in the efficacy of these two EPN species against pine weevil, both in terms of infecting individuals in lodgepole pine stumps and reducing adult emergence from them. They also report that compared with *S. carpocapsae, S. kraussei* persisted in greater numbers within a 10 cm radius around stumps over the course of one year after application. They therefore hypothesised that the adaptation of *S. kraussei* to local climate and soil conditions did not enhance their efficacy against *H. abietis*, but did lead to greater persistence.

In Irish field trials conducted by Dillon et al. beginning in the year 2001, a range of EPN species were manually applied to tree stumps to test their efficacy as potential biocontrol agents against pine weevils. These trials included the commercial strains of *S. carpocapsae*, *S. feltiae* and *H. megidis*, but also indigenous strains of *S. faltiae* and *H. downesi* (Dillon et al. 2006, 2007, 2008a & 2008b). All of

the EPN species used were able to infect pine weevil larvae, pupae and adults in tree stumps, with *H. downesi* (a cruiser species) performing best at infection rates of up to 87 % (Dillon et al. 2006, 2007 & 2008a). As was found in the UK, *S. carpocapsae* performed better than expected for an ambushing species in a cryptic environment and infected up to 64 % of *H. abietis* individuals in pine stumps, though it did not do as well (only approximately 10 % infection) in spruce stumps which harbour fewer weevils and have denser bark than pine stumps do (Dillon et al. 2006, 2007 & 2008a). Both species reduced the number of adults emerging from treated stumps in the season IJs were applied, though *H. downesi* again performed better. The reduction of weevil emergence from spruce stumps by *S. carpocapsae* was not always significant compared with the control (Dillon et al. 2006, 2007 & 2008a) and suppressive effects on adult weevil emergence from tree stumps, especially on sites with pine stumps, are not always sufficient to prevent economically significant damage to seedlings (Griffin et al. 2008).

All of the EPN species and strains that were tested in these studies showed dispersal in or on the stumps as was reflected by pine weevil infection rates within them. Pine weevils were infected at distances of up to 50 cm from the bole of the stump following EPN application and also infected weevils at depths of up to 40 cm below soil level. Infection rates of pine weevils, however, decreased significantly with increasing distance from the bole or the soil surface (Brixey et al. 2006; Dillon et al. 2007). Persistence of EPN on Irish clearfell sites on which tree stumps were treated manually with EPN suspension was high in the first two years (up to 90 % of stumps with EPN), but dropped significantly to about 40 % of stumps in year three after application. By year five after application, only the indigenous strain of *S. faltiae* (strain 4cfmo, originally isolated from soil on an Irish clearfell site) was recovered from treated stumps. This may be an indication that it was better adapted to the local conditions in Ireland compared with both the exotic species *S. carpocapsae* and the indigenous species *H. downesi*, which - though native to Ireland - has a natural distribution in sandy coastal soils (Griffin et al. 1991; Griffin et al. 1999; Dillon, 2003; Rolston et al. 2005; Dillon et al. 2008a). On average, EPN persistence close to the bole of the stump was shown to be four times as high as at 20 cm distance one year after application. Experiments in soil mesocosms that simulated application of EPN to tree stumps also indicated that *S. carpocapsae* and *H. downesi* persistence dropped significantly over time. The number of positive samples at the greatest distance tested (20 cm) increased, however, as time passed. No evidence for spread of nematodes from treated to untreated areas was found (Dillon et al. 2008a). The authors concluded that the risk of establishment of the exotic species *S. carpocapsae* was low.

In light of the field trial results from the UK and Ireland, in 2007 the Irish forestry company Coillte, under advisory from the UK Forestry Service, decided to begin large-scale trials in which

commercially produced infective juveniles of *S. carpocapsae* were applied to all tree stumps on selected clearfell sites in Ireland (Anon. 2007). The ecological risk presented by such large scale application of EPN hinges greatly on persistence and spread on and from treated clearfell sites after application (van Lenteren et al. 2003).

The **aims** of this chapter were to:

- assess the level of EPN persistence in the soil and under the bark of tree stumps treated with IJs to control the large pine weevil. Based on those results and other published data that suggests weevil numbers are higher in pine stumps than in spruce stumps (Leather et al. 1999; Griffin et al. 2008; Aoife Dillon, personal communication) it was predicted that EPN persistence would be greater around pine stumps than spruce stumps, irrespective of the soil type (Dillon et al 2006, 2007, 2008a). Moreover, it was also hypothesized that if the applied EPN were recycling predominantly in pine weevil hosts as intended and not-non-target soil invertebrates, IJs should thus be present under the bark of treated tree stumps to a similar or greater degree than in the soil surrounding the stumps. This was tested by collecting soil and bark samples from around treated tree stumps on several coniferous clearfell sites (four weeks to 24 months after EPN application) and detecting EPN within them by baiting samples with waxmoth larvae (*G. mellonella*) in the laboratory.

- investigate whether there was any spread of EPN on the treated sites. Dispersal of EPN after application was predicted to be to be generally limited to within 1 m of treated stumps and to decrease with increasing distance from stumps (Downes & Griffin 1996; Smits 1996; Dillon et al 2008b). To investigate the dispersal of nematodes, soil samples collected around tree stumps were taken at four distances (0, 20, 40 and 60 cm) from the bole of the treated stumps to track the spread of EPN from the point of application. On three sites, 100 soil samples taken randomly across three sites two years after EPN application were also screened for EPN presence by baiting them with *G. mellonella*.

- assess whether there was any spread of EPN from sites treated with *S. carpocapsae* into adjoining areas. Long-range dispersal of EPN after application is largely dependent on phoresis and therefore should be low (Downes & Griffin 1996; Kruitbos et al. 2009). To test whether dispersal off-site had occurred, soil samples were collected in areas adjacent to treated clearfell sites one and two years after EPN application and screened for nematodes by baiting the samples with waxmoth larvae in the laboratory.

3.2 Materials and Methods

3.2.1 Soil sampling

a) Sites with manual application of EPN to stumps (Glendalough and Oakwood)

Each stump was sampled once every time a site was visited. At Glendalough, 44 stumps were sampled (15 treated with *S. carpocapsae*, 15 with *H. downesi*, 14 tap water controls) and 60 stumps were sampled at Oakwood (20 stumps each with *S. carpocapsae*, *H. downesi* and tap water control). Both sites were visited within a week of each other in November 2007 (14th at Oakwood and 20th at Glendalough, respectively) and again in July 2008 (29th at Oakwood and 20th at Glendalough). Four soil core samples and four bulk soil samples were taken at each sampled stump. Soil core samples were taken by pushing a 50 ml plastic tube (2.9 cm inner diameter; Sarstaedt; Nürnbrecht Germany) into the soil to a depth of approximately 5 cm, resulting in an approximately half-filled tube. Loose litter was brushed aside before taking a core sample. Core samples were taken directly at the bole of the stump at four aspects around it spaced in 90° angles. Aspects at each stump were aligned with stationary landmarks on the site (e.g. a road, stand of trees etc.) to ensure that all stumps were sampled at corresponding locations around the bole of a stump. In addition to these core samples, bulk samples consisting of approximately 250 g of soil were collected with a trowel at 1 m distance from the bole at the same aspects at which core samples were taken. Brush and litter were not included in these samples and sampling depth was approximately 15 cm. Bulk samples were collected in 0.5 L resealable plastic bags (Fig 3.1).

b) Sites with all stumps treated using a spray rig (Lackenrea, Glendine, Ballymacshaneboy, Knockeen, Kilworth)

The sites at Lackenrea and Glendine were sampled on three occasions, five months after EPN application to tree stumps on the site (November 2007), one year after EPN application (June 2008) and two years after EPN application (June 2009). Each time sites were sampled within one week of each other. The sites at Knockeen and Ballymacshaneboy were each sampled once in June of 2009, two years after EPN had been applied. The site at Kilworth was also sampled only once in July of 2009, four weeks after EPN had been applied to the stumps on this site.

On each of the sampled sites, soil cores were taken at 30 treated stumps per visit. At Lackenrea and Kilworth, pine and spruce stumps were included in these 30 stumps and at Kilworth, the 30 stumps were split between areas of the site treated with *S. carpocapsae* and *H. downesi* (15 stumps in each area). Additionally, at Lackenrea and Glendine a new set of 30 stumps was sampled each year in 2008

and 2009 in addition to resampling the set of stumps first sampled 2007, resulting in two separate data sets with N = 30 each per site for those two years. A portion of the site at Lackenrea was disturbed by forestry work between the samplings of 2007 and 2008 and as a consequence, eight stumps from the stump set that was to be resampled could not be located, reducing the size of the respective data sets in 2008 and 2009. Stumps for sampling were selected at 5 to 15 m intervals along a diagonal transect crossing the rows of stumps on each site (except at Knockeen, where sumps were not sampled in transects but as encountered across the site due to topographical constraints). The new sets of stumps to be sampled in 2008 and 2009 at Lackenrea and Glendine were selected along diagonals in parallel but no less than 10 m distant from the transect sampled in the year previous. Stumps chosen for sampling were at least two and at most five rows apart. No other restrictions were made when selecting stumps for sampling. To assess the persistence and local spread of applied EPN around treated stumps, sixteen soil cores were collected from each stump. The basic sampling protocol was similar to that described for the manually treated sites (3.2.1a). Four aspects at 90° angles were sampled at each stump and aspects were aligned to a stationary feature on the respective site. At each aspect, one soil core was taken directly at the bole, with one more each taken at 20 cm, 60 cm and 80 cm distance along a straight line extending away from the bole (Fig 3.1).

Plate 3.2: A Sitka spruce stump sampled at Lackenrea in 2008 (left) and a view of the site

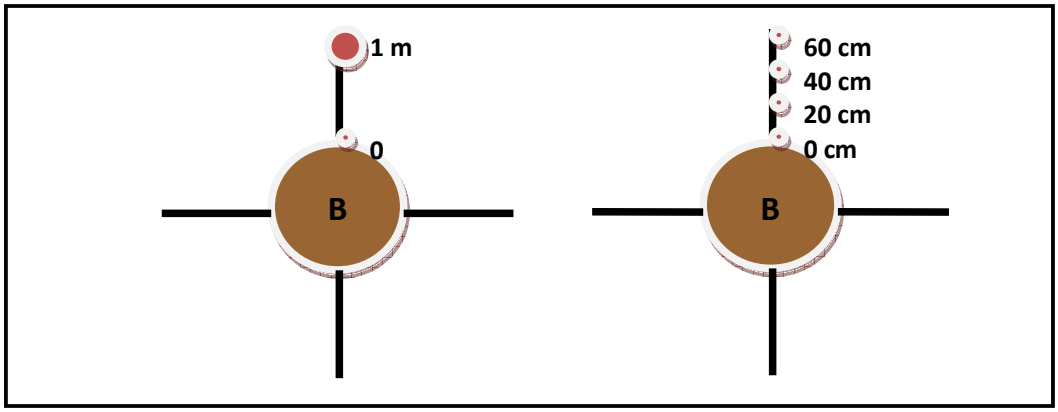


Fig 3.1: Arrangement of soil samples taken around stumps on sites manually treated with EPN (**left**) or treated with a spray rig (**right**). Black lines represent aspects and red dots indicate soil cores (small) and bulk samples (large) at each aspect. B = bole of stump.

In addition to the soil cores collected around tree stumps, 100 soil cores were taken at randomly chosen locations between stumps on each of four of the visited sites (Lackenrea, Glendine, Ballymacshaneboy, Knockeen) to assess the general spread of EPN within a treated area. The only criteria for these samples were that they be taken at locations no closer than 1 m to any treated stump and at distances at least 5 m apart from each other. These random core samples were collected across the entire site.

3.2.2 Bark sampling

To determine whether EPN were persisting under the bark of stumps to which they had been applied, an area of approximately 100 cm^2 bark was pried off the bole at the soil horizon at each aspect of newly sampled stumps in 2008 and 2009. They were transported to the laboratory in a resealable plastic bag.

3.2.3 Off-site sampling

To investigate whether EPN had spread off treated sites after application, soil samples were collected in the environs adjacent to the sites (where accessible) at Lackenrea, Glendine and Ballymacshaneboy. On each side of a site that was accessible, these samples were collected along two parallels running alongside the edge of the site (5 m and 10 m outside the last row of stumps) (Fig 3.2). Samples were also taken along three transects extending away from the edge of the site at a right angle on each of the sides sampled (Fig 3.2). Each parallel was subdivided into four segments of equal length (i.e. if a side was 400 m in total length, each segment was approximately 100 m long). Along each of these segments, 20 subsamples of soil (approximately 20 g each) were collected at evenly spaced intervals and pooled in a resealable plastic bag, resulting in approximately 400 g of bulk soil sample per segment. The length of each interval was determined by the length of each segment (e.g. if a segment was 60 m long, sampling intervals were 60 m/20 = 3 m)

A transect was located at each of the three points where two segments met. Soil samples were collected at four distances from the edge of the site along each transect (5 m, 10 m, 50 m, 100 m). This meant that at the location of each transect, areas at 5 m and 10 m distance from the last row of stumps were covered twice: once as part of the segments meeting there and once for the transect. Care was taken not to collect individual subsamples for a parallel segment or transect at the same spot, however. Along each transect, 10 subsamples of approximately 40 g each were collected along 5 m lines running at a right angle to the transect at each of the four distances from the edge of the site (5 m, 10 m, 50 m, 100 m). At each distance, these samples were pooled, thus resulting in four 400 g bulk samples for each transect. In total, for each side of a site where a complete set of four segments and three transects could be sampled, this protocol resulted in 2 x 4 bulk samples from the

segments and 3 x 4 bulk samples from the transects, yielding a total of 20 bulk samples per side (Table 3.1). Due to dense vegetation or obstructions such as roads or streams, sampling was not possible on all sides of each site (Fig 3.2). All soil samples were returned to the laboratory within 24 h of sampling and stored at 4°C for no more than one week before being baited.

Table 3.1: Number of transects and segments sampled off-site on sites treated with spray rig delivered EPN. Exposure of each side is given.

Site	*Exposure*	*Number of transects sampled*	*Number of segments sampled*
Lackenrea	South	3	4
	West	3	4
	East	1	1
Glendine	South	3	4
	East	3	4
	North	3	4
Ballymacshaneboy	North	3	4
	East	3	4

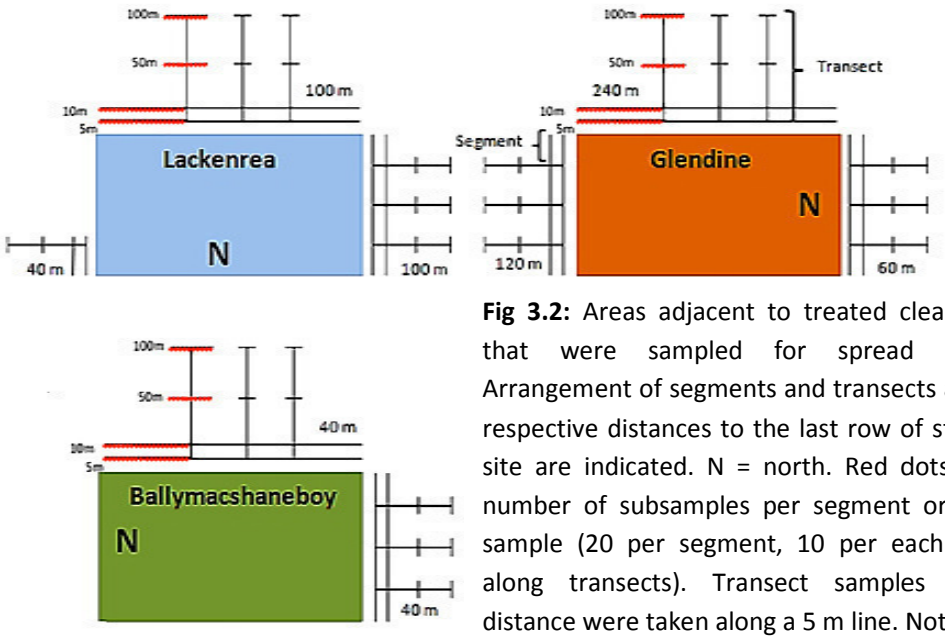


Fig 3.2: Areas adjacent to treated clearfell sites that were sampled for spread of EPN. Arrangement of segments and transects as well as respective distances to the last row of stumps on site are indicated. N = north. Red dots indicate number of subsamples per segment or transect sample (20 per segment, 10 per each distance along transects). Transect samples at each distance were taken along a 5 m line. Not to scale.

3.2.4 Baiting of soil samples

EPN in soil samples were detected by baiting with final instar waxmoth larvae (*Galleria mellonella*) in the laboratory. Soil core samples were baited by placing a single waxmoth larvae in a shallow depression made in each sample directly under the lid of the 50 ml tube containing it. The lid was replaced and screwed on loosely to allow some ventilation. Samples were arranged in random order on the bench top in a grid 20 tubes by 24 tubes per site (480 tubes in total). Cores were incubated with the lid facing downward (i.e. the bait insect was located at the bottom with the soil core above it) to ensure that the soil sample was not fragmented and in contact with the bait insect. The tubes were checked for bait insect mortality after seven days, at which point all bait insects were replaced for a second baiting period of seven days (same conditions). Dead and live bait insects were retained and placed in separate 24 well plates (one insect per well). Live insects were incubated at 20°C and checked for EPN infection seven days after removal from soil cores.

Bulk samples were split evenly into 80 ml subsamples, each of which was placed in a 200 ml plastic tub (8.5 cm diameter; 'Econo' by Hutamaki). Ten waxmoth larvae were added to each tub on top of the sample. Five holes were punched into the lid used to close each tub with a needle for ventilation. Tubs were stacked in random order on the bench top in stacks four tubs high and incubated at room temperature. A plastic tray was placed on top of each grid of stacks (each grid was four by five stacks in dimension = 80 tubs) to prevent differences in evaporation or ventilation among tubs. Bait insects in each tub were checked for mortality after seven days. Live bait insects from each subsample were placed in a 5 cm Petri dish which was incubated at 20°C for seven days after which time they were assessed for delayed mortality due to EPN infection. The baiting procedure was repeated once.

3.2.5 Scoring of soil cores and bulk samples for EPN presence

Soil samples were scored as either positive or negative for EPN presence based on whether or not a bait insect from either the first and/or second baiting was infected with EPN. Insects infected with EPN were identified by cadaver colouration and consistency. Waxmoth larvae killed by *S. carpocapsae* assume a cream colour and are limp, whereas those infected with *H. downesi* turn yellow, orange or red and also luminesce. They also generally have a more solid consistency. In all cases where cadaver appearance was inconclusive, cadavers were tentatively scored as negative for EPN but retained for incubation and later dissection to confirm presence or absence of EPN. In addition to scoring soil samples for EPN presence, infected bait insects from samples collected at Glendine and Ballymacshaneboy in 2009 (year 2 after application of nematodes) were dissected immediately after removal from the soil sample and the adult nematodes in each cadaver were counted.

Coloration of bait insect cadavers was inconclusive for some of the soil samples collected on the two manually treated sites (Glandalough and Oakwood). These cadavers were placed on White traps and emerging IJs were collected one, two and three weeks after first emergence to allow IJ length measurements and the subsequent establishment of a laboratory culture of these EPN isolates.

As a reference for IJ length, IJs emerging from waxmoth larvae infected with laboratory cultures of *S. carpocapsae* or *S. feltiae* (strain 4cfmo) were collected in parallel. IJ morphometrics and adult nematode features (spicule morphology etc.) were used to identify isolates to species where possible. For length measurements, approximately 500 IJs were pipetted onto a glass slide in 1 ml of tap water. IJs were killed prior to measurement by placing the slide on a Stuart CB162 stir-heater set to 80°C for approximately 30 seconds (Bibby Scientific Ltd; Stone, UK). This caused IJs to straighten, thus facilitating precise measurements of length. The length of 30 IJs was then measured to the closest 10 µm using a Nikon Optiphot-2 microscope with an ocular reticule (Nikon; Tokyo, Japan). Adult stages of EPN isolates for morphological examination were obtained by infecting waxmoth larvae with isolate IJs as described in 2.2 and dissecting cadavers two to three days later.

Dead bait insects from off-site soil baiting (see 3.2.4) were dissected to confirm EPN identity based on male spicule morphology. Where more than one bait insect was infected in a tub, an infected cadaver was placed on a White trap (Kaya & Stock 1997) and emerging IJs were collected one week after first emergence to confirm EPN identity by IJ length.

3.2.6 Baiting of bark samples

a) Direct baiting of individual bark samples

Four small sections of bark (approximately 4 cm^2 each) were randomly selected from the bark samples collected at each stump. Any dirt adhering to the outer side of the bark sample was removed with a small paintbrush. A waxmoth larva was placed into each of the two chambers of a perspex slide previously prepared as described for oviposition experiments involving parasitoid wasps (see 6.2.2). One bark section was fastened over each chamber with masking tape, the outer side of the bark facing up. Two slides per stump were prepared, for a total of four baited samples per stump. Each slide was placed in a 9 cm Petri dish that was sealed with Parafilm®. For each site, Petri dishes with samples were stacked in random order with six dishes per stack and an empty dish at each end. They were incubated at 20°C for five days, after which time the waxmoth larvae were removed and infection with EPN was recorded. All cadavers that showed a coloration indicating *S. carpocapsae* infection were scored as positive. Those where mortality occurred but coloration was inconclusive were dissected and checked for the presence and identity of nematodes based on male spicule morphology.

b) Baiting of pooled bark samples

The remaining bark collected from each stump was placed in a 250 ml plastic cup and the weight of the sample was determined. Ten waxmoth larvae were added to each cup and the cup was sealed with a single sheet of Parafilm®. Cups were placed on a tray in random order in a 4 x 5 grid and incubated at 20°C. Bait insects were replaced every three days and the number of infected insects was recorded based on cadaver colouration. This procedure was repeated for each sample until no further infection of bait insects was recorded.

3.2.7 Soil core spiking

To estimate the accuracy of the baiting method used to detect EPN presence in soil core samples, a set of soil cores was spiked with laboratory cultured *Steinernema carpocapsae* IJs. Soil cores were selected from those randomly sampled in 2009 on three sites (Lackenrea, Ballymacshaneboy, Knockeen) (3.2.1). All cores selected had scored negative for nematodes over the 14 day baiting period and thus were considered to be free of nematodes. Live IJs were individually counted as they were taken up in 100 µl of tap water for application. Nematodes used for this experiment were four weeks old. A total of 40 cores were selected from each site. Of these, ten cores from each site were spiked by applying a single IJ to the surface of the soil sample within them and a further ten cores received ten IJs to the surface of the sample ('surface' treatment, see Fig 3.3). A small hole was melted into the side of each of the remaining 20 soil cores from each site and one or ten IJs were applied to the soil sample within the core through this hole ('centre' treatment, see Fig 3.3). The hole was then sealed with a small piece of adhesive (BluTack™, Barker Inc, Fort Wayne, USA). All cores thus spiked with EPN were incubated for 48 h at 20°C to allow IJs to disperse in the soil sample. The cores were then baited with waxmoth larvae and scored for EPN presence the same way soil cores collected in the field had been (see 3.2.4 and 3.2.5).

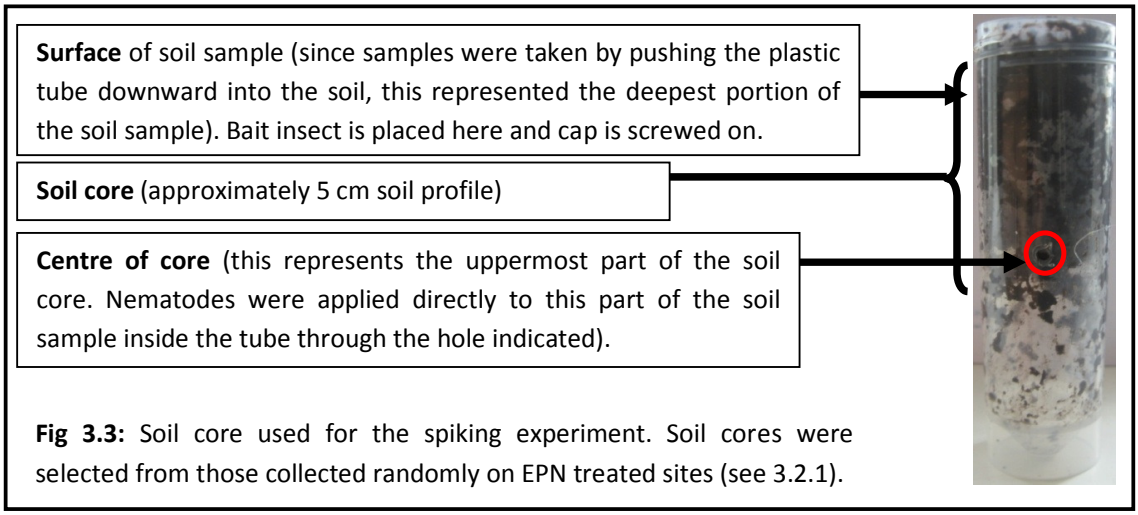


Fig 3.3: Soil core used for the spiking experiment. Soil cores were selected from those collected randomly on EPN treated sites (see 3.2.1).

3.3 Results

3.3.1 Persistence of EPN in soil around manually treated stumps

a) Glendalough

EPN occurrence in the samples collected on this site was very low. Out of 176 soil cores and 176 bulk samples collected in each of the two years the site was sampled, bait insects from only three soil cores and one bulk sample were infected with EPN. All of these positive samples were collected one year after EPN application, no samples scored positive the following year. The bait insect cadaver from one of the positive spoil cores was luminescent and of red colouration, indicating infection with *H. downesi*. The core had been collected from a stump originally treated with this EPN species. The cadaver appearance of bait insects from the remaining two cores and one bulk sample were consistent with infection by *Steinernema* spp. To identify these isolates (labelled isolates 6-4, 27-1 and 22-4 according to source stump) the length of IJs and adult morphology was compared with that of laboratory reared *S. carpocapsae, S. feltiae* (strain 4cfmo) and *S. bicornutum* (see 3.2.5).

There was a highly significant difference in the mean length of IJs of the three field isolates, *S. carpocapsae* and *S. feltiae* for each week of emergence (One-way ANOVA; week 1: $F = 209.16$, $DF = 4$, $P < 0.001$; week 2: $F = 185.27$, $DF = 4$, $P < 0.001$; week 3: $F = 143.19$, $DF = 4$, $P < 0.001$, $N = 30$ for all data sets). Tukey's test ($\alpha = 0.05$) detected a highly significant difference between all Glendalough isolates and *S. carpocapsae* (Table 3.2). Only IJs from isolates 6-4 and 27-1 were significantly different in length from *S. feltiae* IJs in each week of emergence. There was no significant difference in length between IJs from isolate 22-4 and those of laboratory cultured *S. feltiae* in week 3 of emergence. IJs of isolate 22-4 were significantly longer than all other isolates in corresponding weeks of emergence (Table 3.2). Within infected waxmoth larvae, all three unidentified strains showed clear sexual dimorphism and morphology consistent with *Steinernema* spp. Male spicule morphology of none of the isolates matched that of *S. carpocapsae* or *S. feltiae* as observed in males from laboratory culture or as published by Adams & Nguyen (2002). Spicule morphology was otherwise inconclusive as to the identity of the isolates.

Since morphological and morphometric parameters for none of the three isolates matched those of the species that had originally been applied at Glendalough (*S. carpocapsae* and *H. downesi*), they were not considered as indicative of EPN persistence. The unidentified isolates were subsequently cultured in the laboratory as described in section 2.2 and subjected to Restriction Fragment Length Polymorphism (RFLP) analysis of their rDNA ITS fragment for identification (see Chapter IV).

Table 3.2: Host cadaver characteristics and IJ length measurements for unidentified *Steinernema* spp. isolates collected at **Glendalough** one year after EPN application with IJ measurements of *S. carpocapsae* and *S. feltiae* (4cfmo) from laboratory culture and published lengths (from Adams & Nguyen 2002) for comparison (bottom three rows). Mean values within each week of emergence (N = 30) that are labelled with the same letter are not significantly different from each other (Tukey's test for each week of emergence, α =0.05). N = 90 for IJ length range.

Species	*EPN species applied to source stump*	*Cadaver appearance*	*Mean length of IJs in µm ± SE*			*Length range of IJs in µm*
			Week 1	***Week 2***	***Week 3***	
Steinernema sp. (isolate **6-4**)	*H. downesi*	Cream	**775 a** *± 8.17*	**741 a** *± 8.04*	**756 a** *± 8.76*	650 - 800
Steinernema sp. (isolate **22-4**)	*H. downesi*	Grey/Cream	**837 b** *± 8.14*	**878 b** *± 12.80*	**856 b** *± 8.45*	720 - 990
Steinernema sp. (isolate **27-1**)	*H. downesi*	Grey/Cream	**767 a** *± 9.49*	**744 a** *± 9.00*	**758 a** *± 9.94*	600 - 910
S. carpocapsae	-	Cream	**573 c** *±7.73*	**592 c** *± 7.29*	**581 c** *± 9.52*	450 - 700
S. feltiae (4cfmo)	-	Grey	**909 d** *±7.73*	**916 d** *± 8.71*	**861 b** *± 10.62*	720 - 1020
S. carpocapsae	-	-			558	438 - 650
S. feltiae	-	-			849	736 - 950
S. kraussei	-	-			951	797 – 1102

b) Oakwood

Out of the 240 soil cores and 240 bulk samples that were collected in each of the two years at Oakwood, EPN were detected in only two cores, both from a control treatment stump sampled two years after EPN application. The cadavers were of an appearance consistent with *S. carpocapsae* infection. IJs of this isolate were significantly different in length from those of *S. carpocapsae* in week one of emergence (One-way ANOVA: F = 5.53, DF = 2, P = 0.005, N = 30 per data set), but not in week two (One-way ANOVA; F = 1.52, DF = 2, P = 0.224, N = 30 per data set) (Table 3.3). Measurements were only available for weeks 1 and 2 after first emergence as the number of emerging IJs from cadavers carrying the field isolates was too low to permit measurement in week three. Spicule morphology resembled that of *S. carpocapsae* (Adams & Nguyen 2002). Based on IJ length comparison and spicule morphology these EPN isolates were classified as *S. carpocapsae*.

Table 3.3: Source stump, cadaver coloration of infected bait insects and IJ length of unidentified EPN isolates collected two years after EPN application at **Oakwood**. An asterisk indicates a significant difference in average IJ length to cultured *S. carpocapsae* emerging in the same week (One-way ANOVA with Tukey's test [α 0,05]). N = 30 per mean for each week of emergence, N = 60 for IJ length range.

			Mean length of IJs (µm) ± SE		
Species	*Source stump*	*Cadaver appearance*	***Week 1***	***Week 2***	*Length range of IJs (µm)*
Steinernema spp. (isolate 17-2)	Tap water control	Cream	**596*** *± 3.26*	**606** *± 4.32*	550-680
Steinernema spp. (isolate 17-3)	Tap water control	Cream	**598*** *± 4.06*	**598** *± 4.23*	530-660
S. carpocapsae	-	Cream	**573** *± 7.73*	**592** *± 7.29*	450 – 700

3.3.2 Soil characteristics of sites treated with a spray rig

Though no physical measurements of soil parameters such as water potential, pH, particle size and composition or organic content were taken, some basic observations about the appearance and properties of the collected soil samples were made. Soil cores collected from most sites categorized as featuring mineral soil (Lackenrea, Kilworth and Ballymacshaneboy) were similar across sites in that they usually consisted of a sandy to silty loam which, judged by coloration, contained little organic matter. Soil cores collected on these sites usually were estimated to be of low to intermediate moisture content. Soil cores collected at Glendine mainly consisted of peat. The site was located on blanket bog on a hilltop in central Ireland (see Table 2.4). Soil samples collected there were dark brown to black in colour, indicating a large proportion of organic matter. On all three occasions the site was visited, standing water was present on the site, usually restricted to the troughs running in between the rows of stumps. Consequently, samples collected along aspects crossing these troughs contained high amounts of water, often to and above the point of saturation. Soil cores collected at Knockeen (classified as a peat soil site) were very dry. They consisted almost entirely of organic material that was highly particulate.

3.3.3 Persistence of EPN in soil around stumps treated with a spray rig

a) Glendine and Lackenrea

These two sites were sampled five months, one year and two years after EPN application against pine weevils in tree stumps, thus giving an impression of EPN persistence over time. Moreover, the site at Lackenrea featured both Sitka spruce (*Picea sitchensis*) and lodgepole pine (*Pinus contorta*) and thus

allowed a direct comparison of persistence based on stump species. Glendine was stocked with lodgepole pine only (Tables 3.4 and 3.5).

At Glendine, (peat soil and pine stumps) approximately 11 % of soil cores were positive for EPN five months after stumps on the site had been treated. This proportion was slightly higher seven months later, both for samples collected around the same set of stumps (12.5 %) and samples collected around a new set of 30 stumps (13.5 %) (Table 3.4). The proportion of stumps around which at least one soil core was found to score positive for EPN also increased from roughly 77 % after five months to 87 % after twelve months (resampled stumps set and new stump set). The proportion of cores scoring positive for EPN was lower at Lackenrea at both time points and for both stump species present on that site (e.g. 2.2 % for pine stumps 5 months after EPN application and 3.1 % in the resampled stump set or 3.3 % in the new stump set seven months on) (Table 3.5). In year two after EPN application, the proportion of soil cores and stumps scoring positive for EPN at Glendine had fallen considerably from the previous year (e.g. a decrease from 11 % to 6.7 % in the stump set that was repeatedly sampled). The change in EPN presence over time was found to be significant at Glendine for both resampled stumps and newly sampled stumps (resampled stumps: $\chi^2 = 9.770$, DF = 2, P = 0.008; new stumps: $\chi^2 = 19.627$, DF = 1, $P < 0.001$, for N see Table 3.4). With regards to the proportion of stumps at which any EPN were detected, the change approached significance for the resampled stumps ($\chi^2 = 5.724$, DF = 2, P = 0.057; N = 30 for each year) and was highly so for the newly sampled sets ($\chi^2 = 16.586$, DF = 2, $P < 0.001$; N = 30 for each year).

At Lackenrea (mineral soil with spruce and pine stumps), stump species had no significant effect on the proportion of soil cores or stumps scoring positive for EPN at any of the three sampling times (Fisher's exact test, $P < 0.05$, for N see Table 3.5). When comparing the number of positive soil cores from spruce and pine stumps combined for all three sampling times, a significant change over time was found for both the repeatedly sampled stumps ($\chi^2 = 8.062$, DF = 2, P = 0.018; for N see Table 3.4) and new stumps ($\chi^2 = 8.769$, DF = 2, P = 0.012; for N see Table 3.4). No EPN presence at all was detected in soil samples collected around spruce stumps two years after application at Lackenrea, a significant change from both of the two previous samplings (Fisher's exact test, $\alpha = 0.017$; 5 months vs. 1 year: P = 0.017, 5 months vs. 2 years: P = 0.017; for N see Table 3.5). Likewise, there was a significant change in the proportion of stumps around which at least one soil core scored positive at Lackenrea over the two years of sampling, both for the resampled stumps ($\chi^2 = 6.669$, DF = 2, P = 0.036; N = 30 for each year) and the new sets of stumps ($\chi^2 = 9.545$, DF = 2, P = 0.008, N = 30 for each year). Only a few samples collected around pine stumps scored positive two years after application of EPN at Lackenrea (representing 1.3 % of cores in the newly sampled stump set for that year and 0.4 % in the repeatedly sampled set) (Table 3.4 and Table 3.5).

Table 3.4: Percentage of soil cores from **Glendine** scoring positive for EPN presence five months, one year and two years after EPN application to stumps and percentage of stumps around which at least one core scored positive. All stumps were lodgepole pine. Within each group of stumps (resampled or new), values labelled with the same letter are not significantly different from each other between sampling times (χ^2 – test, Bonferroni-adjusted α = 0.017, lowercase letters used for resampled stumps, capital letters used for newly sampled stumps. The five month data set was compared with both resampled and newly sampled stumps)

Glendine (peat soil)				
Time since EPN application	*Stump set*	*N (cores/ stumps)*	*Percentage and (number) of cores positive for EPN*	*Percentage and (number) of stumps positive for EPN*
5 months	-	480/30	**11.0** (53) a A	**76.7** (23) a A
1 year	Repeated	480/30	**12.5** (60) b	**86.7** (26) a
	New	480/30	**13.5** (65) A	**86.7** (26) A
2 years	Repeated	480/30	**6.7** (32) a	**60** (18) a
	New	480/30	**5.2** (12) B	**40** (12) B

Table 3.5: Percentage of soil cores from **Lackenrea** positive for EPN presence five months, one year and two years after EPN application to stumps and proportion of stumps around which at least one core scored positive. SS = Sitka spruce, LP = lodgepole pine. Within each group of stump sets (resampled or new) values labelled with the same letter are not significantly different from each other between sampling times (Fisher's exact test, α = 0.017, lowercase letters used for resampled stumps, capital letters used for newly sampled stumps). No significant differences were found for pine stumps. The 5 month data set was compared with both resampled and newly sampled stumps.

Lackenrea (mineral soil)					
Time since EPN application	*Stump set*	*Stump species*	*N (cores/ stumps)*	*Percentage and (number) of cores positive for EPN*	*Percentage and (number) of stumps positive for EPN*
5 months	-	*LP*	320/20	**2.2** (7)	**30** (6)
		SS	160/10	**3.1** (5) a A	**40** (4) a A
1 year	Repeated	*LP*	256/16	**3.1** (8)	**31** (5)
		SS	96/6	**3.1** (3) a	**33** (2) a
	New	*LP*	240/15	**3.3** (8)	**40** (6)
		SS	240/15	**3.3** (8) A	**40** (6) A
2 years	Repeated	*LP*	256/16	**0.4** (1)	**6.3** (1)
		SS	96/6	**0** (0) a	**0** (0) a
	New	*LP*	240/15	**1.3** (3)	**13.3** (2)
		SS	240/15	**0** (0) A	**0** (0) B

When comparing Lackenrea cores collected only from pine stumps to those collected at Glendine (new sets of stumps only), the latter had consistently higher proportions of positive cores and stumps for each of the three time points sampled and these differences were significant in all three instances (5 months: $\chi^2 = 21.697$, DF = 1, $P < 0.001$; 1 year: $\chi^2 = 18.301$, DF = 1, $P < 0.001$; 2 years: $\chi^2 = 6.707$, DF = 1, P = 0.010; for N see Tables 3.4 and 3.5). Overall, at least one soil core each from 29 of the 30 stumps sampled five months after EPN application and then again in the subsequent two years scored positive for EPN at Glendine in at least one of the years the site was visited. The same was true for only 8 of the 16 pine stumps sampled each of those years at Lackenrea. This difference was highly significant (Fisher's exact test, $P < 0.001$; N = 30 each).

b) Ballymacshaneboy, Knockeen and Kilworth

Stumps on these sites were only sampled for EPN presence once, either two years after EPN application (Ballymacshaneboy and Knockeen) or four weeks after application (Kilworth). At Knockeen and Ballymacshaneboy the proportion of soil cores scoring positive for EPN was low two years after EPN application (0.9 % and 2.9 %, respectively) (Table 3.6). Results from Knockeen at this time after application were similar to those for Lackenrea, the only other site featuring spruce stumps in this study. Persistence at Ballymacshaneboy two years after EPN application (2.9 % of 480 cores and 36.7 % of 30 stumps sampled scoring positive for EPN presence) approached the levels found at Glendine recorded at the same time point after application (Table 3.6). No significant difference between persistence at Glendine and Ballymacshaneboy was found in the proportion of cores scoring positive ($\chi^2 = 3.234$, DF = 1, P = 0.072, N = 480 cores for both sites), or stumps for which at least one core scored positive ($\chi^2 = 0.071$, DF = 1, P = 0.791) at this time.

The highest frequency of EPN presence in the study was recorded for samples collected at Kilworth four weeks after nematodes had been applied. Almost 18 % of the 128 cores collected around pine stumps treated with *S. carpocapsae* were positive for EPN and the same was true for approximately 13 % of the of the 128 cores collected around stumps treated with *H. downesi* (Table 3.6). EPN presence was much lower in samples from spruce stumps (*S. carpocapsae*: 6.3 %, *H. downesi*: 3.6 %). A statistical comparison with other sites in the study was not carried out since Kilworth was the only site on which samples were taken so shortly after EPN application. However, statistical analysis indicated that EPN species did not have a significant effect on the proportion of positive cores (pine/***S.c.*** vs. pine/***H.d.***, $\chi^2 = 0.335$, DF = 1, P = 0.563; spruce/***S.c.*** vs. spruce/***H.d.***, $\chi^2 = 0.492$, DF = 1, P = 0.483), but that stump species did (**pine**/*S.c.* vs. **spruce**/*S.c.,* $\chi^2 = 9.666$, DF = 1, P = 0.002; **pine**/*H.d.* vs. **spruce** /*H.d.*, $\chi^2 = 8.880$, DF = 1, P = 0.003, Bonferroni-adjusted $\alpha = 0.0250$). No significant difference was found when comparing stumps that scored positive along these lines (Fisher's exact test, $\alpha = 0.05$).

Table 3.6: Percentage of soil cores collected at **Ballymacshaneboy, Knockeen** and **Kilworth** which scored positive for EPN and percentage of stumps around which at least one core scored positve. The proportions of stumps from which at least one core scored positive are also given. SS = Sitka spruce, LP = lodgepole pine. *S.c.* = *Steinernema carpocapsae*, *H.d.* = *Heterorhabditis downesi*.

Site (soil type)	*Time since EPN application*	*Stump species*	*EPN species applied*	*N (cores/ stumps)*	*Percentage and (number) of cores positive for EPN*	*Percentage and (number) of stumps positive for EPN*
Ballymac-shaneboy (mineral)	2 years	*LP*	*S.c.*	480/30	**2.9** (14)	**36.7** (11)
Knockeen (peat)	2 years	*SS*	*S.c.*	432/27	**0.9** (4)	**11** (3)
Kilworth (mineral)	4 weeks	*LP*	*S.c.*	128/8	**18** (23)	**87.5** (7)
		SS	*S.c.*	112/7	**7** (6.25)	**57.1** (4)
		LP	*H.d.*	128/8	**13.3** (17)	**100** (8)
		SS	*H.d.*	112/7	**3.6** (4)	**42.9** (3)

3.3.4 EPN numbers invading bait insects in soil cores

The number of adult nematodes was determined in each of the bait insects that had been infected in samples collected two years after EPN application at Glendine and Ballymacshaneboy (Fig 3.4). On average, approximately ten adult nematodes were found in bait insect cadavers from 0 cm cores collected at resampled Glendine stumps two years after EPN application. The means for new stumps sampled at Glendine and for stumps at Ballymacshaneboy was slightly lower at approximately eight adult nematodes per cadaver. A comparison of these data sets yielded no significant differences either among sites or among the repeatedly sampled and newly sampled stump sets at Glendine (only 0 cm distance soil cores included; One-way ANOVA, F = 0.69, DF = 2, P = 0.507, N = 17 for Glendine resampled stumps, N = 11 for Glendine new stumps and N = 9 for Ballymacshaneboy) (Fig 3.4).

Since only a small number of soil cores collected at distances from the bole of 20 cm or greater at scored positive at Ballymachsaneboy and Glendine in 2009, data was combined for the two sites (0 cm, 20 cm and 40 cm, no samples positive at 60 cm distance) (Fig 3.5). There was a significant decrease in the median number of nematodes per cadaver from 0 cm to 40 cm distance (M.-W. U-test, W = 635.0, P = 0.002), but there was no significant difference when the median numbers at 0 cm and 20 cm (M.-W. U-test, W = 644.0, P = 0.039 [Bonferroni-adjusted $\alpha = 0.017$]) or 20 cm and 40 cm (M.-W. U-test, W = 111.5, P = 0.126) were compared.

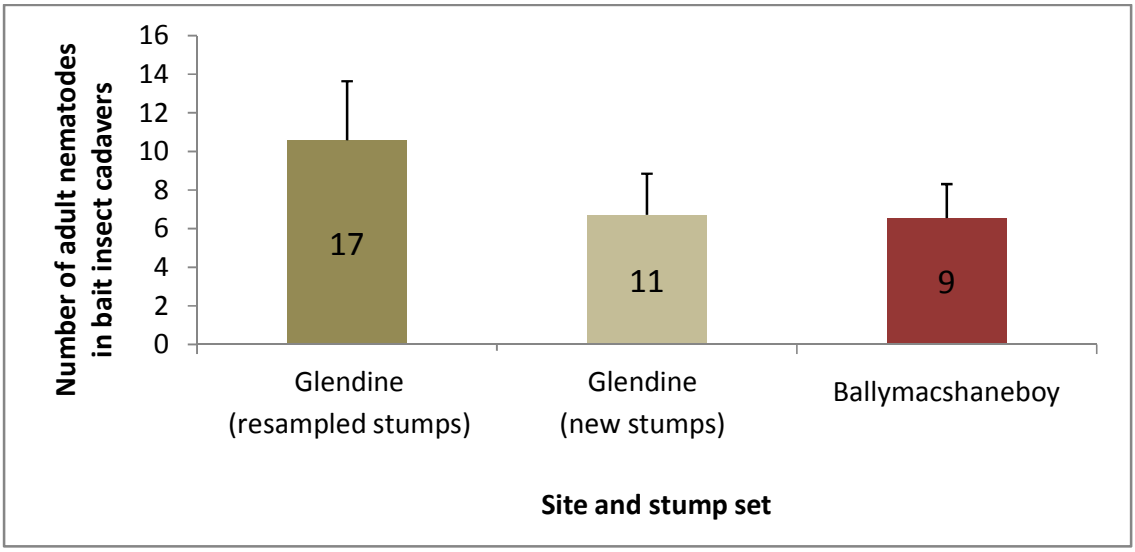


Fig 3.4: Mean number of *S. carpocapsae* adults found in bait insect cadavers retrieved from soil cores collected at 0 cm distance at **Glendine** and **Ballymacshaneboy** two years after EPN application to these sites. Error bars represent standard error and the numbers in each bar represent N.

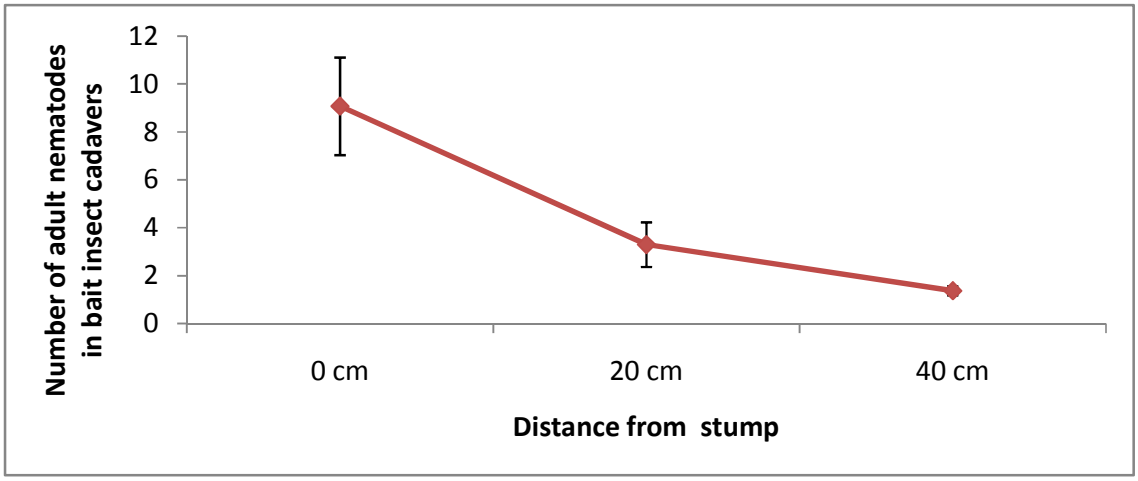


Fig 3.5: Mean number of *S. carpocapsae* adults found in bait insect cadavers retrieved from soil cores collected at 0 cm, 20 cm and 40 cm distance at **Glendine** two years after EPN application to these sites (data from resampled and new stumps sets combined). Error bars represent standard errors. N = 28 for 0 cm, N = 10 for 20 cm and N = 8 for 40 cm.

3.3.5 EPN spread from tree stumps

a) Glendine and Lackenrea

The proportion of soil cores collected at Glendine in which EPN were detected decreased consistently and exponentially with increasing distance from the bole of the stump for each of the times each site was visited (Fig 3.6). This decrease was observed both for stumps repeatedly sampled over the two year period and the new sets of stumps sampled one and two years after EPN application and was

significant in all cases (χ^2 – test, P ≤ 0.002) (Table 3.7). By the third year of sampling, no cores taken at a distance of 60 cm from the bole of the stump scored positive on either site. Results for Lackenrea are shown in (Table 3.8).

A decrease in the percentage of soil cores in which EPN were detected was also observed in each year samples were collected at Lackenrea. For instance, one year after EPN had been applied, the percentage of positive samples at the newly samples set of pine stumps decreased from 10 % at the bole to 3.3 % at 20 cm distance and 0 % beyond that. None of the soil cores collected at 60 cm distance from the bole of the stump was positive for EPN two years after application. Since the total number of positive samples for this site was low for each of the times it was sampled and within each subset of samples (from pine or spruce stumps), statistical analysis was not possible.

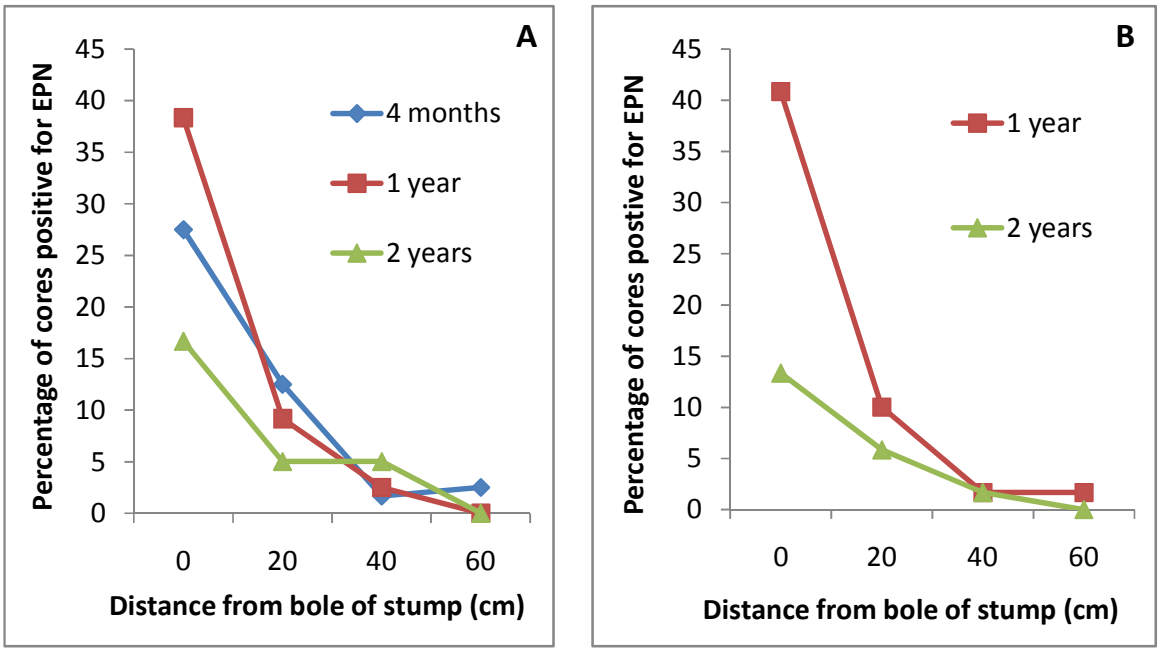


Fig 3.6: Proportion of soil cores positive for EPN collected at **Glendine** in the two years after EPN application to site. Distance from the bole of the stump is plotted along the x-axis. **A:** repeatedly sampled stumps. **B:** new sets of stumps sampled in year one and year two after EPN application.

Table 3.7: Statistical test results (χ^2 –tests) for comparisons of the proportion of positive spoil cores from **Glendine** at each of the four distances from treated stumps at which samples were collected. N = 120 at each distance, four distances (0 cm, 20 cm, 40 cm, 60 cm) included in each test.

Data set	**Test result**
Resampled stumps (5 months)	χ^2 = 53.003, DF = 3, **P < 0.001**
Resampled stumps (1 year)	χ^2 = 102.552, DF = 3, **P < 0.001**
Resampled stumps (2 years)	χ^2 = 28.929, DF = 3, **P < 0.001**
New stumps (1 year)	χ^2 = 106.534, DF = 3, **P < 0.001**
New stumps (2 years)	χ^2 = 12.981, DF = 3, **P = 0.002**

Table 3.8: Percentage of soil cores collected at **Lackenrea** which scored positive for EPN at each of four distances from the bole of the stump. Data is further grouped by time of sampling and stump species. SS = Sitka spruce, LP = Lodgepole pine.

	Percentage of cores positive in % (repeatedly sampled stumps/new stumps)					
	LP			**SS**		
Distance from bole of stump	***5 months*** *N = 80*	***1 year*** *N = 64/60*	***2 years*** *N = 64/60*	***5 months*** *N = 40*	***1 year*** *N = 24/60*	***2 years*** *N = 24/60*
0 cm	3.8	6.3/10.0	1.0/1.7	5.0	4.2/6.7	0/0
20 cm	1.3	0/3.3	0/1.7	5.0	8.3/5.0	0/0
40 cm	2.5	0/0	0/1.7	2.5	0/0	0/0
60 cm	1.3	2.1/0	0/0	0	0/1.7	0/0

b) Ballymacshaneboy, Knockeen and Kilworth

Of the four cores from Knockeen scoring positive for EPN, two had been taken at 20 cm distance from the stump, and one each at 40 cm and 60 cm distance (N = 108 each). At Ballymacshaneboy, twelve of the 14 positive cores collected had been taken at 0 cm distance, with a further one core each at 20 cm and 40 cm distance from the bole of the stump (N = 120 each). No statistical comparison was possible due to low numbers of positive cores.

Cores returned from Kilworth showed a decrease in EPN presence with increasing distance from the bole of the stump. The decrease was particularly pronounced around pine stumps to which *S. carpocapsae* had been applied, with a drop from roughly 60 % positive cores to only about 10 % positive cores within the first 20 cm (Fig 3.7). This trend was found irrespective of stump species (pine or spruce) or the applied nematode species (*S. carpocapsae* or *H. downesi*). Due to the low number of replicates, data from pine and spruce stumps was combined for each of the nematode species applied. χ^2 – tests revealed that the decrease in EPN prevalence was highly significant for both *S. carpocapsae* and *H. downesi* as distance from the stump increased (χ^2 = 46.791, DF = 3, $P < 0.001$ and χ^2 = 19.670, DF = 3, $P < 0.001$, respectively).

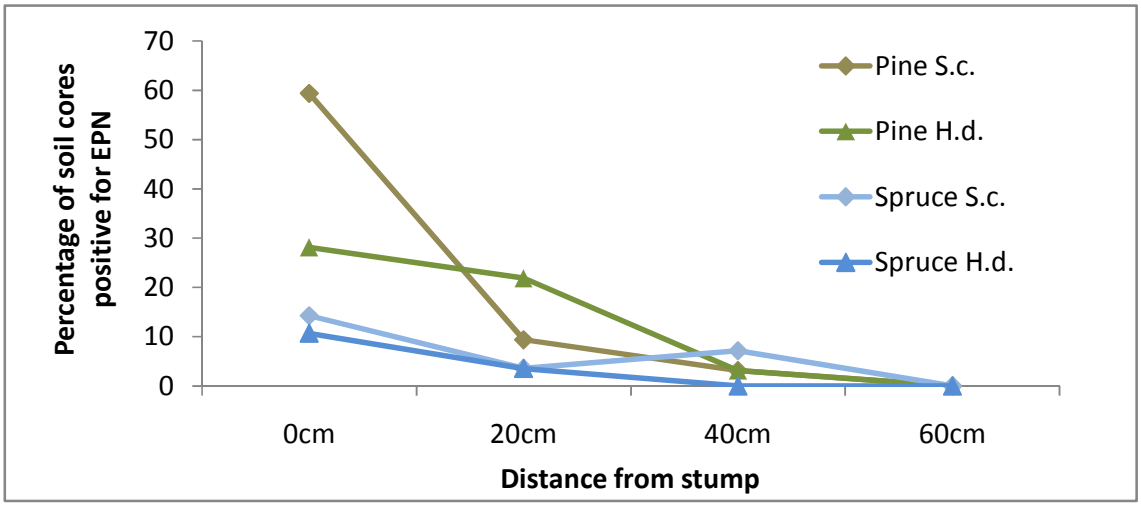


Fig 3.7: Percentage of soil cores positive for EPN collected at **Kilworth** four weeks after EPN application to stumps. Distance from the bole of the stump is plotted along the x-axis. Each line represents data from a subset of stumps (spruce or pine) treated with either *S. carpocapsae* (S.c.) or *H. downesi* (H.d.).

3.3.6 EPN persistence under bark on sites treated with a spray rig

a) Lackenrea and Glendine

The prevalence of EPN under the bark of treated stumps broadly reflected the results obtained from soil baiting. As was seen for soil samples (see Tables 3.4 and 3.5), the proportion of stumps with either directly baited or bulk baited bark samples scoring positive for EPN at Glendine decreased from approximately 77 % to 43 % from year one to year two after EPN application and this decrease was significant (χ^2 = 6.944, DF = 1, P = 0.008; N = 30 stumps for each year of sampling) (Table 3.9). There was no significant difference in persistence between bark and soil at Glendine one and two years after EPN application (one year: χ^2 = 1.002, DF = 1, P = 0.317, two years: χ^2 = 0.069, DF = 1, P = 0.793; N = 30 stumps for each year of sampling). The low number of only three positive bark samples found at Lackenrea over the two-year period prohibited statistical testing.

Table 3.9: Percentage of stumps with bark samples positive for EPN collected at **Lackenrea** and **Glendine**. SS= Sitka spruce, LP = Lodgepole pine. N = 30 for Glendine stumps and N = 15 for each stump species for Lackenrea. Number of positive samples in brackets.

			Stumps with at least one of the samples scoring positive		
Site	*Time since EPN application*	*Stump species*	*Directly baited bark sample*	*Bulk bark sample*	*Any bark sample*
Lackenrea	1 year	LP	**6.7** (1)	**6.7** (1)	**6.7** (1)
		SS	**6.7** (1)	**6.7** (1)	**6.7** (1)
	2 years	LP	**6.7** (1)	**6.7** (1)	**6.7** (1)
		SS	**0** (0)	**0** (0)	0 (0)
Glendine	1 year	LP	**60.0** (18)	**66.7** (20)	**76.7** (23)
	2 years	LP	**26.7** (8)	**36.7** (11)	**43.3** (13)

Since bark bulk samples were repeatedly baited with ten waxmoth larvae every three days until no further infection occurred, the number of infected bait insects per positive bulk sample over the entire baiting period could be compared. At Glendine, there was a slight decrease in the number of infected bait insects per 100 g of bulk bark sample from year one to year two after EPN application (Fig 3.8). This difference was, however, not significant, nor was there any difference between the number of infected waxmoth larvae per 100 g of bark between bulk samples from Glendine and Ballymacshaneboy in this respect (K.-W.-test; H = 1.23, DF = 2, P=0.539, N = see Table 3.11 for Ballymacshaneboy results). Only data from bark samples in which any EPN were detected were included in these statistical tests.

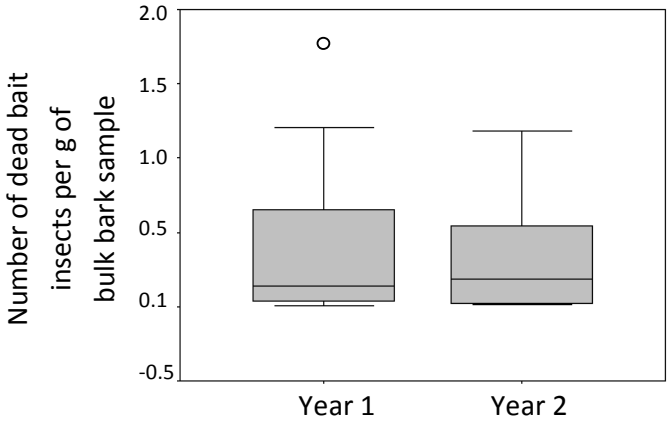


Fig 3.8: Median number of infected bait insects per 100 g of bark sample from stumps sampled at **Glendine** as recorded during successive baitings. Whiskers represent upper and lower quartiles, lines in boxes represent median. N = 20 for year 1 and N = 11 for year two. Only data from bulk samples positive for EPN included. The circle indicates an outlier.

It was notable that some bark bulk samples collected both one and two years after application of EPN to stumps yielded relatively high numbers of infected bait insects. One sample collected in the second year produced a total of 80 infected bait insects in eleven successive baitings. Similar results were found for some samples collected in year one. There was no significant connection between soil and bark samples from the same stump scoring positive among Glendine samples in either of the two years (P = 0.225 in year one and P = 0.078 in year two, Table 3.10).

Table 3.10: Tabulation of data from 30 tree stumps for which bark and soil samples were collected at **Glendine** in 2008 and 2009, one and two years after EPN application.

		Soil sample		
	Bark sample	*Positive*	*Negative*	*Test results (χ^2 or Fisher's exact test)*
2008	*Positive*	21	2	**P = 0.225**
	Negative	5	2	
2009	*Positive*	8	5	χ^2 = 3.096, DF = 1
	Negative	5	12	**P = 0.078**

b) Ballymacshaneboy, Knockeen, Kilworth

No bark samples from Knockeen scored positive for EPN. Bark samples from ten stumps at Ballymacshaneboy that were directly baited did score positive for EPN and bulk samples from twelve stumps did so (N = 30). In total, bark samples baited either directly or in bulk scored positive for EPN in 13 out of 30 stumps sampled at Ballymacshaneboy. This was the same number of stumps that had scored positive at Glendine when it was sampled that year. No significant difference between persistence in soil and under the bark was found at Ballymacshaneboy (χ^2 = 0.278, DF = 1, P = 0.598; N = 30). The median number of infected bait insects per 100 g of bulk bark sample from this site was 35.5 (N = 13 bulk samples with EPN) and as mentioned earlier (see 3.3.6a), this was not significantly different from the median for Glendine bulk bark samples. Just as was found for bulk samples from Glendine, several of the bulk bark samples, however, produced infected bait insects for several successive baitings, in one case for a total of 68 infected bait insects over the course of ten baitings.

Only a single directly baited bark sample from spruce stumps sampled at Kilworth four weeks after EPN application was positive for EPN. None of the bulk samples from these stumps were positive. Five of the eight pine stumps sampled that had been treated with *S. carpocapsae* yielded bark samples containing EPN and the same was true for six of the eight pine stumps that had been treated

with *H. downesi* IJs. There was no significant difference between these proportions (Fisher's exact test, P = 1). There was no significant difference between the median of infected bait insects per gram of bulk bark sample found over the course of successive baitings of bulk bark samples from pine stumps treated with the two different EPN species (see Fig 3.9).

Some of the bulk bark samples collected at Kilworth produced a very high number of infected bait insects, the maximum amount being 125 over 17 successive baitings. There was a significant connection between the presence of EPN in soil samples and bark samples for stumps at both Kilworth and Ballymacshaneboy (Fisher's exact test, P = 0.014 and χ^2 – test, P = 0.001, respectively; Table 3.11).

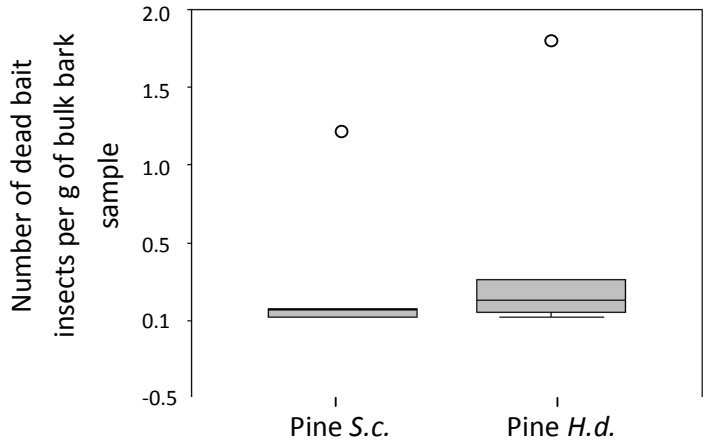


Fig 3.9: Median number of infected bait insects per 100 g of bulk bark sample from pine stumps sampled at **Kilworth** as recorded during successive baitings. Results for stumps that had been treated with *S. carpocapsae* (S.c., N = 5) and *H. downesi* (H.d., N = 6) are given. Whiskers represent upper and lower quartiles, circles indicate outliers.

Table 3.11: Tabulation of data from 30 tree stumps for which bark and soil samples were collected at **Kilworth** and **Ballymacshaneboy** in 2009, four weeks and two years after EPN application, respectively. Data for both EPN species (*S. carpocapsae* and *H. downesi*) and both stump species (spruce and pine) was combined.

Site	Bark sample	Soil sample		*Test results*
		Positive	*Negative*	*(χ^2 or Fisher's exact test)*
Kilworth	*Positive*	11	0	**P = 0.014**
	Negative	11	8	
Ballymacshaneboy	*Positive*	8	1	χ^2 = 10.866, DF = 1
	Negative	5	16	**P = 0.001**

3.3.7 Combining data for persistence across sites and regression of persistence data against pine weevil emergence

a) Soil samples

For the sampling year 2008, persistence data for Knockeen, Ballymacsahneboy, Glendine and Lackenrea was combined across sites for both stump species (pine and spruce, three and two sites, respectively) and also for each of the two main soil types that were distinguished (mineral and peat, two sites each) (Fig 3.10). With respect to soil type, the proportion of cores positive for EPN was roughly twice as on peat sites than it was on mineral sites and this difference was significant (χ^2 = 6.250, DF = 1, P = 0.012; N = 1667 and 976). No significant difference was found, however, in the proportion of stumps with at least one positive core on the two soil types (31 % on peat, 23 % on mineral soil; χ^2 = 1.123, DF = 1, P = 0.289; N = 108 and 61). By contrast, EPN presence was higher in soil cores collected around pine stumps compared with spruce stumps and this was highly significantly so (χ^2 = 21.021, DF = 1, P < 0.001; N = 1934 and 768). The same was true for pine stumps and spruce stumps with at least one positive soil core (χ^2 = 15.523, DF = 1, P < 0.001; N = 121 and 48).

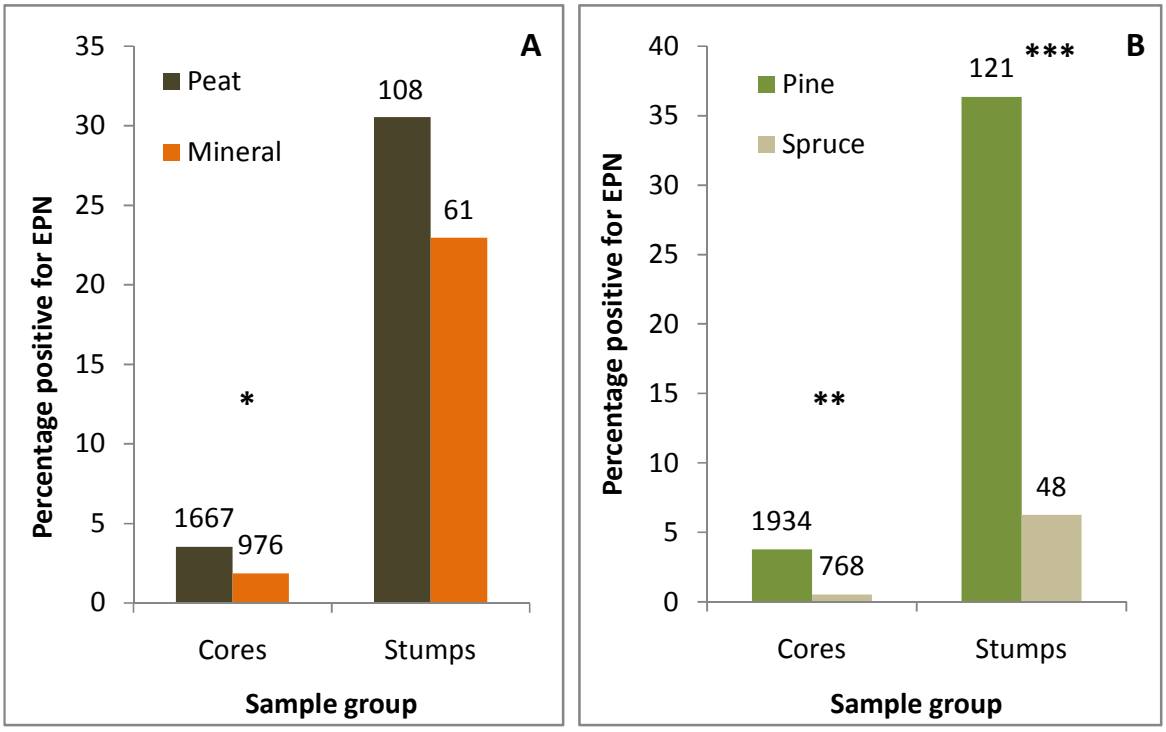


Fig 3.10: Overall proportion of cores positive for EPN presence and proportion of stumps with at least one core scoring positive for EPN presence collected in 2008, two years after EPN application, at **Ballymacshaneboy, Glendine, Knockeen** and **Lackenrea**. Graph **A** shows results grouped by soil type on sites, graph **B** shows results grouped by stump species. Number above bars is N, an asterisk above two bars indicates a significant difference between the two (χ^2 – test, * P < 0.05, ** P < 0.01; *** P < 0.001).

Since the number of site replicates (i.e. for soil and stump type) was low and the collected data might have been unrepresentative, additional analysis was conducted by regressing the percentages of positive soil cores and stumps per site as recorded in 2009 against the mean number of adult weevils emerging from untreated control stumps in 2008 as an indicator of pine weevil populations within stumps on these sites (Lackenrea [spruce: N = 13; pine: N = 14], Glendine [N = 19], Ballymacshaneboy [N = 19] and Knockeen [N = 15] included; for weevil emergence data see Appendix A.1) (Fig 3.11). Emergence data were collected by Aileen Foster as part of the Abate project funded by Coford (Griffin et al. 2008). For regression, emergence was log transformed (visual examination of data indicated a logarithmic increase in EPN presence with weevil emergence) and percentages for cores and stumps scoring positive were arcsin transformed. To avoid negative values on the x-axis, each value for emergence was transformed by adding one to each data point before transformation.

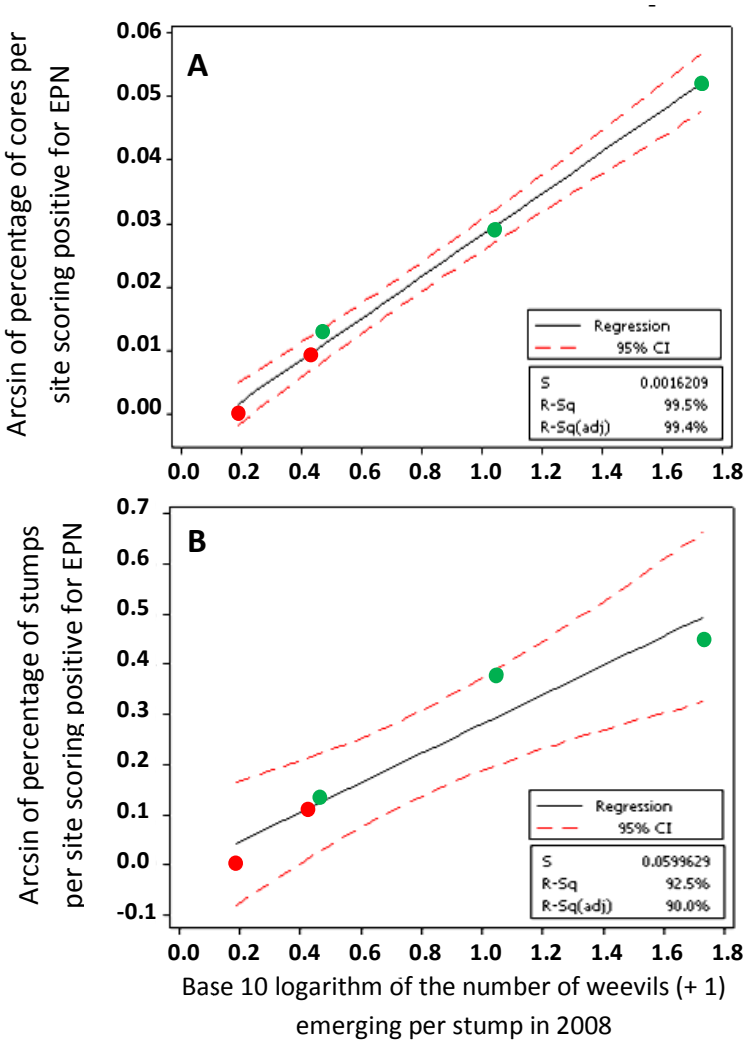


Fig 3.11: Linear trendlines for regressions of the percentage of soil cores (**A**) or stumps (**B**) scoring positive for EPN against the mean number of adult weevils emerging from untreated tree stumps at **Lackenrea**, **Glendine**, **Ballymacshaneboy** and **Knockeen**. Dashed red lines indicate 95 % confidence intervals. Green data points represent data for pine stumps, red data points for spruce stumps. N = 5.

There was a highly significant positive relationship between the percentage of positive soil cores in 2009 and the mean number of adult weevils emerging in 2008 (Coef. = 0.0328, T = 25.21, R^2 = 0.99, F = 635.59, DF = 1, P < 0.001; N = 5) and also between positive stumps and emerging weevil numbers (Coef. = 0.293, T = 6.08, R^2 = 0.93, F = 36.97, DF = 1, P = 0.009; N = 5).

a) Bark samples

The data for stumps with at least one infected bait insect found in bark samples (bulk samples or directly baited samples) was also combined as described above for soil samples (Fig 3.12). As can be seen, persistence under the bark was almost identical for each of the two soil types with approximately 23 % of samples scoring positive for both peat and mineral soil (χ^2 = 0.005, DF = 1, P = 0.946; for N see Fig 3.12), but was significantly affected by stump species (35 % in pine, 0 % in spruce, χ^2 = 0.005, DF = 1, P < 0.001; for N see Fig 3.12).

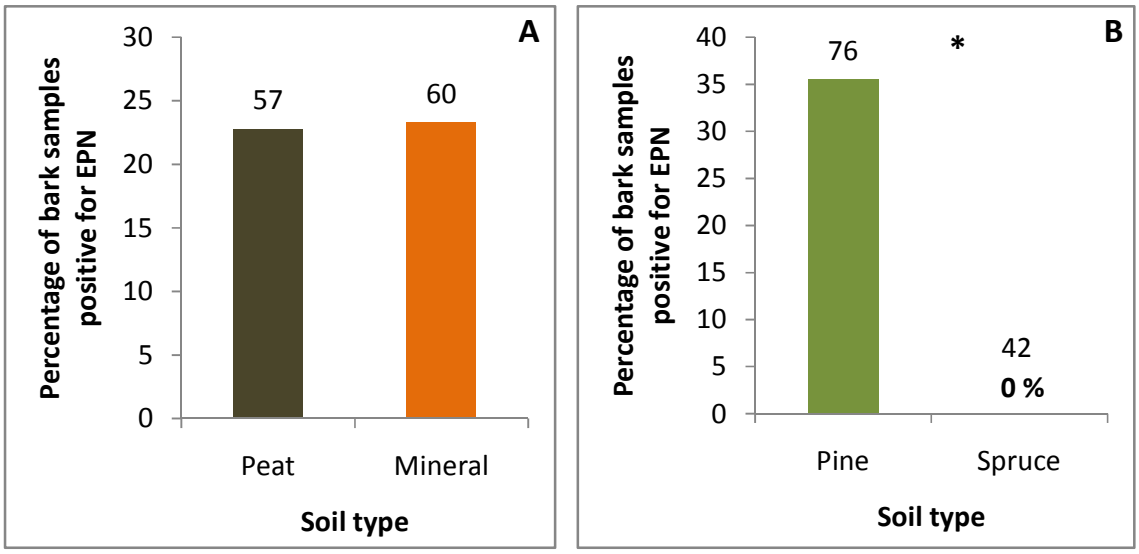


Fig 3.12: Overall percentage of stumps with at least one bark sample scoring positive for EPN presence in 2008, two years after EPN application, at **Ballymacshaneboy, Glendine, Knockeen** and **Lackenrea**. Graph **A** shows results grouped by soil type on sites, graph **B** shows results grouped by stumps species. Number above bars is N, an asterisk above two bars indicates a significant difference between the two (χ^2 – test, P < 0.001).

When the proportion of tree stumps from which at least one bark sample scored positive for EPN in 2009 was regressed against the number of weevils emerging in 2008 as described above for soil samples, a significant positive relationship was found (Coef. = 0.344, T = 3.81, R^2 = 0.83, F = 14.55, DF = 1, P = 0.032; N = 5) (Fig 3.13).

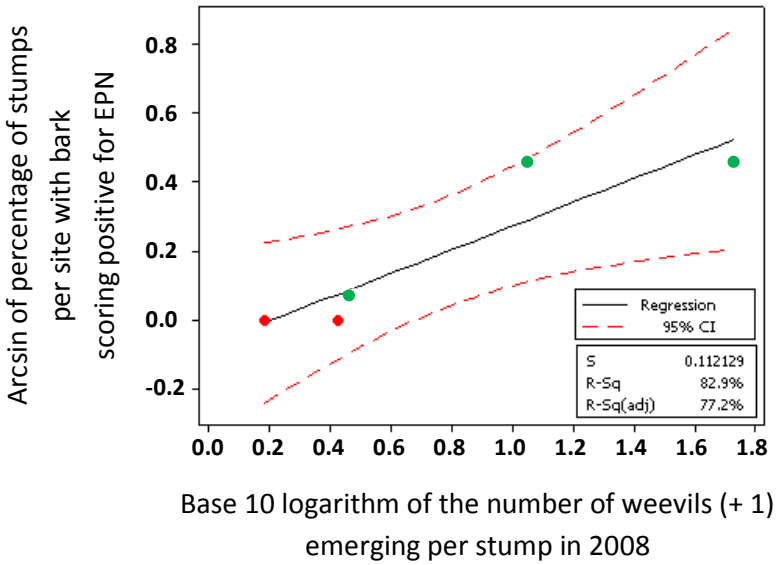


Fig 3.13: Linear trendline for regression of the percentage of stumps with at least one bark sample scoring positive for EPN against the mean number of pine weevils emerging from untreated tree stumps at **Lackenrea**, **Glendine**, **Ballymacshaneboy** and **Knockeen**. Dashed red lines indicate 95 % confidence intervals. Green data points represent data for pine stumps, red data points for spruce stumps N = 5.

3.3.8 Spread of EPN on and from treated sites

Of the 100 random soil cores that were taken on each of the four sites treated with nematodes in 2007, EPN were detected in only a single core collected at Glendine. No EPN were detected in soil samples collected in areas adjacent to the site at Ballymacshaneboy two years after EPN application. At Lackenrea, only a single infected insect was found in baitings of off-site soil samples (640 subsamples of 20 g each were collected around the edge of this site one and two years after EPN application). The positive sample was among those collected in year two after EPN application in a transect sample (Northernmost transect on Western side of site, see Fig 3.2) taken at 10 m distance from the edge of the site. At Glendine, 1200 subsamples of 20 g each were collected at this site each year it was sampled. EPN IJs were detected in samples from two transects samples originating from one transect and one parallel segment samples collected at Glendine in year one and a single parallel segment in year two after EPN application (Table 3.12 and Fig 3.14). Length measurement of IJs emerging from infected cadavers as well as male spicule morphology indicated that the EPN detected in all samples were *S. carpocapsae*.

Table 3.12: List of parallel segment and transect bulk samples in which *S. carpocapsae* were detected at **Glendine** one and two years after *S. carpocapsae* had been applied to stumps on the site. See Fig 3.13 for precise location of transects and segments listed.

Time since EPN application	*Side of site*	*Sample source*	*Distance from edge of site*	*Number of positive subsamples*	*Number of infected bait insects (N = 10 per subsample)*
	South	Segment 3 (S3)	10 m	1	1
1 year	South	Transect 3 (T3)	10 m	3	14
	South	Transect 1 (T1)	5 m	3	4
2 years	South	Section 3 (S3)	10 m	3	3

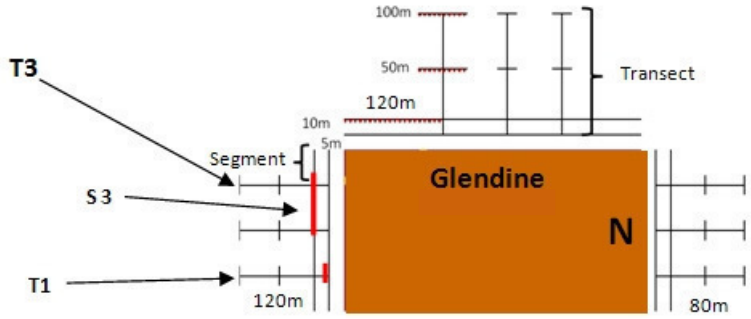


Fig 3.14: Schematic view of off-site samples collected at **Glendine**. Red lines on left indicate areas where bulk samples were collected that scored positive for EPN in year one or two after EPN application. Designation of transects and segments given in Table 3.12 as indicated.

3.3.9 Soil core spiking

When ten IJs of *S. carpocapsae* were applied to the surface of the soil sample in the core, all but one of the cores from the three sites scored positive for IJ presence (Fig 3.15). The number of positive cores was approximately halved when only one IJ was applied in this fashion. When IJs were applied to the centre of a core, the number of cores that scored positive was lower than when IJs were applied at the surface across all source sites and doses (Fig 3.16). The greatest difference was found in cores collected at Knockeen. When ten IJs were applied to the surface of the soil sample, 100 % of cores from this site produced infected bait insects, while this was the case for only four out of the ten cores (40 %) to which this number of IJs had been applied at the centre of the tube containing it. No more than two cores out of ten scored positive (Knockeen and Ballymacshaneboy) when only one IJ was applied at the centre of a soil sample.

To test for the possible effect of the source sites and their respective soil types on these results, the two most divergent data sets within each dosage group were compared using Fisher's exact test (e.g. cores sourced from Lackenrea and Knockeen receiving 10 IJs at the centre were compared etc.). Since no significant differences were found, data was subsequently grouped across sample sites by IJ dose (Fig 3.17). There was a significant difference among treatments when data was combined in this way (χ^2 = 39.932 , DF = 3, P < 0.001). The proportion of soil cores scoring positive irrespective of source site was higher for both doses when nematodes were applied to the surface of the core, a significant effect (10 IJs: χ^2 = 10.417, DF = 1, P = 0.001; 1 IJ: χ^2 = 8.864, DF = 1, P = 0.003) and more cores scored positive when 10 IJs were applied, a significant difference from the single IJ dose (centre application: χ^2 = 13.611, DF = 1, P < 0.001; surface application: χ^2 = 15.022, DF = 1, P < 0.001).

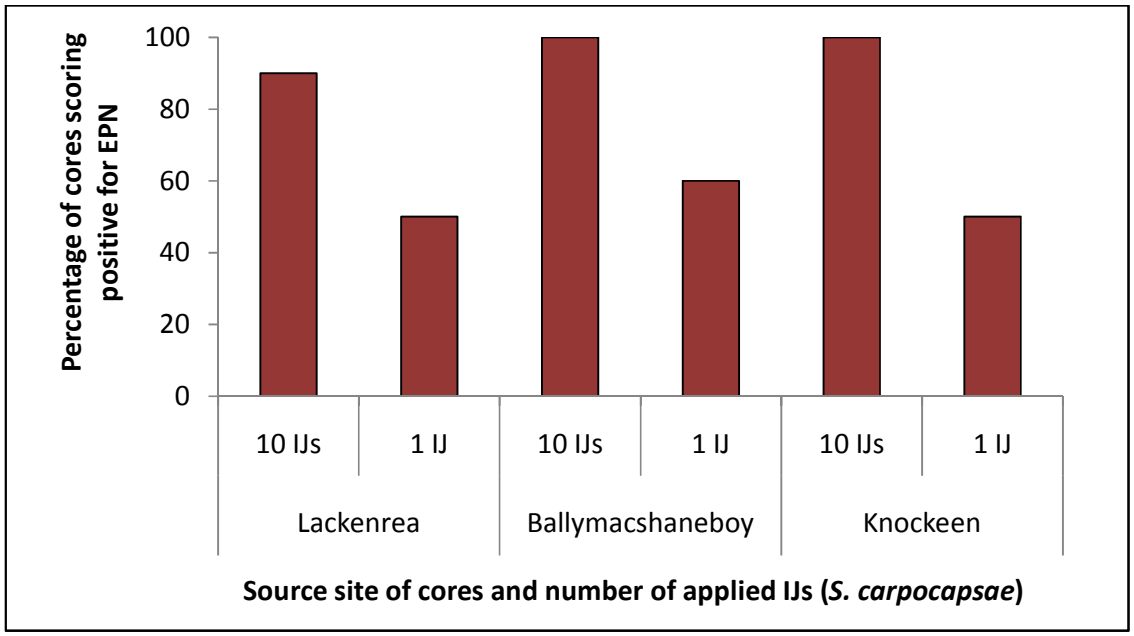


Fig 3.15: Proportion of spiked soil cores sourced from three sites scoring positive for nematode presence as detected by infection of waxmoth larvae. Either 10 IJs or 1 IJ of *S. carpocapsae* had been applied directly to the surface of the soil sample inside the tube. N = 10 for each bar.

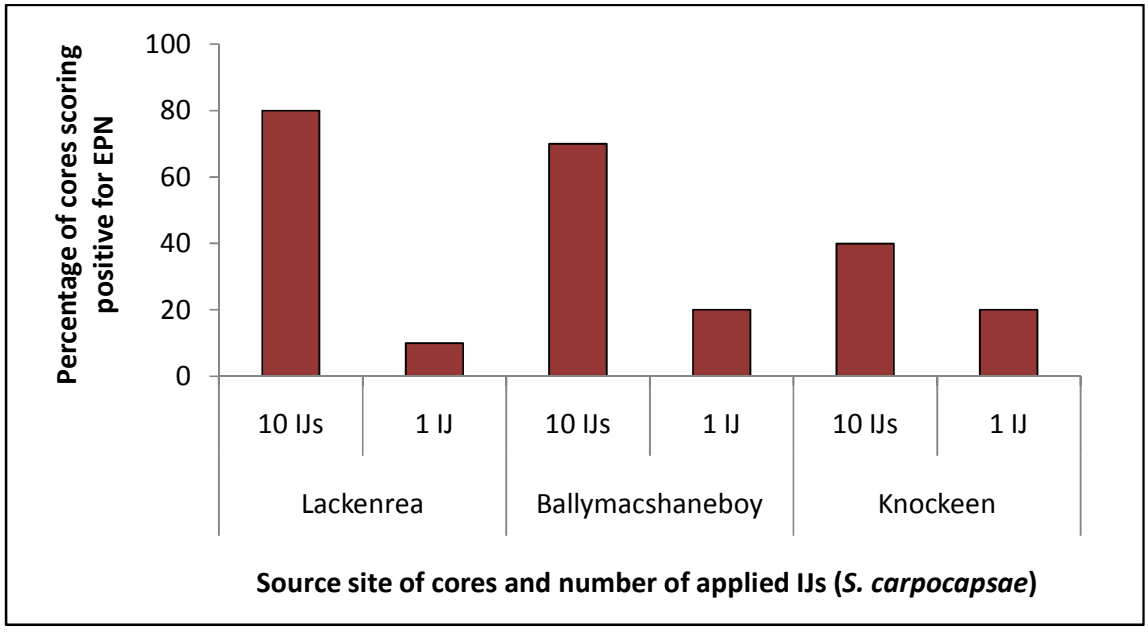


Fig 3.16: Proportion of spiked soil cores sourced from three sites scoring positive for nematode presence as detected by infection of waxmoth larvae. Either 10 IJs or 1 IJ of *S. carpocapsae* were applied to the centre of the core through a small hole melted in its side. N = 10 for each bar.

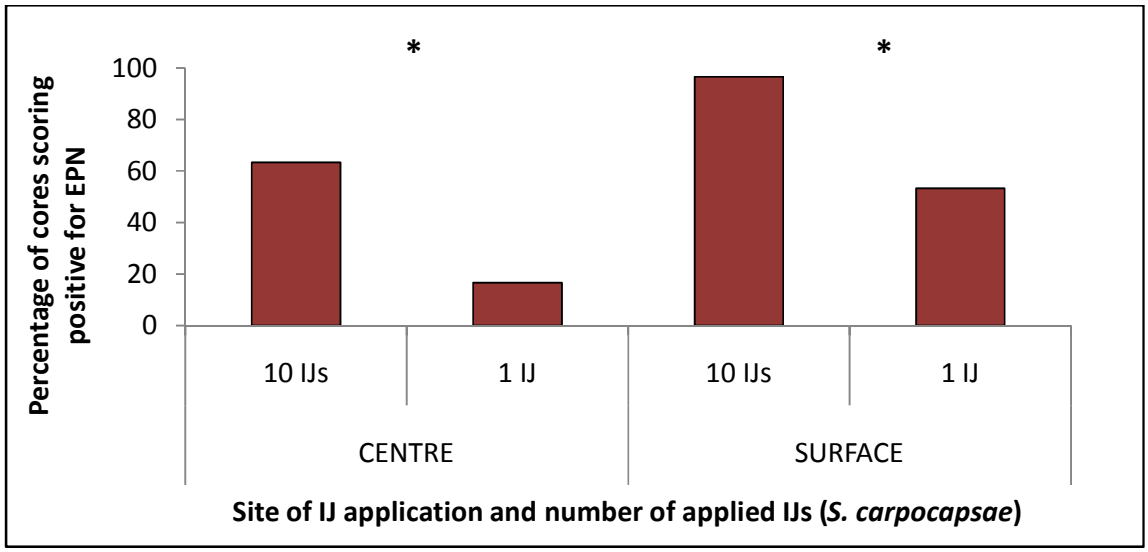


Fig 3.17: Proportion of spiked soil cores scoring positive for nematode presence as detected by infection of waxmoth larvae. Either 10 IJs or 1 IJ of *S. carpocapsae* were applied to surface of the soil sample or to the centre of the core through a small hole melted in its side. N = 30 for each bar. An asterisk above a pair of bars indicates a significant difference between the respective data sets (χ^2 – test, $P < 0.001$).

3.4 Discussion

The data presented in this study demonstrate that with the baiting method used, it was possible to detect single *S. carpocapsae* IJs in spiked soil samples, especially when IJs were applied at the surface of the sample. Fan et al. (1991) have previously been able to demonstrate that two successive baiting periods of six days accounted for 75 % of the total number of nematodes recovered from soil samples when using *G. mellonella* as bait, though less than 40 % of the originally applied number of nematodes was recovered within that period (*Steinernema* spp., spiking doses ≥ 10 IJs). The success rate of the baiting technique used in the present study was not significantly affected by the soil type of a sample (peat or mineral). However, when applying 10 IJs at the centre of the soil core the proportion of cores scoring positive was lowest for cores from Knockeen (peat soil). While the difference to other sites was no significant, a higher number of replicates may well have yielded a significant difference. This is somewhat surprising as Kruitbos et al. (2010) have recently demonstrated that the movement and infectivity of *S. carpocapsae* in peat is good, especially when compared with sand. Dillon et al (2008a) likewise reported good dispersal of *S. carpocapsae* in mesocosm experiments using peat substrate.

During baiting, it was noted that the soil samples taken from Knockeen were of particularly low humidity, consisting of dry and loose peat flakes. This may have adversely affected IJ survival and infectivity in these cores. Koppenhöfer et al. (1995) have found that IJ pathogenicity of *S. carpocapsae* is reduced at soil water potentials above or below an optimal range of approximately -0.1 MPa and -0.01 MPa. At a relative soil humidity of 25 %, *S. carpocapsae* IJs survive for as little as two days and are less infective than at higher humidity levels (Kung et al. 1991). Since the percentage of positive cores sourced from Knockeen was affected only when IJs were applied at the centre, it seems likely that it was the movement of IJs to the host through the soil sample that was impeded and thus responsible for the reduction in EPN detection in spiked cores in relation to the other sites. Soil samples from the other site included in the present study that featured a peat soil (Glendine) were noted to be of much greater moisture content than those collected at Knockeen, often to the point of saturation. The higher water content of samples from this site may have reduced IJ mortality due to desiccation and made movement for IJs easier in those samples, but it may also have caused increased IJ death from hypoxia (Molyneux & Bedding 1984). In soils of hypoxic character, mortality of *S. carpocapsae* IJs can be as high as 70 % within two weeks of storage (Kung et al. 1990b). Soil cores from Ballymacshaneboy, Lackenrea and Kilworth often appeared to contain a considerable amount of clay and sand, resulting in soil texture that is known to benefit survival of IJs, but may reduce the movement and infectivity of *S. carpocapsae* IJs in particluar (Portillo-Aguilar et al. 1999; Kruitbos et al. 2010). Waxmoth larvae used for baiting were almost always infected in cores spiked

with 10 IJs, but in only about half of those spiked with a single IJ. This suggests that IJs in field-collected soil cores which contained only very few or a single nematode may not have been detected in all cases, thus causing persistence to be underestimated overall. However, since *S. carpocapsae* is a dioeceous species, individual IJs may infect a host, but will not be able to reproduce within it (Poinar 1993; Nguyen & Adams 2002). Soil areas with low IJ densities can therefore be considered to represent less of a concern with regards to persistence of nematode populations.

The fact that cores spiked at the surface produced significantly more infected bait insects than those spiked at the centre was not surprising. A portion of the IJs applied at the centre of the core may have died on their way to the waxmoth bait at the surface of the sample, or some IJs simply failed to reach it within the 14 days of baiting. Laboratory studies have shown that as an ambusher species (i.e. the IJs usually do not forage actively in search of a host insect but instead wait for one to pass by), *S. carpocapsae* movement can be very limited and that - depending on soil texture - IJs of the species may only migrate only a few cm over the course of several days (Georgis & Poinar 1983; Schroeder & Beavers 1987). It has been observed that IJs of *S. carpocapsae* tend to migrate upwards in the soil (Schroeder & Beavers 1987). Since cores were incubated in an inverted position with the bait insect at the bottom (this ensured that soil samples were not fragmented and in contact with the bait insect), some IJs may have migrated upward in the sample rather than downward, toward the insect. Attraction of IJs to host movement may have offset this effect to a degree (Torr et al. 2004). It can be assumed, however, that these factors applied in roughly equal measures to samples from all sites and thus would not have affected the relative success of EPN detection.

IJs used for spiking were reared *in vivo* in waxmoth larvae in the laboratory, whereas those detected in soil samples from the field had most likely recycled in pine weevil larvae or some other host on-site or had simply survived since the time of application. This may have had a different effect on IJ survival, mobility and infectivity of these two groups of IJs. However, to what extent this may have occurred cannot be estimated from the present results. While EPN were not detected in all spiked cores, especially when only a single IJ was applied, it can be concluded that the baiting technique used was satisfactory and fulfilled the main requirement of producing results that allowed comparisons between sites and over time. Due to the relatively high recovery rate of EPN from spiked cores containing soil collected at Lackenrea and Ballymacshaneboy, it can also be surmised that EPN persistence on these sites was not likely to be underestimated to any significant degree. Finally, one of the two sites for which soil core characteristics appeared to be least favourable for EPN survival (Glendine) was also the site with the highest proportion of positive soil cores for each time point at which samples were taken. Thus, any underestimation of EPN presence in samples from this site is not expected to have had a significant impact on the overall comparative interpretation of

results. Soil cores from Knockeen, the site with the lowest recorded EPN presence in soil samples, also seemed to be among those with the least favourable conditions for IJ survival and bait insect infection. This will be taken into account when discussing the differences in EPN persistence among sites. When tracing the persistence of EPN over time, the sampling method (sampling the same stumps repeatedly or selecting a new set of stumps each year) did not appear to have a significant effect on results, suggesting that disturbing the soil around tree stumps and removing a portion of the soil along with some of the nematodes it harboured did not affect persistence.

Except for the site at Kilworth, which was sampled only four weeks after application of EPN, the highest rates of persistence, whether expressed as the proportion of soil cores or the proportion of stumps with EPN present, was found at Glendine and Ballymacshaneboy, both sites which featured lodgepole pine stumps, but on different soil types (peat and mineral soil, respectively). Persistence was high five and twelve months after application of EPN, a result matching previous reports from pine stumps manually treated by Dillon et al. (2008a) on two clearfell sites in Ireland. EPN detection around stumps at Glendine and Ballymacshaneboy had, however, dropped to low levels of approximately 30 to 40 % of stumps only two years post-application, one year earlier than a similar drop was found by Dillon et al. (2008a). This difference in persistence may be explained by EPN having less opportunity or less time to reproduce in pine weevil hosts in sites included in the present study.

The more weevils are infected by EPN after application, the higher EPN reproduction is expected to be and the higher the number of IJs in the soil in the following year. However, differences in the timing of EPN application with respect to the felling date or stump species (both of which may affect how many weevils are available for EPN recycling in stumps) are unlikely to account for the difference in persistence between the present study and that by Dillon et al. (2008a), since sites in both studies were felled 16 to 18 months before EPN application and all featured pine stumps (Dillon et al. 2006; Dillon et al. 2008a). Dillon et al. (2007) also have reported that differences in timing of EPN application of least two months (11th of April, 6th of May or 6th of June) did not have a significant effect on the infection rate of pine weevils in tree stumps. However, stumps on sites included in this study were treated in early to mid July (6th of July at Glendine and 20th of July at Lackenrea; Aoife Dillon, personal communication), at least one month later than those included in the study by Dillon et al. (2008a) in which persistence was higher two years after EPN application. While this may not have adversely affected the initial rate of pine weevil infection after application, it may have shortened the period of time in which EPN were able to multiply and infect new hosts (pine weevil or otherwise) before the winter, thus reducing the number of IJs produced overall and thereby also reducing persistence two years after application in this study compared with the published figures

(Dillon et al. 2008a). The timing of sampling at Lackenrea and Glendine in 2007 (November) was such that EPN populations at this time had probably been slightly reduced due to the suboptimal temperatures for their reproduction, movement, infectivity and survival at that time of year (Kaya 1993; Campbell et al. 1995; Brown & Gaugler 1997). This could explain why EPN presence on these two sites increased between the first and second sampling, as the latter occurred in the middle of summer when EPN would have been more likely to have already recycled.

As was predicted, the level of persistence of nematodes on the clearfell sites that were treated with *S. carpocapsae* on a large scale seemed to be influenced primarily by the stump species on the site rather than the soil type. This trend was found when data for all sites two years post-application was combined, but was most striking when the number of weevils that had emerged from untreated stumps on sites in the previous year was regressed against persistence data. Positive soil cores were significantly less abundant around Sitka spruce stumps in 2009 than they were around pine stumps and emergence of adult weevils in 2008 from pine stumps was higher than that for spruce stumps. Even when taking into account that EPN presence in Knockeen soil cores may have been twice as high as recorded (based on core spiking results as discussed above), this would have held true. A strong difference between the two stumps species was also observed in persistence of EPN under bark. The bark represents a more sheltered environment that also resulted in more uniform samples (i.e. similar moisture content and texture among samples from different sites). Just as for the soil samples, persistence under bark was best predicted by stump species and the number of weevils emerging from stumps in 2008. Moreover, in agreement with the predictions made, persistence under the bark per stump was greater than that in soil at Glendine and Ballymacshaneboy. EPN applied for inundative biological control frequently recycle in natural populations of host insects, preferably the target pest, and host density is one of the key factors determining long-term persistence of EPN after inundative application (Kaya 1993; Gaugler 1988; Smits 1996; Susurluk and Ehlers 2008). *S. carpocapsae* can complete its reproductive cycle in *H. abietis* larvae in the field and a single host cadaver can produce more than 85,000 IJs (Pye and Burman 1978; Dillon 2003). In the present study, *H. abietis* infected with EPN at the Kilworth site were found to contain reproducing adult nematodes four weeks after application (author's observation).

Therefore, the most likely explanation for the results presented here appears to be that the nematodes primarily recycled in *H. abietis* individuals under the bark of tree stumps after application and that a portion of the emerging IJs then migrated into the surrounding soil, replenishing the population there. This is also supported by the fact that repeated sampling of stumps had no effect on persistence. Differences in soil characteristics among sites could then affect both IJ persistence and baiting accuracy, which may be why soil type did seem to have a limited effect on persistence

around stumps, but not under the bark. Data on pine weevil emergence and IJ persistence for *S. carpocapsae* on individual clearfell sites has been published previously, but in those studies either only one tree species was investigated or the sampling methods used do not allow direct comparison of results among them (Torr et al. 2007; Dillon et al. 2008a). The connection between weevil abundance in tree stumps as reflected by the number of emerging weevils may also serve to explain why persistence was similarly low around pine and spruce stumps at the only site in this study that contained both: Lackenrea. Weevil emergence on this site was very low for both stump species in 2008 (1.9 weevils on average per pine stumps and 0.5 per spruce stump), indicating that there was little opportunity for EPN to recycle under the bark. In 2007, weevil emergence was higher but still similar for both stump species (29 weevils on average per pine stumps and 16.2 per spruce stump). Weevil numbers in spruce stumps were relatively high on this site compared with others (e.g. 0.5 weevils emerging per stump at Knockeen in 2007) which would result in the persistence around spruce stumps being similar to that around pine stumps on this site.

Weevil numbers in spruce stumps that are interspersed with pine stumps on a clearfell site are expected to be higher than those in monoculture spruce stumps as adult weevils are primarily attracted to clearfell sites by pine stumps, their preferred host (Tunset et al. 1993; Aoife Dillon, personal communication). When comparing emergence from spruce stumps at Lackenrea and Knockeen, emergence in 2008 was low on both sites, with slightly more weevils emerging at Knockeen in that year on average (mean of 0.5 and 1.7, respectively). Weevils emerged from spruce stumps at Lackenrea, however, in numbers far greater than from stumps at Knockeen in 2007 (16.2 vs. 0.5, respectively). Since development of *H. abietis* in spruce takes longer than it does in pine stumps, it could be argued that emergence data collected for the two stumps species after the same period of time post-felling will under-represent *H. abietis* populations in spruce (Bejer-Petersen et al. 1962). The data showed that emergence had peaked in 2007 at Lackenrea, but not Kockeen, where it increased from a mean of 0.5 to a mean of 1.7. This was still low when compared with sites with pine stumps (e.g. 52.9 weevils emerging per stump at Glendine in 2008) and would not be expected to significantly increase further as the time since felling progresses further (Dillon et al. 2008b). Therefore, any distortion derived from asynchronous emergence of weevils from pine and spruce stumps should be minimal.

Soil type also seemed to have a very limited effect on EPN persistence. The proportion of positive cores (but not stumps) from peat sites was significantly higher than that from mineral sites, but this effect was found to be less significant than that of stump species when data was combined across sites. This slightly higher persistence recorded in peat soils may have simply been due to a slight detection bias. As discussed above, *S. carpocapsae* may find it easier to move through a peat soil,

especially if it is suitably moist (Kruitbos et al. 2010). To some extent, underestimation of EPN presence in Knockeen samples due to their dryness should have corrected for this, however.

Even though the connection between EPN in bark and soil samples was not always significant in the present study, the high proportion of stumps at Glendine, Ballymacshaneboy and Kilworth with EPN IJs in their bark – often at densities at or above a hundred IJs per 100 g bark sample as indicated by repeated baiting – does lend support to the hypothesis that EPNs were predominantly recycling through pine weevils within the bark. The fact that spruce bark is generally less porous and much denser than pine bark in the first few years following the cutting of trees (author's observation) may have restricted IJ movement and therefore also the recycling rate of EPN within the bark (Portillo-Aguilar et al. 1999). Infection rates of pine weevil larvae by *S. carpocapsae* in spruce stumps can, however, be as high as in pine stumps in some cases, indicating that the bark characteristics cannot fully account for the difference in persistence observed in the present study (Brixey et al. 2006; Dillon et al. 2006 & 2008a). Interestingly, while the percentage of stumps at Glendine for which EPN were detected in the bark decreased over time in tandem with the proportion of stumps for which EPN were detected in the soil, there was no significant decrease in the number of infected waxmoth larvae per positive bulk bark sample. The number of pine weevils in tree stumps in Ireland peaks one to two years after felling, falling off rapidly thereafter as adult weevils emerge. Numbers of weevils per pine stump may also vary considerably within a site (by up to two orders of magnitude) (Dillon et al. 2007; Griffin et al. 2008; Christopher Williams, personal communication). Together with the bark persistence data collected in the present study, this might indicate that as long as a certain density of *H. abietis* is present in the stump to sustain regular recycling of the nematodes, the population density of the latter remains high. When the number of weevils per stump falls below a threshold level, the nematode population collapses. Thus, the temporally limited nature of weevil populations in tree stumps should provide a natural limit to EPN persistence on clearfell sites if recycling in non-target insects is infrequent.

Connections between the occurrence of EPN recycling after inundative application and host density also have not been investigated in detail, though EPN densities usually decrease rapidly after application (Gaugler 1988; Smits 1996). Local epizootics of EPN are known to occur, but their connection to host density is poorly understood at this point (Peters 1996; Smits 1996). In a turfgrass environment, no connection between *S. carpocapsae* and a prevalent host insect (*Popilla japonica*) was found (Campbell et al. 1995). However, Mráček et al. (2000) have reported that *Steinernema* spp. in the Czech Republic may use aggregations of overwintering sawfly and winter moth to multiply. Since the bark of tree stumps offers a sheltered, buffered and contained environment that generally provides EPN with a large supply of *H. abietis* hosts, persistence in this substrate can be

expected to be much greater than in the surrounding soil, where host density (mostly non-target hosts) may be much lower and nematodes are more exposed to biotic and abiotic stressors. As Begley (1993) and others have pointed out, cryptic habitats such as those occupied by plant-boring insects are perfectly suited for infective juvenile survival and efficacy (Gaugler 1988; Glazer 1992; Begley 1993; Arthurs et al. 2004). Unlike exposed environments, in which conditions are not only more harsh, but in which host finding success may be reduced due to the greater expanses of foraging area to be covered, cryptic environments that provide direct routes to the host (e.g. larval frass galleries) or restrict movement to two dimensions, rather than three (e.g. movement of IJs between bark and bole of stump) should favour EPN recycling and with it, persistence (Moore 1970; Lindegren 1981; Cottrell & Shapiro-Ilan 2006).

Almost no persistence of EPN was found on the sites treated manually (Oakwood and Glendalough), even as early as one year after application (Oakwood). As is discussed in Chapter IV, the identity of the EPN isolates collected at Oakwood could not be confirmed by morphological measurements or molecular methods, though it could be conclusively shown that they did not belong to *S. carpocapsae.* This meant that only three of the soil samples on these sites were positive for EPN species that had been originally applied. Stumps on both of these sites were Sitka spruce. Dillon et al. (2007) monitored pine weevil emergence and population structure of untreated stumps on these sites in the first year after EPN application at Glendalough and in the first two years at Oakwood. Approximately 20 adult weevils emerged on average per stump at Oakwood in year one after application and weevils inside stumps were found to be at more advanced stages of development than they were in spruce stumps on other sites included in that study (Dillon et al. 2008b). Data on weevil emergence from stumps in Glendalough are not given by the authors, but emergence is reported to have been so low as to make monitoring of this parameter unnecessary for year two after application. This corroborates the hypothesis that overall, stump species and not soil type is the most informative predictor for EPN persistence if other factors like timing of application and felling date of stumps are equal (Dillon et al. 2006 & 2007).

Persistence due to the recycling of *S. carpocapsae* in non-target hosts may also have occurred to some degree. The more non-target hosts are affected in this way, the greater the risk of an adverse effect of EPN application on the local ecosystem of the clearfell site (van Lenteren et al. 2003). While it is generally lower than in mature forests, insect diversity in successional or plantation forests, on clearfell sites and on boreal blanket bogs can be high and insects may be abundant in these ecosystems (Sippola et al. 2002; Spitzer & Danks 2005; Niemela & Koivula 2007). For example, Fahy and Gormally (1998) report that carabid beetle diversity on an Irish coniferous clearfell site was lower than in a mature oakwood stand, but that it was higher than in a mature coniferous plantation.

Similarly, Magurran (1985) found that although moth species richness and diversity was lower in coniferous plantation sites compared with relic woodland, the former did harbour a large number of moth species. When non-target beetles were trapped from tree stumps treated with *S. carpocapsae* on Irish clearfell sites (including some of the sites represented in this study), the result gave no indication of EPN application having a significant effect on either beetle abundance or diversity, even when analysis was restricted to wood-associated beetles (Griffin et al. 2008). This data would suggest that the nematodes were not reproducing in non-target hosts to any significant extent, especially when considering that this data encompasses insects that were in close proximity to treated tree stumps, in some cases developing within them (e.g longhorn beetle species *Asemum striatum*). Low incidence of recycling in non-target hosts following inundative biological control is the most common outcome reported in those studies that investigate it (Bathon 1996; Smits 1996).

Persistence of EPN around stumps sampled at Kilworth only one month after EPN application already displayed a clear difference between pine stumps and spruce stumps for both EPN species applied. A sizeable proportion of the IJs detected in soil samples were probably survivors of the originally applied batch, as IJs can survive in soil for several weeks (Poinar & Hom 1986; Kung et al. 1990a; Dillon et al. 2008a). The difference in persistence between the stump species is, however, broadly in line with the hypothesis advanced so far regarding EPN persistence in and around pine and spruce tree stumps. No data on the weevil population on this particular site are available, but a cursory inspection of pine and spruce stumps on the site suggested that weevil numbers were higher in pine stumps and that infection was prevalent in treated stumps (author's observation). Generational time in laboratory hosts such as waxmoth larvae (*G. mellonella*) is around two weeks for *S. carpocapsae* and three weeks for *H. downesi.* In saproxylic larvae of *R. bifasciatum,* a cerambycid beetle also found on coniferous clearfell sites, generation time is much more variable, but usually emergence occurs within three weeks of infection (see Chapter V). Infection of *H. abietis* by applied *S. carpocapsae* and *H. downesi* was found to occur within only four to five days and IJs were seen emerging from some *H. abietis* larvae in the field within four weeks of EPN application (Dillon 2003; Dillon et al. 2006). This means that the four weeks that passed between EPN application and soil sampling at Kilworth would have allowed for at least some recycling of nematodes in *H. abietis*.

At Kilworth, EPN were significantly more likely to be found in soil samples if they were also present in bark samples from the same stump. This may simply reflect a correlation due not to EPN recycling, but rather application accuracy (i.e. some stumps were treated more accurately, thus having higher EPN numbers in soil and bark, while others might have been missed altogether). This would, however, not explain why almost all stumps with EPN in the bark were pine stumps, unless bark porosity and density differences between spruce and pine stumps made for major difference in EPN

penetration of the stump. Substrate texture has been shown to be important for EPN dispersal, survival and infectivity (Georgis & Poinar 1983; Molyneux & Bedding 1984; Portillo-Aguilar et al. 1999; Kruitbos et al. 2010). These differences in bark texture may have affected bulk sample baiting efficacy, but direct baiting in which waxmoth larvae were in direct contact with the bark should have controlled for this and showed similar results to bulk baitings. The high persistence rate at Kilworth was in agreement with persistence rates observed by Dillon et al. (2008a) one month post-application. Just as previously reported, there was no significant difference in persistence between *S. carpocapsae* and *H. downesi* at Kilworth, Glendalough or Oakwood (Dillon et al. 2008a).

Overall, persistence in the soil recorded in the present study were no higher than previously reported and in some cases lower than anticipated (Torr et al. 2007; Dillon et al. 2008a). Though sampling was only conducted up to year two after application, there is no reason to think that the downward trend in persistence that was observed for the sites that were repeatedly sampled will not continue in the subsequent years, as observed by Dillon et al. (2008a). When compared with previous studies in which application of EPN produced *in vivo* was manual, the present results also show that application method (manual or via spray rig) and production method (*in vitro* for most sites in this study compared with *in vivo* for previous studies) does not significantly affect persistence rates (Wright et al. 1993; Brixey et al. 2006; Dillon et al. 2006 & 2007).

As expected, a significant tendency for outward spread from the stump was not observed on any of the sampled sites. The presence of EPN in soil cores seemed to drop exponentially with increasing distance from a stump at Glendine and Ballymacshaneboy. The number of adult nematodes per infected bait insect also decreased with increasing distance from the stump. At Kilworth, only four weeks after application of EPN, the number of positive cores decreased linearly for most stump/nematode combinations. Since Ballymacshaneboy and Glendine featured different soil types, but Kilworth shared soil characteristics with Ballymacshaneboy, it seems unlikely that soil was the decisive factor, even though its texture. Moisture content and pH can affect EPN survival and movement considerably (Kung et al. 1990a & b, 1991; Koppenhöfer et al. 1995; Portillo-Aguilar et al. 1999; Kruitbos et al.2010). It appears more probable that, just as persistence of EPN around stumps was dependent on their reproduction in *H. abietis*, so was their spatial distribution. Early on after application IJs are expected to be more evenly distributed as they migrate in all directions from the point of application (in this case a circle around the stump). Several studies have shown that IJs can disperse rapidly (Poinar & Hom 1986; Downes & Griffin 1996; Dillon et al. 2008a). Movement rates of up to 33 cm per day have been reported in maize plantations, but are generally thought to be much lower. In a study conducted on loamy soil, IJs had traversed a horizontal distance of 46 cm from the point of application after two weeks (Poinar & Hom 1986; Jabbour & Barbercheck 2008). IJs may also

use roots and other substrates in the soil to disperse (Ennis et al. 2010). As their energy reserves decrease, however, IJs may slow their movement or even enter an immobile state (Lewis et al. 1997 & 2006). Many IJs will also die from exposure to the conditions in the field (Gaugler 1988).

In the present study, IJs were found at distances of up to 60 cm from the bole of the stump to which they had been applied. In the absence of suitable hosts, migrated IJs will not be able to establish away from the stump. Therefore, as time progresses (especially during periods of harsh abiotic conditions, for instance during winter frost), EPN presence will be reduced in the soil while EPN sheltered in stumps will be able to survive in greater numbers and subsequently recycle in *H. abietis* hosts the following season. As IJs then gradually migrate into the soil surrounding the stump, IJ numbers close to the stump would constantly be replenished, while numbers away from the stumps would grow at a more gradual rate, due mainly to IJ migration. This is reflected in the results presented here: at Glendine, IJ presence had increased directly at the stump from the first to the second sampling, but had decreased at 20 cm distance to the bole while remaining low at 40 cm and 60 cm. Presence of EPN at distances of 40 cm and 60 cm from the stumps at Kilworth was roughly equal for *H. downesi* and *S. carpocapsae*. Published data suggest that heterorhabditid and steinernematid species show similar vertical and horizontal dispersal in soils (Rovesti et al. 1991; Dillon et al. 2008a).

Whatever the precise mechanisms of dispersal and persistence away from the stump may be, the decrease in EPN presence with increasing distance from the site of application (the stump) as reported here is in agreement with previous findings of studies in which *S. carpocapsae* was applied in other settings (Poinar & Hom 1986; Downes & Griffin 1996; Jabbour & Barbercheck 2008), as well as studies in which *S. carpocapsae* was applied against the large pine weevil (Torr et al. 2007; Dillon et al. 2008a). Since all the stumps on most of the sites included in this study were treated with EPN, it was not possible to assess the spread of EPN from treated to untreated stumps in detail. However, at Oakwood at least one instance of phoresis was found, as *S. carpocapsae* IJs were detected in soil samples collected around a stump that had not been treated with EPN. Dillon et al. (2008a) report that incidence of such dispersal is low. Since only a single one of the 300 cores that were taken from areas between stumps in this study on all of the sites treated on a large scale scored positive for IJs, the same can be assumed in the present scenario.

There was some evidence to suggest that EPN were spreading to adjoining areas from the sites to which they were applied. A low number of bait insects was infected within an off-site sample from Lackenrea. At one location at Glendine, EPN were found at up to 10 m distance from the edge of the site in both years of sampling. The number of infected bait insects in samples from this area was considerably lower in year twohowever, which would indicate that EPN were not establishing at this

location. It should be noted that at Glendine, the site these EPN were found at, was separated from the site by a narrow drainage trench 1.5 m deep, at the bottom of which a stream of water approximately 30 cm wide was running downhill. Though IJs can swim and survive in water, they usually settle quickly and it seems improbable that enough IJs were able to traverse this obstacle to allow reproduction on the far side (Gray & Lissmann 1964; Bathon 1996). Therefore, transport of IJs by a host insect (phoresis) or some other animal seems to be a more probable explanation for their spread in this instance (Timper et al. 1988; Downes & Griffin 1996). Parkman et al. (1993 & 1996) report that following application of *S. scapterisci* to control mole crickets in Florida, infected insects were collected as far as 23 km from the nearest site of application. In field trials, *S. scapterisci* were transported up to 150 m by infected mole crickets within one year. Lacey et al. (1995) have reported dispersal of *S. glaseri* IJs either on the cuticle or within the hemocoel of *Popilla japonica*. Infected beetles contained enough nematodes to allow reproduction in many cases and phoretic dispersal in the field was at least 50 m. Attachment of IJs to adults of *H. abietis* has also been reported in laboratory assays (Kruitbos et al. 2009). The site at Glendine was located on a blanket bog on an exposed hilltop and few flying insects were encountered during soil sampling on the site (author's observation). Beetle diversity can, however, be high on coniferous clearfell sites, including those situated on bogs or heathlands, thus providing ample opportunity for dispersal by carabids and other mobile beetles (Butterfield et al. 1995; Fahy & Gormally 1998; Spitzer & Danks 2005). IJs could also be directly transported from treated tree stumps by emerging saproxylic insects. Most saproxylic beetles developing in tree stumps emerge from the top of the stump, the area that is most exposed and thus unlikely to accommodate a large number of IJs as it is most prone to dessication and UV irradiation (author's observation; Gaugler et al. 1992; Glazer 1992; Shapiro-Ilan et al. 1996). Since, however, only two IJs are required to initiate reproduction in a host, some emerging beetles or other insects may carry sufficient IJ loads from stumps to cause occasional phoretic dispersal of EPN.

An alternative dispersal route for IJs may be provided by other animals frequenting the site, some of which may be more mobile than insects. At Glendine, groups of feral goats, wild deer and rabbits were encountered foraging on the site during soil sampling. Animal trails strewn with fecal pellets could be discerned running between tree stumps and throughout the adjoining peatland. Rabbits were also spotted at Lackenrea. Frogs were frequently encountered on all sites that were sampled. At the Featherbed site, also treated with *S. carpocapsae* but not sampled for persistence, sheep from adjoining farmland were frequently seen foraging on or around the edges of the site. IJs may cling to these animals, either directly on their skin or within dirt particles adhering to their feet. It is not known if any of these routes were taken by the EPN found off-site at Glendine and Lackenrea, but it can be surmised that on sites that are foraged by vertebrates including farm animals, the risk for EPN dispersal within and from the treated area is higher compared with sites with little animal activity.

Overall the results of this study indicate that long-range spread and subsequent off-site establishment of *S. carpocapsae* is not a major risk associated with EPN use against the large pine weevil.

Most commonly, the spread and prolonged persistence of inundatively applied biological control agents is not desired, mainly because it may increase the risk of non-target effects (van Lenteren et al. 2003). If the persistence is, however, spatially restricted to the target location (i.e. the area of greatest target host density), then persistence for an extended period of time may protract the controlling effect and reduce the need for repeated application without simultaneously causing damage to non-target hosts (Kaya 1993; Klein & Georgisi 1992; Smits 1996). The data presented here points toward the conclusion that EPN persistence is limited by the availability of pine weevil larvae as hosts and decreases with time. In some cases where weevil density is low, EPN numbers in and around stumps can drop to undetectable levels within a year of application. Dillon et al. (2008a) found that persistence on high-density sites stocked with pine stumps is not expected to exceed four or five years. The presence and in some cases high abundance of IJs under the bark may provide an inoculum that continuously controls pine weevil populations as long as they are present within stumps. This will, however, be of little use if emergence in the year of replanting the site with new seedlings is not reduced to an extent that will reduce feeding damage by adults on seedlings below an economically acceptable level. Due to the current timing of replanting (two years after felling) and the migration capabilities of weevils, long-term control effects on weevil populations would only be of sufficient impact if biological control of EPN were used on a massive scale incorporating most of the clearfell sites in Ireland (Örlander et al. 1997; Långström & Day 2004). Future studies investigating field persistence of EPN applied against the pine weevil should incorporate the screening for EPN reproduction in target hosts and persistence in bark to shed further light on the interplay between EPN recycling, dispersal, local population dynamics and persistence.

In addition to the results presented here, what little data have been published on EPN persistence on clearfell sites so far is supportive of the notion that EPN species (including exotic ones) are not likely to persist for any extended period of time after application to an environment they are not adapted to (Gaugler 1988; Smits 1996; Georgis et al. 2006; Torr et al. 2007; Dillon et al. 2008a). Though it may seem counter-intuitive, in some scenarios where prolonged persistence is not desirable, especially in cases dealing with pests with time-limited and spatially confined populations like the pine weevil, it may be safer to inundatively apply an exotic species of EPN, the IJs of which are effective at locating, killing and even reproducing within the target host, but less so at surviving when exposed to conditions in the target habitat, rather than using a locally adapted species that presents no significant superiority in finding and killing hosts, but may persist for much longer.

CHAPTER IV

Using restriction fragment length polymorphism to detect hybridisation in *Steinernema feltiae* (Nematoda: Rhabditida) and identify nematodes isolated in the field

4.1 Introduction

When considering inundative biological control, the possible risks associated with the introduced organisms should be taken into account (Ehlers & Hokkanen 1996; van Lenteren et al. 2003; Bigler et al. 2006). Since commercially produced nematodes are usually non-native to the habitat that is being targeted for application, they may compete with and have impacts on any local EPN species present in the area (Millar et al. 2001). When EPN that are to be applied are of the same species as populations in the target habitat, but originate from a separate strain or isolate, one possible risk of the inundative application of EPN is the hybridisation of indigenous and introduced strains (Bathon 1996; van Lenteren et al. 2003; Dillon 2008a). Hybrid strains may have fitness advantages over the indigenous population. It is also possible that the introduced strain will out-compete the local one and ultimately lead to its extinction. Little information on the impact of introduced exotic EPN strains and species is available, though so far results of field trials in which EPN persistence and spread has been monitored that they rarely persist for longer than one or two years (Smits 1996; van Lenteren et al. 2003). When Millar et al. (2001) introduced the exotic EPN species *Steinernema riobrave* into corn field soil populated by *H. bacteriophora* and *S. carpocapsae*, they found that the exotic species suppressed subsequent detection of *H. bacteriophora*, but not *S. carpocapsae*. Neither of the two indigenous species disappeared completely. Gaugler et al. (1997b) reported that a transgenic strain of *H. bacteriophora* that was designed to be more resistant to heat exposure did not persist for any longer than the wild-type when both were applied in a turfgrass habitat.

This problem is exacerbated by the fact that species distinction within the two families containing EPN (*Heterorhabditidae* and *Steinernematidae*) is not always clear. The history of nomenclature and the designation of phylogenetic relationships are complex and the subject of ongoing debate (Hominick et al. 1997; Adams & Nguyen 2002; Spiridonov et al 2004; Nadler et al. 2006). It can be very time consuming to individually examine many dozens of samples and it may also require expensive equipment (e.g. scanning electron microscope) and a great deal of expertise and experience with the morphology of EPN species (Hominick et al. 1997; Adams & Nguyen 2002). For

these reasons, more objective and less labour intensive molecular identification techniques are now used in parallel with or in lieu of classical morphological identification or cross-breeding experiments (Stock et al. 2001; Floyd et al. 2002; Yoshida 2003; Spiridonov et al 2004; Stock et al. 2004).

Restriction Fragment Length Polymorphism (RFLP) is one of the best established and published methods used for genotyping and comparing EPN species and strains (Hominick et al. 1997; Reid et al. 1997; Yoshida 2003). DNA can be extracted from single nematodes or bulk samples of infective juveniles (Nasmith et al. 1996; Hominick et al. 1997; Szalanski et al. 2000). A short segment of DNA (usually around 1 kb in length) is then amplified. Since the target DNA sequence has to be variable enough to allow distinction of different species, but the primers have to be very specific, non-coding sequences couched within highly conserved regions of the genome are the ideal choice for this kind of analysis (Vrain et al 1992; Joyce et al. 1994; Nasmith et al. 1996; Hominick et al. 1997). Nuclear ribosomal DNA (rDNA) codes for the manufacture of ribosomes and is present in multiple tandemly repeated copies within eukaryotic genomes. Within this area of the genome, the highly variable internal transcribed spacer region (ITS) is situated between the conserved 18s and 26s rDNA genes has frequently been used in molecular phylogenetic studies and the comparison of sequence data (Hominick et al. 1997; Jian et al. 1997; Reid et al. 1997; Hazir et al. 2003). Though 'ITS region' is the overall term for this region, it actually consists of two spacer regions (in EPN: ITS1, 243-306 bp and ITS2, 274-375 bp), separated by a short ribosomal gene (5.8s, in EPN: 154-156 bp) (Spiridonov et al. 2004). Specific primers situated in the 18s and 26s genes bracketing this region have been published (Vrain et al. 1992).

Once ITS DNA has been amplified (the exact length of the PCR product varies between species, but is usually around 1 kb in length) it can be subjected to restriction enzyme digest and the digested product can be visualised via agarose gel electrophoresis (Hominick et al. 1997). The resulting banding pattern indicates the number of recognition sites present in the ITS region specific to each restriction enzyme and sequence differences between EPN strains or species are indicated by differing banding patterns. Hominick et al. (1997) have conducted RFLP analysis of ITS samples from most recognised members of the genus Steinernema using a range of 17 different restriction enzymes. They found that a relatively small set of only four restriction enzymes (*Alu* I, *Hinf* I, *Rsa* I, *Dde* I) allowed a differentiation between the species described at the time and recommended these for use in identifying unknown isolates. ITS variability has also been sufficient to detect intraspecific differences between populations of the same EPN species, including *Steinernema feltiae* (Yoshida et al. 2003; Spiridonov et al. 2004).

S. feltiae is one of the nematode species that has been tested in field trials against the large pine weevil in Ireland (Dillon et al. 2006; Dillon et al. 2007). The species was first described by Filipjev in 1934 and is distributed globally, with records on all continents save Antarctica (Hominick 2002). The species appears to be common in Britain and Ireland and has been isolated from a number of habitats in Scotland, England, Northern Ireland and the Republic of Ireland (Blackshaw 1988; Boag et al. 1992; Griffin et al. 1991; Gwynn & Richardson 1996; Dillon 2003). Dillon (2003) reported *S. feltiae* on Irish clearfell sites. One of the isolates, collected on a clearfell site in County Mayo, has been maintained in culture at NUI Maynooth and has been designated *S. feltiae* strain 4cfmo. As part of the trials conducted by Dillon et al. in the subsequent years designed to test the efficacy of various EPN species against the large pine weevil, *S. feltiae* of the commercially produced strain EN02 were applied to tree stumps on clearfell sites. This strain was shown to significantly reduce the number of adult pine weevils emerging from tree stumps (Dillon et al. 2006; Dillon et al. 2007; Dillon et al. 2008a). The question arose as to whether or not the exotic strain EN02 would hybridise with or displace any local populations of *S. feltiae* on clearfell sites to which they were applied, an issue that would have to be resolved if informed predictions were to be made about risks associated with the application of the exotic strain.

It was found that four to five years after application of exotic EPN species to tree stumps, only *S. feltiae* nematodes were present in soil samples collected around tree stumps, indicating that none of the other EPN species (*S. carpocapsae, H. downesi* and *H. megidis*) that had been applied in the trial had displaced the indigenous *S. feltiae* populations on this site. Moreover, when the genotype of these *S. feltiae* isolates was analyzed using Amplified Fragment Length polymorphism (AFLP), with which genotypes can be compared using hundreds of loci across the entire nuclear genome, they were found to be closest to the indigenous strain 4cfmo, regardless of which strain (4cfmo or EN02) or species (*S. feltiae, S. carpocapsae, H.downesi* or *H. megidis*) had been applied to the respective stump (Dillon et al. 2008a). However, only a limited number of samples were available for analysis and since no *S. feltiae* were found on the site when it was sampled before application, hybridisation was most likely not a factor covered by that study.

The original **aim** of this chapter was to test whether the exotic *S. feltiae* strain EN02 hybridizes with the native Irish strain 4cfmo in the field and to investigate the possibility of competition between the commercially produced and indigenous strains of *S. feltiae* and any effects these factors might have on the persistence of the strains themselves and the genetic material introduced by the exotic strain. Based on the results published by Dillon et al. (2008a), it was predicted that hybridization would be infrequent and would not give rise to a hybrid strain that featured the local genetic adaptation of the native strain but contained significant amounts of exotic genetic material originating from the exotic

strain, thus leading to a shift in the local *S. feltiae* gene pool and a possible increase in persistence and spread of nematodes as a consequence.

To test these hypotheses, field trials in which both strains would be applied alone and in mixture to tree stumps harbouring pine weevil larvae were envisioned. Soil samples would then be collected over the following months and the genotype of any isolated *S. feltiae* would be assessed and compared with each of the two parent strains using the AFLP method already used by Dillon et al. (2008a). To conclusively detect hybridisation events and their frequency, it would be necessary to analyse the DNA of individual nematodes as opposed to bulk DNA extracts from IJs, as was done in the previous study.

Unfortunately, largely due to the fact that the AFLP method required a greater starting concentration of DNA than could be extracted from individual nematode samples, it was not possible to initiate these trials as planned. In the process of rectifying the methodological problems, the RFLP method was evaluated as a possible alternative to AFLP in assigning *S. feltiae* individuals to either of the two parent strains or a potential hybrid strain.

Results of these efforts are presented here, as are the results of RFLP analysis of the three unidentified strains isolated from samples collected on clearfell sites during the course of trials presented in Chapter III (see 3.3.1). RFLP results for the unknown isolates were compared against RFLP digests of *S. carpocapsae* and *S. bicornutum* cultured in the laboratory as well as *in silico* digests of published ITS sequences of *S. affine*, *S. kraussei* and *S. bicornutum* (these species were chosen as references based on their morphological similarity to the unknown isolates and a geographic distribution, either of which made them potential candidates for the unknown strains).

4.2 Materials and Methods

4.2.1 Hybridisation of *Steinernema feltiae* strains

To generate hybrid nematodes, final instar waxmoth larvae were exposed to a single IJ each of the two strains examined – *S. feltiae* strain EN02 and *S. feltiae* strain 4cfmo. Filter paper (Whatman No 1) was used to line 40 plastic reaction tubes (0.5 ml each, Eppendorf; Cambridge, UK), each tube with a perforated lid. Each tube received a single IJ from each strain in 25 µl of tap water and a single waxmoth larva (*G. mellonella*) was placed into each tube. The tubes were incubated at 20°C for five days after which time the insects were removed. Of those cadavers displaying colouration consistent with EPN infection, half were incubated for another ten days before being placed on individual White traps prepared in 9 cm Petri dishes. Emerging IJs (F2-4) were collected and stored as described in sections 2.2 and 2.3. These IJs were used to infect *G. mellonella* larvae and F2-4 adult nematodes were then obtained by dissecting cadavers three days after infection. The remaining cadavers were dissected and F1 adults were removed for DNA extraction from individual nematodes.

4.2.2 Preliminary experiments and changes to protocols

The original protocol that was intended for use on the hybrid strains was based on the Amplified Fragment Length Polymorphism (AFLP) method. In this method, the entire genome of an organism is subjected to restriction digest and subsequent amplification with short, unspecific primers, allowing the comparison of different species and strains across multiple loci within the genome. Unfortunately, preliminary experiments on DNA extracts from single hybrid nematodes using this method did not yield sufficient quantities of DNA for analysis after amplification. This was most likely due to the low quantities of DNA present in single nematode extracts. In an effort to optimize the extraction procedure, it was decided to perform PCR on single nematode DNA extracts and make adjustments to the extraction protocol based on PCR yields. The internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) repeat unit was selected for these PCR reactions, as primers and amplification protocols for it have been published (Vrain et al. 1992, Hominick et al. 1997). Initial PCR reactions were not consistent with varying degrees of fidelity and product yields. It was therefore decided to assess the Restriction Fragment Length Polymorphism (RFLP) method as an alternative to AFLP by making use of the ITS DNA obtained via PCR.

4.2.3 Reagents and incubation conditions

All reagents were supplied by Sigma Aldrich (Dublin, Ireland) unless otherwise stated and all lysis and DNA extraction incubations, PCR reactions and enzymatic digests were carried out in an Eppendorf Mastercycler (Eppendorf, Cambridge, UK).

4.2.4 DNA extraction from nematodes

a) Nematodes used for extraction

DNA was extracted from individuals of *S. feltiae* strain 4fcmo, *S. feltiae* strain EN02, their hybrid (4cfmo x EN02) as well as unidentified Glendalough *Steinernema* spp. isolates 6-4, 22-4 and 27-1. Waxmoth larvae (between 10 and 20) were exposed to approximately 3000 IJs of the respective nematode strains applied in 3 ml of tap water in a 9 cm Petri dish lined with three layers of filter paper (Whatman No 1). These dishes were incubated at 20°C for three to five days until adult nematodes were required for DNA extraction. If DNA extraction was not carried out within five days, the cadavers were frozen at -20°C for later use. DNA was also extracted from individuals of *S. carpocapsae* and *S. bicornutum* (strain IRA7 isolated in Iran by Naser Eivasian) and subjected to digestion following PCR (the latter species was chosen as a reference for three reasons: the length of IJs of two of the unknown strains, 6-4 and 27-1, fell within the range published for *S. bicornutum*, specimens of the species have been found in Europe and its spicule morphology also resembled that of these unknown isolates). This would allow a direct comparison of the banding patterns of the unidentified isolates to those of known strains.

b) Mass DNA extraction from infective juveniles

DNA was extracted from >50,000 infective juveniles using the Qiagen DNEasy 50 spin column kit for tissue samples (Qiagen; Crawley, UK). The manufacturer's protocol for DNA extraction from animal tissues was used and DNA from 25 - 100 mg of IJs were extracted with each column. DNA concentration in the elute was determined using a Nanodrop 1000 spectrophotometer (Mason; Dublin, Ireland) and a portion of the eluate was diluted with sterile ddH_20 to 10 ng/µl where DNA concentration in the pure eluate was > 10 ng/µl.

c) DNA extraction from single nematodes

DNA was extracted from single adult females in accordance with the protocol recommended by Hominick et al. (1997). The nematodes were picked from a cadaver dissected in ddH_20 with a scalpel using a platinum wire mounted in the tip of a glass pipette (the wire was fixed in place by breaking off the pipette tip and melting the glass as the wire was inserted into the broken tip). All reagents were sterile and implements used were sterilised between handling each nematode by dipping in 100 % ethanol and subsequent flaming. Each nematode was transferred to a 15 µl drop of lysis buffer (50 mM KCl, 10 mM Tris pH 8.3, 2.5 mM $MgCl_2$, 0.45 % Igepal, 0.45 % Tween 20, 0.01 % gelatine and 60 µg/ml proteinase K) placed on a sterile glass slide. Nematodes were cut up with a scalpel in the drop and transferred from the slide to a sterile plastic 200 µl reaction tube with 10 µl of the lysis

buffer. In some cases, nematodes were placed directly in a 10 µl drop of lysis buffer in a sterile plastic 200 µl reaction tube and broken up with a pipette tip inside the tube.

The tubes were then frozen at -72°C for at least ten minutes, before being incubated at 65°C for 1 h (lid at 65°C), followed by a final incubation at 95°C for 10 minutes to inactivate the proteinase (lid at 95°C). Debris was spun down into a pellet in a Sigma 1-15 microcentrifuge (Sigma Centrifuges; Shropshire, UK) set to 14,000g for 2 min. 5 µl of lysate supernatant was subsequently used as a template for PCR reactions. Lysates were stored at -20°C until use.

4.2.5 PCR protocol

PCR master mixes were prepared in 1.5 ml reaction tubes and reactions were carried out in 200 µl reaction tubes in a total volume of 50 µl. The master mix was prepared on ice and for each template sample reaction ingredients were added according to the recipe presented in Table 4.1. To optimize the PCR reaction, a range of concentrations was tried for each ingredient in separate PCR reactions. These ingredients are highlighted with an asterisk in the table and concentration ranges are indicated. Suppliers for all ingredients are also given.

Table 4.1: PCR reaction ingredients with stock solution concentrations and final concentrations given. Asterisks indicate an ingredient for which a range of final concentrations were used to optimize the reaction. The ranges of these concentrations are given. Note that template DNA amounts given only apply to bulk DNA extracts prepared with the Qiagen DNEasy kit.

Component	Amount added	Final concentration	Supplier (Product)
PCR Buffer (5 x/10 x)	10 µl/5 µl	1 x	Promega /Sigma Aldrich
$MgCl_2$ (25 mM)*	2-8 µl	1-4 mM	Promega/Sigma Aldrich
dNTP Mix (ATGC) 10 mM	1 µl	0.2 mM	Promega/Metabion
Primers (Forward + Reverse) (5mM)*	1-5 µl each	0.1-0.5 µM	MWG Operon
Taq Polymerase (5 u/ µl)	0.25 µl	0.025 u/µl	Promega (GoTaq Flexi)/Sigma Aldrich (Taq polymerase)
ddH_20	19.75 µl/24.75 µl	-	-
TOTAL VOLUME	45 µl	-	-
Template DNA (1 ng /µl-200 ng /µl)*	5 µl	(5 ng-1 µg)	-

A volume of 45 µl of master mix was transferred to each reaction tube and 5 µl of supernatant from single nematode lysates or 5 to 100 ng of mass extraction DNA in were added in 5 µl of elution buffer. For some reactions DNA extracts of *Caenorhabditis briggsae* and *C. elegans* prepared with the DNAEasy Kit were used as a control template (supplied by Prof Ann Burnell of Nematode Genetics at NUI Maynooth).

4.2.6 Primer sequence

Primers used were specific for the ITS region of the rDNA repeat unit as published by Vrain et al. (1992). The primer sequence is specific to the highly conserved ribosomal genes (18S and 26S) flanking the ITS region and is suitable for use on all EPN species. The sequence of forward and reverse primers is given in Table 4.2. Primers were supplied by Eurofins MWG Operon (Ebersberg, Germany). Depending on the EPN species providing the template DNA, the product yielded by these primers is expected to be 1,000 to 1,100bp in length (Hominick et al. 1997).

Table 4.2: Primer sequences used in PCR reactions (Vrain et al. 1992).

Primer	Sequence (5' to 3')
18S (Forward)	TTGATTACGTCCCTGCCCTTT
26S (Reverse)	TTTCACTCGCCGTTACTAAGG

4.2.7 PCR reaction cycle

PCR reactions were carried out according to the program given in Table 4.3. To optimize the PCR reaction, a range of durations and temperatures were programmed for some steps. These are highlighted with an asterisk in the table and the respective ranges are given. The lid was set to 95°C throughout the reaction. PCR products were stored at -20°C if not used directly for gel electrophoresis.

Table4.3: PCR reaction cycles. Steps in the program that were varied are indicated with an asterisk and ranges are given.

Step	**Number of cycles***	**Temperature**	**Duration**
Initial denaturation	1	95°C	10 min
Denaturation	32 – 40	95°C	30 sec
Annealing*		45 - 65°C	60 sec
Elongation		72°C	90 sec
Final elongation	1	72°C	5 min
Hold	1	4°C	Open ended

4.2.8 Gel electrophoresis of PCR samples

A volume of 5 µl of each PCR product was run with 1 µl of 5 x loading buffer (Bioline; London, UK) each on a 1% agarose gel (w/v) (Invitrogen; Carlsbad, USA) for 0.5 h at 10 V/cm. Agarose gels were prepared with 1 x TAE buffer and the gel chamber was filled with 1 x TAE buffer. Gels were pre-stained by adding 10 µl of ethidium bromide (1 mg/1 ml) or 10.000 x Syb*Rsa*fe (Invitrogen) per 100 ml of molten gel. Ladders used for comparison were the mi-1 kb DNA Marker Go and mi-100 bp+ DNA Marker Go provided by Metabion (Martinsried, Germany). The gel was poured when agarose had cooled to a temperature of approximately 60 °C.

4.2.9 Restriction enzyme digest

PCR products were selected for restriction based on amplification success as revealed by gel electrophoresis. Only samples showing a clear and distinct band in the gel corresponding to the full target ITS region (approximately 1 kb in length) were used. Restriction enzymes with corresponding buffers were provided by Fermentas (St. Leon-Rot, Germany). Four enzymes recommended for identification of nematode species and/or strains by Hominick et al. (1997) were used: *Rsa* I, *Hinf* I, *Alu* I, *HpyF3* I (= *Dde* I). The target sequences for these enzymes are given in Table 4.4. An aliquot of 10 µl of PCR product were used for each digestion and added to a 200 µl reaction tube containing 7.5 µl of ddH2O, 2 µl of reaction buffer (10 x) and 0,5 µl of restriction enzyme, resulting in a total reaction volume of 20 µl. Samples were incubated at 37°C for 16 h and digested material was applied to gel electrophoresis directly afterward.

Table 4.4: Target base pair sequences of restriction enzymes used for RFLP analysis of *S. feltiae* strains and their hybrids as well as unidentified Glendalough isolates. ^ = restriction point.

Restriction enzyme	Target sequence
Alu I	5'...A G^C T...3' 3'...T C^G A...5'
Hinf I	5'...G^A N T C...3' 3'...C T N A^G...5'
HpyF3 I (=*Dde* I)	5'...C^T N A G...3' 3'...G A N T^C...5'
Rsa I	5'...G T^A C...3' 3'...C A^T G...5'

4.2.10 Gel electrophoresis of digested samples

All 20 µl of each restriction product was run with 2 µl of 5x loading buffer each on a 3 % agarose gel (w/v) for 1. 5h at 5 V/cm. Agarose gels were prepared with 1 x TAE buffer and the gel chamber was filled with 1 x TAE buffer. Gels were pre-stained by adding 10 µl of ethidium bromide (1 mg/ml) or 10.000 x Syb*Rsa*fe per 100 ml of molten gel. Ladders used for comparison were the mi-1 kb DNA Marker Go and mi-100 bp+ DNA Marker Go provided by Metabion. The gel was poured when agarose had cooled to a temperature of approximately 60 °C. Gels were photographed with a Kodak DC90 ZOOM Camera (Kodak; Hemel Hempstead, UK) and illuminated with a TFX-35M UV transilluminator (Invitrogen) or illuminated and photographed in a Syngene G:Box (Mason). Bands in each gel were detected and visualized using the Syngene GeneTools software package (Mason).

4.2.11 *In silico* restriction of ITS sequences obtained from GenBank

While restriction patterns for most *Steinernema* spp. have been published (e.g. Nguyen et al. 2001 and Hominick et al. 1997), precise data on banding patterns and individual fragment sizes is not available for all species within the genus. Reference data for most species (including *S. carpocapsae*, *S. feltiae*, *S. bicornutum* and *S. intermedium*) were taken from Hominick et al. (1997), Nguyen et al. (2001), Yoshida et al. (2003) and Spiridonov et al. (2004). Reference data for RFLP banding patterns of two additional species, *S. affine* and *S. kraussei,* and the species *S. bicornutum*, for which IJ lengths that have been published matched the size range of IJs of the unidentified Glandalough isolates were obtained by simulating digestion with the four restriction enzymes used. To this end, a complete ITS rDNA sequence published by Spiridonov et al. (2004) for the geographically most relevant isolate (i.e. isolated closest to Ireland) of each of the three species was located in GenBank (http://www.ncbi.nlm.nih.gov/sites/entrez?db=nucleotide) and then subjected to *in silico* digestion with the web-based tool NEBCutter V2.0 (New England Biolabs, http://tools.neb.com/ NEBcutter2/index.php).

4.2.12 Unidentified field isolates

The three EPN isolates from the field site at Glendalough (see 3.3.1) were cultured in *G. mellonella* larvae as described in 2.2 and DNA was extracted from IJs (bulk extraction) as well as adults (individual extraction) of these isolates according to the protocols described for RFLP analysis of *S. feltiae* strains and hybrids. The ITS region of the extracted DNA was amplified and RFLP was performed on the amplified products as described above.

4.3 Results

4.3.1 Optimisation of DNA extraction and ITS amplification

Amplification of the ITS region of the rDNA via PCR from single nematode extracts presented numerous significant obstacles. Initially, protocol reliability was low and amplification success varied markedly from one set of extracts to the next, despite the respective extraction and amplification protocols being identical. When using GoTaq-Polymerase supplied by Promega, the PCR success rate was approximately 10 % for extracts from single adults and only approximately 50 % for bulk extracts of IJs obtained using the DNEasy spin column kit (data not shown). PCR reliability was similarly low for all EPN species or isolates at this stage. Amplification of control samples using *Caenorhabditis briggsae* and *Caenorhabditis elegans* DNA also produced inconsistent results, indicating that the PCR reaction portion of the protocol was inefficient and unreliable. This hypothesis was further supported by the fact that the method of single nematode DNA extraction (cutting with scalpel or mashing with pipette tip) did not affect PCR success rate. Most PCR reactions generated at least some product often including a substantial amount of 'smear', indicating unspecific or incomplete amplification of target DNA or contamination of samples. Typical PCR reaction results at this stage as visualized by gel electrophoresis are presented in Fig 4.1.

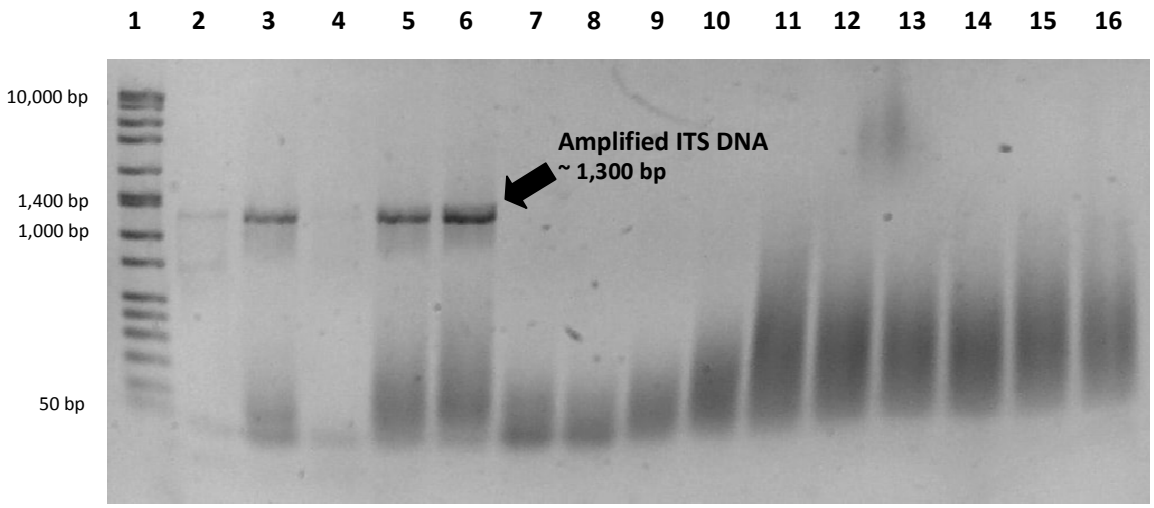


Fig 4.1: PCR results from five control samples (*C. briggsae*, lanes 2 to 6) and single female extracts of *S. feltiae* strain 4cfmo (lanes 7 to 17). Lane 1 contains DNA ladder (Sigma Aldrich DirectLoad™ Wide Range DNA Marker) and fragment lengths of select bands in the ladder aregiven on left in bp. The amplified ITS DNA product is indicated with an arrow in lane 6. ~ = approximately.

Efforts to enhance the fidelity and success rate of the PCR reaction by reducing or increasing the amount of some ingredients (primers, $MgCl_2$) were not successful, nor were changes in the PCR reaction protocol. Specifically, reactions along a gradient of annealling temperatures did not consistently increase specificity of reactions, nor did increasing or decreasing the amount of template DNA. PCR reactions on bulk extract DNA, however, seemed to have a higher success rate than those using template DNA extracted from individual nematodes. Changing suppliers for ingredients did not consistently enhance PCR reactions, with one exception. Once the Taq polymerase used in the reactions was changed from Promega GoTaq Flexi DNA Polymerase to Sigma Taq polymerase, PCR reaction accuracy and reliability increased substantially to approximately 50 – 75 %. Consequently, this polymerase was used for all subsequent reactions. The PCR recipe chosen as the most reliable based on these results is presented in Table 4.5. This recipe was used for all reactions for which results are presented and discussed in this chapter. The reaction steps used for all PCR products for which results are presented and discussed are listed in Table 4.6. An example of a successful PCR reaction using template DNA extracted from single female nematodes is presented in Fig 4.2.

Table 4.5: Optimised PCR reaction recipe with stock solution concentrations and final concentrations. Template DNA amounts given only apply to bulk DNA extracts prepared with the DNEasy kit.

Component	**Amount added**	**Final concentration**	**Supplier (Product)**
PCR Buffer (10 x)	5 µl	1 x	Sigma Aldrich
$MgCl_2$(25 mM)	4 µl	2 mM	Sigma Aldrich
dNTP Mix (ATGC) 10 mM	1 µl	0.2 mM	Metabion
Primers (Forward + Reverse) (5 µM)	5 µl each	0.5 µM	MWG Operon
Taq Polymerase (5 u/µl)	0.25 µl	0.025 u/µl	Sigma Aldrich (Taq polymerase)
ddH_20	24.75 µl	-	-
TOTAL VOLUME	45 µl	-	-
Template DNA (1 ng /µl-200 ng /µl)*	5 µl	(5 ng-1 µg)	-

Table 4.6: Optimised PCR reaction steps.

Step	**Number of cycles**	**Temperature**	**Duration**
Initial denaturation	1	95°C	10 min
Denaturation	40	95°C	30 sec
Annealing		45°C	60 sec
Elongation		72°C	90 sec
Final elongation	1	72°C	5 min
Hold	1	4°C	Open ended

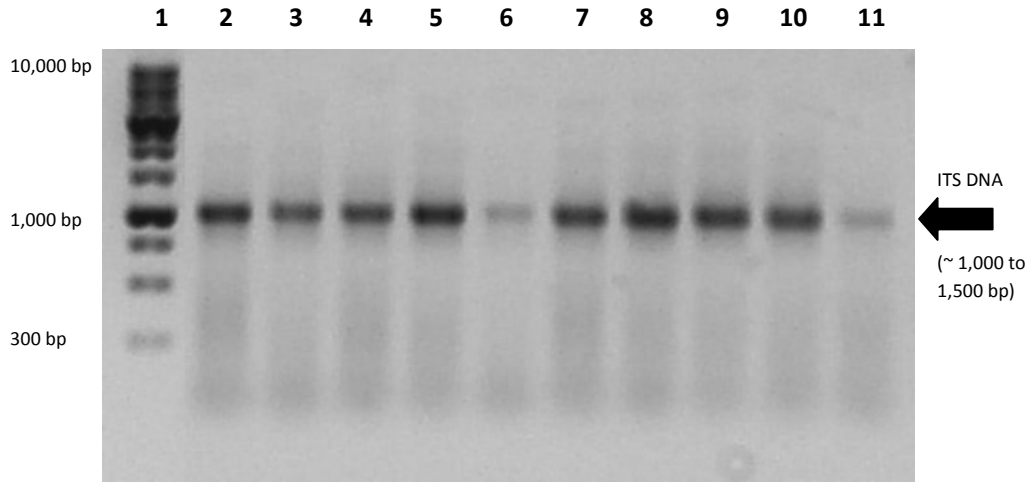


Fig 4.2: PCR results of DNA extracted from ten single adult female nematodes of *S. feltiae* strain 4cfmo (lanes 2 to 11 from left). Lane 1 contains DNA ladder (Metabion mi-1 kb Marker Go), lengths of selected bands of the ladder given on left in bp, the arrow on the right indicates the fragment length of amplified ITS DNA. ~ = approximately.

PCR reactions using the finalized protocol outlined above were not consistently successful for all isolates, however. PCR reactions on single nematode extracts from *S. feltiae* strains EN02 and 4cfmo and their hybrids, but also the unidentified field isolate 22-4 had a higher success rate (approximately 50 to 75 %) than those performed on extracts from field isolates 27-1 or 6-4 (approximately 5 to 10 %).

4.3.2 Hybridisation

Steinernema feltiae strains EN02 and 4cfmo hybridised and produced fertile offspring, but, reproduction occurred in only in a small proportion of *G. mellonella* larvae that had been exposed to an IJ from each strain (approximately 5 %). Whether this was due to low success rates of IJs invading the host, failure to mate or poor reproductive success of the strains was not investigated.

4.3.3 RFLP banding patterns of the ITS region in *S. feltiae* strains 4cfmo, EN02 and their hybrids

Using the optimized PCR protocols (see 4.5), it was possible to amplify the ITS region of at least 20 individual females from each *S. feltiae* strain. These females were taken from *G. mellonella* larvae cadavers infected with IJs from several separate laboratory culture generations of each strain that was investigated (EN02 and 4cfmo), thus representing a broad range of the genetic diversity within the population of the strains that was cultured in the laboratory. Four separate hybrid strains were generated and it was possible to compare the RFLP pattern of the ITS regions of both F1 adults and later generation (F2-4) nematodes to those of the two parent strains. All banding patterns presented here were derived from single nematode DNA extracts, except where stated otherwise.

When scoring for matches in banding patterns (i.e. by comparing fragment lengths among strains or species), it was taken into account that fragment lengths represented the mean of measurements across multiple lanes and sometimes multiple electrophoresis gels and some inaccuracy in the measurement of fragment lengths was to be expected. A match in fragment lengths between strains or species was therefore accepted in all cases in which fragment length was no more than 20 bp apart (this was based on the observation that length differences between fragments from the same strain of *S. feltiae* within the same gel generally ranged from 0 – 15 bp and differences in the mean length of such fragments across multiple gels ranged from 0 – 20 bp, see Table 4.7).

a) Parent strains

Since not all PCR reactions yielded amounts of ITS DNA sufficient to allow full and clear visualisation, it was not possible to derive complete banding patterns from all samples that were subjected to enzymatic digest. Results from the first set of female nematodes for which RFLP was carried out indicated that two of the four restriction enzymes employed to digest the ITS region of the two parent strains *S.feltiae* 4cfmo and *S. feltiae* EN02 produced distinct banding patterns that would allow individual nematodes to be assigned to either strain. These enzymes were *Hinf* I and *HpyF3* I (= *Dde* I). Based on this observation, digestion of ITS DNA from female nematodes of the two parent strains and their hybrids was subsequently restricted to these two enzymes. As the number of RFLP replicates for each strain increased, intra-strain variation was found to be high in both parent strains. Two distinct banding pattern variants resulted for both parent strains when ITS DNA was digested with *Hinf* I and three distinct banding pattern variants were found when digesting strain 4cfmo with *HpyF3* I (= *Dde* I) (one for strain EN02 with this restriction enzyme) (Table 4.7). An example of intraspecific variation for strain EN02 in banding patterns is given in Fig 4.3.

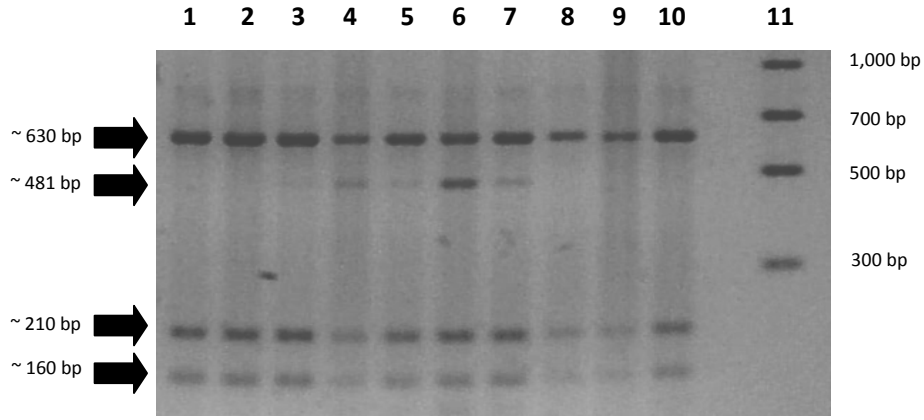


Fig 4.3: Banding patterns of ITS DNA from ***S. feltiae*** **strain EN02** after digestion with restriction enzyme *Hinf* I (lanes 1 to 10). Lane 11 contains a DNA ladder (Sigma Aldrich DirectLoad™ Wide Range DNA Marker), lengths of selected bands of the ladder given on right in bp. Arrows on the right indicate the length of restriction fragments. ~ = approximately.

Restriction with *Hinf* I resulted in a higher number of fragments for each of the two *S. feltiae* strains, with at least three distinct fragments appearing per sample (Table 4.7). Each of the strains produced two different banding patterns when digested with this enzyme. Two of these banding patterns were very similar between the two strains (4cfmo *Hinf* I A and EN02 *Hinf* I B) and both were patterns commonly found in both strains (78 % and 33 % of samples, respectively). The other patterns that were found for the strains when using this enzyme were unique in each case (4cfmo *Hinf* I B and EN02 *Hinf* I A), but only the smallest of all fragments produced by *Hinf* I with a mean size of 123 bp was found exclusively in strain 4cfmo (see 4cfmo *Hinf* I B). The sum of fragment sizes exceeded the expected size of 1000 bp for the full ITS region in three out of the four variants produced by *Hinf* I.

Digestion with enzyme *HpyF3* I (=*Dde* I) yielded three variant banding patterns for strain 4cfmo, while all 16 digestions of ITS DNA from strain EN02 produced the same banding pattern consisting of only two fragments. While the variant banding patterns A and B found for strain 4cfmo were distinct from that of strain EN02, variant C overlapped with it, with both of the fragments found for strain EN02 being present in this variant in addition to the two fragments also represented in variant B (Table 4.7).

b) Hybrids

As expected, ITS region DNA extracted from adult hybrid females that belonged to the F1 generation and was digested with *Hinf* I and *HpyF3* I (= *Dde* I) yielded banding patterns that included the fragments seen in both of the parent strains (Table 4.8). Due to the intra-strain variation in banding patterns described above for the parent strains, these banding patterns were, however, not distinctive from all sampled individuals of at least one of the parent strains. The longest band of the *Hinf* I variant B for the F1 hybrid generation (664 bp) did not appear in either of the parent strains based on the criteria laid out for fragment comparisons (i.e. more than 20 bp difference in length). However, since this fragment was only present in two out of the five F1 hybrids analysed and the length difference between parents and filial generation was slight, this fragment could not be used as a diagnostic band for reliably identifying hybrids.

Though gel electrophoresis results for hybrid nematodes of subsequent generations (F2-4) were poor and only one band was detectable for each restriction enzyme, it appeared that all five of the F2-4 nematodes sampled had reverted to a banding pattern similar to one of the two parent strains. Bands were faint and though the analysis software was unable to detect any additional bands for the F2-4 hybrids, visual examination of the gels and gel photographs did indicate that at least one additional fragment of about 300 bp size was present in F2-4 samples digested with either *Hinf* I or *HpyF3* I (= *Dde* I). Smaller fragments could not be seen or detected. As with the banding patterns

observed in the parent strains, fragment sizes found for hybrids in some cases added up to a total greater than the expected 1000 bp for a full copy of the ITS region.

Table 4.7: Banding patterns of ITS DNA from *S. feltiae* strains 4cfmo and EN02 after digestion with restriction enzymes *Hinf* I and *HpyF3* I (= *Dde* I). All banding pattern variants are shown with images from representative lanes found in gel electrophoresis of digests. An approximate scale of fragment sizes is provided to the left of gel images. Fragments in brackets were not seen in all samples. The sum of the fragment sizes for each enzyme is given, as is the number of samples for which each pattern was found, with numbers in brackets indicating the number of times fragments given in brackets were seen.

Strain	***S.feltiae* strain 4cfmo**					***S.feltiae* strain EN02**		
Restriction enzyme	*Hinf* I	*Hinf* I	*HpyF3* I (=*Dde* I)	*HpyF3* I (=*Dde* I)	*HpyF3* I (=*Dde* I)	*Hinf* I	*Hinf* I	*HpyF3* I (=*Dde* I)
Banding pattern variant	***A***	***B***	***A***	***B***	***C***	***A***	***B***	**-**
1000 bp 600bp 500bp 300bp 200bp								
Fragments in bp (mean length across all samples in which detected)	642 499 225 175	639 494 216 162 123	1092	950 (*64*)	952 677 (*290*) (*69*)	635 214 166	626 481 207 156	662 279
Sum of fragments detected (mean of all samples)	**1514**	**1641**	**1092**	**950 (*1014*)**	**1629 (*1988*)**	**1015**	**1470**	**941**
Number of times observed	18	5	5	12 (*2*)	7 (*1*)	10	5	16

Table 4.8: Banding patterns of ITS DNA from ***S. feltiae*** **hybrids 4cfmo x EN02** after digestion with restriction enzymes *Hinf* I and *HpyF3* I (= *Dde* I). All banding pattern variants are shown with images from representative lanes found in gel electrophoresis of digests. An approximate scale of fragment sizes is provided to the left of gel images. The sum of the fragment sizes for each enzyme is given, as is the number of samples for which each pattern was found. Fragments marked with * were also found in at least one variant of parent strain 4cfmo and those marked with ^ were also found in at least one variant of parent strain EN02.

Hybrid generation	**F1**				**F2-4**	
Restriction enzyme	*Hinf* I	*Hinf* I	*HpyF3* I (=*Dde* I)	*HpyF3* I (=*Dde* I)	*Hinf* I	*HpyF3* I (=*Dde* I)
Banding pattern variant	***A***	***B***	***A***	***B***	**-**	**-**
1000 bp 600 bp 500 bp 300 bp 200 bp						
Fragments in bp (mean length across all samples in which detected) with indication of fragments also present in parent strains (* = 4cfmo, ^ = EN02)	639*^ 495*^ 221*^	664 226*^ 177*^	951* 676*^	965* 694* 287*^ 67*	657	639*^
Sum of fragments detected (mean of all samples)	**1355**	**1067**	**1627**	**2013**	**657**	**639**
Number of times observed	5	2	5	2	5	5

4.3.4 RFLP banding patterns of the ITS region in *Steinernema* spp. isolates from Glendalough

Due to the difficulties with the PCR protocol, only a small proportion of the reactions using template DNA from the *Steinernema* spp. isolates 22-4, 27-1 and 6-4 were successful. Isolates 27-1 and 6-4 presented more problems in the PCR stage than isolate 22-4. In total, it was possible to carry out two

and four complete digests for isolates 27-1 and 6-4, respectively. By contrast, a total of twelve complete digests were possible for isolate 22-4. The results from digests with all four enzymes used (*Hinf* I, *HpyF3* I (=*Dde* I), *Alu* I and *Rsa* I) are presented in Tables 4.10 through 4.12.

The banding patterns produced for isolates 6-4 and 27-1 were almost identical for all enzymes used. This was particularly apparent when digested samples from these two strains were run in the same gel (Fig 4.4). Three of the enzymes used produced multiple fragments in both of the isolates (*HpyF3* I (= *Dde*I), *Alu*I and *Rsa*I) and the sum of fragment sizes exceeded 1000 bp in all cases, though only by 100 - 200 bp. The banding patterns and sum of fragment lengths for both the 27-1 and the 6-4 isolates did not match those of isolate 22-4. Digestion with *Hinf* I produced the most marked difference among the isolates. This enzyme cut the ITS DNA of isolate 22-4 twice, but not at all in either of the other two isolates. *Rsa* I cut the ITS into two fragments for isolates 6-4 and 27-1, whereas for isolate 22-4, three fragments were observed. The only isolate for which any variants in banding patterns for one enzyme were found was isolate 22-4. The enzyme *HpyF3* I (=*Dde*I) failed to cut the ITS DNA in one sample, whereas it cut it twice in the remaining eleven samples.

Steinernema spp. isolate 22-4 did match some of the variants of the Irish strain of *S. feltiae* (4cfmo) for two of the restriction enzymes used (*Hinf* I and *Dde* I). However, none of the fragments found for isolate 22-4 after digestion with *Alu* I and *Rsa* I matched those produced for *S. faltiae* 4cfmo when using these enzymes (Table 4.9).

Table 4.9: Banding patterns of ITS DNA from ***S. feltiae*** **strain 4cfmo** after digestion with restriction enzymes *Alu* I and *Rsa* I (= *Dde* I). Fragment lengths given in bp. N = 2 for each enzyme.

Species	***S. feltiae strain 4cfmo***	
Restriction enzyme	*Alu* I	*Rsa* I
Fragments (mean length across all samples in which detected in bp)	522 331 261	445 281
Sum of fragments detected (mean of all samples in bp)	**1114**	**726**

Table 4.10: Banding patterns of ITS DNA from *Steinernema* spp. **isolate 6-4** originally found in a soil sample collected at Glendalough. Results of digestion with restriction enzymes *Hinf* I, *HpyF3* I (=*Dde* I), *Alu* I and *Rsa* I are presented. An approximate scale of fragment sizes is provided to the left of gel images. The sum of the fragment sizes for each enzyme is given. N = 5 except for *HpyF3* I (= *Dde* I), where N = 4.

Isolate	**Isolate 6-4**			
Restriction enzyme	*Hinf* I	*HpyF3* I (=*Dde* I)	*Alu* I	*Rsa* I
1000 bp 600 bp 500 bp 300 bp 200 bp				
Fragments in bp (mean length across all samples in which detected)	1134	812 240 76	 561 307 161	737 402
Sum of fragments detected in bp (mean of all samples)	**1134**	**1128**	**1029**	**1139**

Table 4.11: Banding patterns of ITS DNA from *Steinernema* spp. **isolate 27-1** originally found in a soil sample collected at Glendalough. Results of digestion with restriction enzymes *Hinf* I, *HpyF3* I (= *Dde* I), *Alu* I and *Rsa* I are presented. An approximate scale of fragment sizes is provided to the left of gel images. All fragments detected are listed. The sum of the fragment sizes for each enzyme is given. N = 3 for each enzyme.

Isolate	**Isolate 27-1**			
Restriction enzyme	*Hinf* I	*HpyF3* I (=*Dde*I)	*Alu* I	*Rsa* I
1000 bp 600 bp 500 bp 300 bp 200 bp				
Fragments in bp (mean length across all samples in which detected)	1145	800 227 69	573 314 157	714 394 (*70*)
Sum of fragments detected in bp (mean of all samples)	**1145**	**1096**	**1044**	**1108** ***(1178)***

Table 4.12: Banding patterns of ITS DNA from *Steinernema* spp. **isolate 22-4** originally found in a soil sample collected at Glendalough. Results of digestion with restriction enzymes *Hinf* I, *HpyF3* I (= *Dde* I), *Alu* I and *Rsa* I are presented. An approximate scale of fragment sizes is provided to the left of gel images. All fragments detected are listed. Fragments given in brackets for *Hinf* I digest were detected in only two samples. The sum of the fragment sizes for each enzyme is given. N = 12 for all enzymes, except for *HpyF3* I (= *Dde* I) variant A, where N = 4, variant B, where N = 8 and variant C, where N = 1. Fragments marked with an asterisk were also present in *S. feltiae* strain 4cfmo (see Table 4.7).

Isolate	***Isolate 22-4***				
Restriction enzyme	*Hinf* I	*HpyF3* I (=*Dde* I)	*HpyF3* I (=*Dde* I)	*Alu* I	*Rsa* I
Variant	-	A	C	-	-
1000 bp 600 bp 500 bp 300 bp 200 bp					
Fragments in bp (mean length across all samples in which detected) (* = also present in *S. feltiae* 4cfmo)	639* 221* 146* (*105*)	789 157 73	1074*	428 290 204	668 206 131
Sum of fragments detected in bp (mean of all samples)	**1006**	**1019**	**1074**	**922**	**1005**

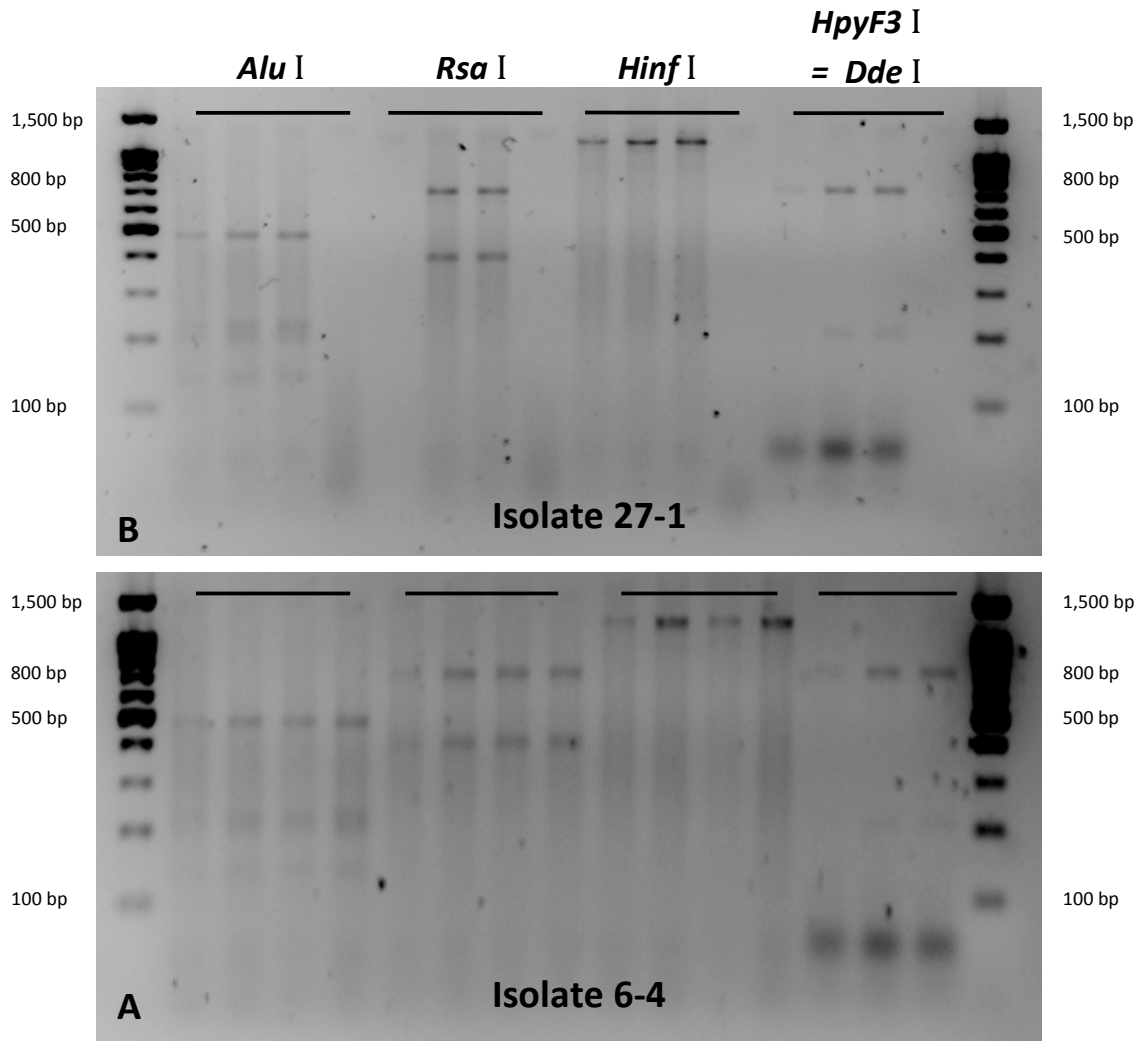


Fig 4.4: Gel electrophoresis of ITS DNA digested with four restriction enzymes. **A**: *Steinernema* spp. **isolate 27-1**. **B**: *Steinernema* spp. **isolate 6-4**. In each image, lanes 1 and 18 (17 for 6-4) contain a 100 bp ladder (Metabion). Lanes 2-5: *Alu* I, Lanes 6-9: *Rsa* I, lanes 10-13: *Hinf* I, lanes 14-17 (14-16 for 6-4): *HpyF3* I (= *Dde* I). Empty lanes indicate that DNA amounts insufficient for visualisation. The length of selected bands of the DNA ladder are given on the left and right sides. For restriction fragment lengths refer to Tables 4.10 and 4.11).

None of the banding patterns for the two *Steinernema* spp. matched that found for *S. feltiae* strain 4cfmo for the four restriction enzymes used (Tables 4.7 and 4.9 though 4.11). The intensity of bands after digestion of ITS DNA from *S. carpocapsae* and *S. bicornutum* was low and only one band was detectable in each of the four digests of *S. bicornutum* samples (ITS product for these species was generated using template from IJ bulk DNA extracts using the DNEasy Kit). Consequently, the sum of fragments did not reach the full length expected for the ITS region in these digests. The results obtained were, however, sufficient to allow a comparison between these species and the Glandalough isolates. None of the isolates matched either *S. carpocapsae* or *S. bicornutum* in their banding patterns (Table 4.13). For each isolate, at least three of the restriction enzymes cut the ITS

DNA at sites different from the ones in these two EPN species as reflected in the length of detectable fragments.

Table 4.13: Fragment lengths for *S. carpocapsae* and *S. bicornutum* after digestion of ITS product. Results of digestion with restriction *Hinf* I, *HpyF3* I (= *Dde* I), *Alu* I and *Rsa* I are presented. All fragments detected are listed. The sum of the fragment sizes for each enzyme is given. N = 2.

Species	***S. carpocapsae***				***S. bicornutum***			
Restriction enzyme	*Hinf* I	*HpyF3* I (=*Dde* I)	*Alu* I	*Rsa* I	*Hinf* I	*HpyF3* I (=*Dde* I)	*Alu* I	*Rsa* I
Fragments in bp (mean length across all samples in which detected) (* = also present in isolate 22-4)	420 312	648 257	460 244 193*	371	422	642	700	815
Sum of fragments detected in bp (mean of all samples)	**732**	**905**	**897**	**371**	**422**	**642**	**700**	**815**

4.3.5 *In silico* restriction of ITS sequences

In silico disgestion of the published ITS DNA sequences of *S. affine* (Accession no. AY230159), *S. kraussei* (Accession no. AY230176) and *S. bicornutum* (Accession no. AY230163) resulted in the banding patterns presented in Table 4.14 (Spiridonov et al., 2004). The *in silico* banding pattern that was found for *S. kraussei* (UK isolate, Nash) was a very close match for isolate 22-4 for three of the four restriction enzymes used (all but *Dd*e I). All but one of the fragments with a length of more than 50 bp that were predicted by the *in silico* digest of the British strain of *S. kraussei* for enzymes Hinf I, Alu I and *Dd*e I were also seen in digests of isolate 22-4, indicating that the restriction sites were located at similar positions along the ITS region of these two *Steinernema* spp (Tables 4.12 and 4.14). Moreover, the total fragment length for isolate 22-4 as reflected by the sum of fragments observed was close to that for *S. kraussei*, especially when taking into account that some of the smaller fragments seen *in silico* (< 50 bp) would not have been detected in the gels that were visualised for the present study. None of the banding patterns for *S. affine* and *S. bicornutum* were consistently good matches for isolates 6-4 and 27-1, however, with only individual bands for some of the restriction enzymes being shared among them (Tables 4.10, 4.11 and 4.14).

Table 4.14: Banding patterns of ITS DNA sequences from *in silico* digests of *S. affine, S. kraussei* and *S. bicornutum*. Accession no. AY230159, AY230176 and AY230163, respectively. Digestion carried out with NEBCutter V2.0 using target sequences of *Hinf* I, *Dde* I, *Alu* I and *Rsa* I. All fragments produced by the simulation are listed. The full length of the published sequence is given. Fragments marked with an * were also detected in isolate 22-4, while those marked with an ^ were also present in isolates 6-4 or 22-7. Fragments in italics would have been too small to be accurately detected by gel electrophoresis and were thus not taken into account when comparing banding patterns.

Species	***S. affine* (AY230159)**				***S. kraussei* (AY230176)**				***S. bicornutum* (AY230163)**			
Restriction enzyme	*Hinf* I	*HpyF3* I (=*Dde* I)	*Alu* I	*Rsa* I	*Hinf* I	*HpyF3* I (=*Dde* I)	*Alu* I	*Rsa* I	*Hinf* I	*HpyF3* I (=*Dde* I)	*Alu* I	*Rsa* I
Fragment lengths in bp (* = also present in isolate 22-4; ^ = also present in isolate 27-1 or 6-4)	863 245	557 196 150^ 86 51^ *49* *19*	480 198 155^ 119 67 62 *20* *7*	692 416^ *22*	658* 219* 148* *10* *9*	937 *49* *34* *19*	429* 278* 197* 102 *33*	663* 200* 139* *23* *14*	378 377 168 62 *31*	676 201 111 *28*	716 177 123	806 208 *2*
Total sequence length in bp	**1108**				**1039**				**1016**			

115

4.4 Discussion

PCR reliability and yield was poor for single-nematode DNA extracts. It was not resolved why this was the case, even after considerable time and effort had been invested in developing a reliable protocol. Attempts to optimise the DNA extraction protocol were confounded by the fact that PCR reactions were not consistent in their product yield or success rate. A given protocol would produce high quantities of relatively pure ITS DNA for one set of DNA extracts, but when the procedure was repeated with a new set of DNA extracts obtained by the same method, yields of the target sequence would be low in quantity or of poor quality. A number of factors are known to inhibit or enhance the stringency and efficiency of PCR reactions, including the magnesium chloride concentration, the concentration of dNTPs and template as well as that of the primers (Kramer and Coen 2006). Super- or suboptimal annealing temperatures are known to decrease specificity of the primers, resulting in lower yields and unspecific amplification, which often appears as a smear in gel electrophoresis of samples (Rychlik et al. 1990). During troubleshooting of the PCR protocol all of these factors were addressed from multiple angles, but to little effect. Since the reactions worked very well in some instances and poorly in others when using the same protocol, it must be concluded that the efficacy of the DNA extraction from individual nematodes was highly variable.

Slight differences in the timing of DNA extraction from single worms may have caused DNA extracts to be of varying quality. Nematodes consist of a genetically predetermined number of cells ('eutely', 959 cells in *C. elegans*), though most species seem to have a variable numbers of cells in the epidermis among individuals (Cunha et al. 1999). If some adult females were sampled at a slightly later stage of their development, thus allowing them to feed and grow for a longer period of time, this may have increased the ratio of lipids, proteins and other compounds to the amount of DNA present in cells. Contamination of DNA samples with lipids, proteins and other remnants of the lysed organism could have reduced the efficacy of PCR reactions (Rådström et al. 2003). While this may account for slight variations in the quantity of amplified DNA and some smear appearing in some samples, it seems unlikely that this would explain the complete failure of multiple sets of reactions, some including dozens of samples. Using DNA extracts from bulk extraction of multiple IJs produced DNA extracts yielded more reliable results, but since hybrid identification necessitated the analysis of DNA from single individuals, bulk extraction did not meet experimental requirements.

Interestingly, once the protocol used for the presented results was established, amplification success differed most strongly between the different unknown *Steinernema* spp. isolates 27-1, 6-4 and 22-4 collected from field sites. Similar to the *S. feltiae* strains used, amplification of ITS DNA from females of isolate 22-4 proved to be comparatively unproblematic, but isolates 6-4 and 27-1 only very rarely

yielded usable quantities of ITS DNA for enzymatic digest, even when using DNA extracted from bulk extraction of IJs (data not shown). These isolates possibly had physiological (e.g. cuticle thickness or cell membrane resilience to lysis) or biochemical properties (e.g. more active DNAses) that resulted in lower yields of DNA from single extraction or poor quality DNA from bulk extraction (bulk extraction DNA was quantified and no consistent difference was found in the total DNA yield from each isolate; data not shown). No mention of difficulties in extracting DNA from particular strains or species of EPN is reported in the pertinent literature and there are no known nematode species for which universal primers such as the ones published by Vrain et al. (1992) failed to work (Hominick et al. 1997; Powers et al. 1997). In the present study, the primers were successfully used to amplify ITS DNA from *C. briggsae* and *C. elegans*.

Due to the methodological problems associated with the PCR reaction and the only intermittent production of sufficient quantities of ITS DNA for analysis, it was not possible to follow a stringent analytical protocol that would have ideally included digestion of ITS DNA with multiple restriction enzymes to facilitate RFLP mapping (assigning a position of each restricition sites within the ITS DNA). Such analysis would also have aided in distinguishing between fragments that resulted from different ITS paralogues. Furthermore, it the interpretation of the results presented as well as the identification of the unknown *Steinernema* spp. would have benefited from having a complete sequence of the ITS region available in each case. No sequence data for *S. feltiae* 4cfmo or EN02 has yet been published. Attempts to clone the ITS region for each of the unknown isolates from single ITS samples to generate sufficient amounts of clean ITS DNA for sequencing were not successful (Gail Maher, unpublished data).

Despite the problems that were encountered, it was not only possible to draw some conclusions about the ITS region of the two *S. feltiae* strains that were investigated and the result of hybridisation between the two strains, but also to make some tentative statements about the identity of the unidentified *Steinernema* spp. isolates.

Two of the four restricition enzymes (*Dde* I and *Hinf* I) recommended for species identification by Hominick et al. (1997) produced different banding patterns in the *S. feltiae* strains investigated in this study. With a substantial proportion of samples from both strains showing different banding patterns for each of the two restriction enzymes (e.g. three distinct patters for *Dde* I digestion of 4cfmo ITS), genetic variation was high within both strains. Intraspecific variation, both genetic and phenotypic, is thought to be common in small soil-bound organisms such as nematodes which – due to patchy distribution and low long-range mobility – may form genetically distinct local populations of differing variability, each adapted to its local environment (McArthur et al. 1988; Harrison et al. 1989; Liu et al.

2000; Stock al. 2000). This need not necessarily be the case, as genetic investigation of globally distributed populations of the nematode *C. elegans* and populations of other nematode species have shown (Hoste et al. 1993; Sivasundar & Hey 2003). Podrucka & Reid (unpublished data) were able to group *S. feltiae* isolates from different regions of Britain based on their genetic variability as detected by RAPD (Random Amplification of Polymorphic DNA), ISSR-PCR (Inter Simple Sequence Repeat) and AFLP methods and found closely clustered populations to be of greater genetic similarity than ones separated by greater geographical distances. When sequences for the ITS region from 24 nominal and 16 putative globally distributed *Steinernema* spp. were sequenced by Spiridonov et al. (2004), intraspecific variation was found to range between 0 % and 3 %. In the phylogenetic trees that were the result of sequence comparison, *S. feltiae* fell within the group of species that showed the highest intraspecific variation (up to 3 % or approximately 33 bp), a group that also included *S. kraussei* (Spiridonov et al. 2004). With this level of intraspecific variation within the ITS region it is not surprising to find divergent RFLP patterns among strains originating from different geographic regions. Only *S. feltiae* isolates from grasslands were included in the study by Spiridonov et al., however, one of which originated from Britain (strain 76), so it cannot be stated with any confidence how strains 4cfmo (isolated from woodland) or EN02 (commercial strain) would have compared.

RFLP analysis is only useful if enzymatic digestion yields distinct banding patterns for each of the genotypes one is trying to distinguish (Nasmith et al. 1996; Hominick et al. 1997). *Hinf* I could not be used as a diagnostic enzyme for assigning a particular nematode to one of the two parent strains, as considerable intrastrain variation for restriction sites was observed for both *S. faltiae* 4cfmo and EN02. About a third of individuals from strain EN02 yielded a banding pattern consisting of four fragments, identical to the one most frequently observed for strain 4cfmo using this enzyme. Enzyme *Dde* I appeared to be a more promising candidate as it produced only one banding pattern for all EN02 samples that were subjected to digestion and this pattern did not match any of the three patterns observed for strain 4cfmo. Unfortunately, some of the 4cfmo samples (approximately 30 %) showed a banding pattern that could not be distinguished from that found for some of the F1 hybrids (also about 30 % of samples). This meant that neither of the two restricition enzymes that produced different banding patterns between the parent strains were reliable in distinguishing F1 hybrids from both parents.

Differences in restriction banding patterns within *S. feltiae* have also been reported in a number of other studies on this nematode. Hominick et al. found that two distinct restriction variants exist for British strains of *S. feltiae* (isolates 76 and 107) for the restriction enzymes *Hinf* I and *Rsa* I (Hominick et al. 1997; Reid et al. 1997). Based on the published gel electrophoresis results as shown in Hominick et al. (1997), the banding patterns of these variants after *Hinf* I digestion, designated A1

and A2, included fragments approximately 620 bp, 220 bp and 160 bp in length (A1) and 480 bp, 200 bp, 130 bp and 100 bp (A2). When compared with the present results for strains 4cfmo and EN02, there was only one complete match with these banding patterns (A2 and EN02 A), but several partial matches were found (A1 and 4cfmo A and B; A1 and EN02 B; A2 and EN02 B). This would indicate that some polymorphism in ITS DNA exists between the Irish *S. feltiae* strain (4cfmo) and the British strains.

The restriction enzyme *Dde* I initially appeared to be the best candidate for distinguishing between the two parent strains of *S. feltiae* (4cfmo and EN02) in the present study. Fragments for both of the British variants A1 and A2 reported by Hominick et al. (1997) for this enzyme are approximately 650 bp and 300 bp. This pattern matched that found for all samples of strain EN02 investigated in this study, but none of those found for the three variants of *Dde* I digestion observed for strain 4cfmo. This and the results from *Hinf* I digestion suggest that Irish strain 4cfmo is distinguishable from British strains of *S. feltiae* using only these two restriction enzymes. Nguyen et al. (2001) published *in silico* restriction fragment patterns for ten *Steinernema* spp. based on their ITS sequence. They only used a single strain per species, so intraspecific variation was not investigated. For the North American *S. feltiae* strain UNK-35 (isolated in South Dakota) they report that three restriction sites are expected for both *Hinf* I (611 bp, 194 bp, 166 bp and 9 bp) and *Dde* I (647 bp, 271 bp, 34 bp and 28 bp). Fragments shorter than 50 bp are usually hard to resolve in gel electrophoresis. Even when taking this into account, these banding patterns do not match either *S. feltiae* strain 4cfmo or EN02 as reported in the present study, indicating that these two enzymes were sufficient to distinguish between a North American and an Irish strain of *S. feltiae* (4cfmo) (Nguyen et al. 2001).

Yoshida et al. (2003) compared banding patterns of the British *S. feltiae* strains A1 and A2 to those of Japanese isolates of *S. feltiae*. They found the same banding pattern differences between the two variants with enzymes *Rsa* I and *Hinf* I as reported by Hominick et al. (1997) and were able to clearly distinguish both variants from all of the Japanese isolates included in the study using the enzymes *Hinf* I and *Dde* I (Hominick et al. 1997; Yoshida 2003). However, Yoshida et al. (2003) found no intraspecific variation among Japanese *S. feltiae* isolates. All Japanese strains yielded only a single fragment of approximately 930 bp when digested with *Dde* I, clearly distinguishing them from the UK variants A1 and A2, both of which showed two fragments after digest with Dde I (Hominick et al. 1997). Based on the results presented here, the Irish strain 4cfmo is a closer match to the Japanese strains than the commercial strain EN02, with variants 4cfmo A and 4cfmo B being complete and partial matches, respectively (Yoshida et al. 2003).

Spiridonov et al. (2004) reported that the different banding patterns they observed in some strains of *S. feltiae* when using enzyme *Dde* I were due to a 10 bp deletion at the single restriction site for *Dde* I present in the ITS regions. A Japanese strain included in that study showed the deletion (though the strain was not represented in the study by Yoshida et al. [2003]). Most of the European strains investigated (e.g. from Russia, Switzerland, Czech Republic) did not show the deletion, including both of the British variants (A1 and A2). Intrastrain variation for the deletion was detected for clones of the Belgian strain *Va*. The authors were also able to show that two Belgian strains of *S. feltiae* differed in this respect, with one featuring the *Dde* I restriction site and the other having lost it due to the 10 bp deletion at this position of the ITS region. When restricted with *Dde* I, the ITS region from these two isolates yielded distinct banding patterns (single fragment of approximately 950 bp length for clone 4 of strain *Va* or two fragments of 650 bp and 300 bp length for strain *N*), each similar to one of the two strains investigated in the present study. Crossing of the two Belgian strains yielded a triple-banded pattern with all three fragments (Spiridonov et al. 2004). The same was observed in the present study in the F1 hybrids of strains 4cfmo and EN02. It seems likely that those individuals of Irish strain 4cfmo that had no restriction site for *Dde* I were of a genotype that featured the deletion described by Spiridonov et al (2004).

The sum of fragments recorded for some of the restriction pattern variants in both *S. feltiae* strains as well as in the unknown isolates 22-7 and 6-4 in this study was considerably greater than what would be expected for the complete ITS region (1000-1100 bp). While some minor divergence from the expected length can be expected due to inaccuracies inherent in determining the fragment length based on DNA ladders, it is possible that the mismatches in at least some cases may be due to incomplete digestion or polymorphism within one individual (ITS DNA is present in multiple copies or 'paralogues' within the genome) (Baldwin et al. 1995; Koch et al. 2003). The evolution of rDNA paralogues has been studied extensively in plants, where it is thought to be concerted, thus reducing the intraindividual variation in rDNA sequences in most genera (Baldwin et al. 1995). In some families, however, divergent paralogues within individuals appear to be common and parent and hybrid strains can feature a number of genetically distinct versions of the rDNA region (Buckler-IV et al. 1997; Koch et al. 2003). Some of the paralogues may be silenced within the individual by methylation, thus constituting pseudogenes that are freed from selection pressures and can evolve at a more rapid pace. In some species, PCR conditions were found to affect which particular rDNA paralogues were amplified in any given reaction (Buckler-IV et al. 1997). If and to what extent these mechanisms apply to the EPN genome is not clear, however. Variation in rDNA or ITS sequences on the species, strain or even individual level appears to be common among some EPN and has been

reported for *S. feltiae* and *S. kraussei* in at least two studies, both of which will be discussed below (Joyce et al. 1994; Hominick et al. 1997; Yoshida 2003; Spiridonov et al. 2004).

When digesting ITS DNA from strain 4cfmo with *Dde* I, some individuals (roughly 50 %) appeared to contain paralogues for both of the sequences (with and without restriction site for *Dde* I), thus resulting in banding patterns that were ambiguous or, in some cases, identical to the F1 hybrid pattern, making a reliable identification of hybrids impossible. Spiridonov et al. (2004) also found that some of the Belgian individuals they investigated (strain Va) contained ITS sequences for both ITS versions (with and without the deletion). When they allowed this strain to hybridise with one that only contained ITS sequences without the deletion, they found that the banding pattern for the resulting hybrids was indistinguishable from the parent strain Va (Spiridonov et al. 2004). These results are in agreement with the observations made for *S. feltiae* strains and the hybrids in the present study. Interestingly, gel photographs presented in Yoshida et al. (2003) suggest that they too found the same intrastrain variation for restrictions with enzyme *Dde* I (i.e. individuals with either two or three fragments after *Dde* I digestion, indicating that these contained ITS paralogues with and without the deletion of the *Dde* I restriction site). Also, restriction of *S. kraussei* with Tru9 I appeared to yield polymorphic banding patterns within one isolate that suggests intraindividual variation among ITS paralogues. The authors do not discuss those apparent findings, however, indicating that they may have been an isolated instance of incomplete ITS digestion (Yoshida et al. 2003). What the published data and the results of the present study suggest is that intraspecific and even intrastrain genetic variability within the ITS DNA of *S. feltiae*, particularly with regard to restriction sites for *Dde* I, is very high compared with other *Steinernema* spp., for which little to no variation in ITS banding patterns has been observed to date (Joyce et al. 1994; Hominick et al. 1997). This finding has confounded any attempts to make confident statements about the identity of *S. feltiae* nematodes (parent or hybrid strain) based on RFLP in the present study.

As the results presented here show, the RFLP pattern for *Dde* I in F1 hybrids represented a combination of the parent genotypes and was thus distinct from some individuals of both parents (though not all, as some individuals of *S. feltiae* strain 4cfmo produced banding patterns that were not distinguishable from F1 hybrids). In the following hybrid generations (F2-4), the banding patterns were, however, indistinguishable from the parent strain EN02. This may simply be due to dilution of some ITS variants within the nematode population as it is cycling through two or more generations in the host cadaver. Also, as discussed earlier, when two distinct paralogues of the rDNA are present in an organism, one of the two may be preferentially amplified during PCR, thus giving the appearance of hybrids in the F1 generation or thereafter being indistinct from one of the parent strains with respect to the ITS sequence (Buckler-IV et al. 1997). Even if other, more expansive methods that

cover more loci within the genome (e.g. RAPD or AFLP) were to be used to identify and trace parental and hybrid strains in the field, extraction of the DNA before nematodes can mate within the bait insect used to extract the nematodes from soil samples would be of vital importance. Once reproduction occurs within a bait insect, it becomes impossible to determine whether the invading individuals were themselves hybrids or members of the originally applied parent strains that had either persisted on the site since application or had recycled in insect hosts on site separately from each other.

RFLP is considered a cheap, high-throughput method for resolving species relationships within EPN genera (Nasmith et al. 1996; Hominick et al. 1997). Yoshida et al. (2003) argue that a small set of only two restriction enzymes (*Hinf* I and *Dde* I) can be sufficient to unambiguously distinguish between strains of *S. feltiae* found on different continents (Yoshida 2003). As the results presented here and as reported by Spiridonov et al. and others demonstrate, intraspecific and even intrastrain variability for the ITS region may in some instances, however, be so high as to make distinctions based on RFLP patterns difficult and in some cases impossible (Hominick et al. 1997; Yoshida 2003; Spiridonov et al. 2004).

With one exception (isolate 22-4 had one variant for *Dde* I), no variants for the unidentified field isolates collected at Glendalough in 2007 were found for any of the four diagnostic restriction enzymes used as per recommendation in Hominick et al. (1997). This is not surpising as the laboratory cultures for each isolate originated from a single soil core taken at the site. This result in a relatively small founder population passing through a genetic bottleneck which would have limited genetic variation in the laboratory cultures (Dillon et al. 2008a). Restriction fragments added up to a total length of around 1000 bp for all isolates, indicating that digestion was complete and only a single paralogue of the ITS region was present in the samples.

Mean IJ length for strain 22-4 was significantly lower than that of laboratory-reared *S. feltiae* (strain 4cfmo) in the first two weeks of emergence, but not in the third (Table 3.2, Chapter III). IJ length for this isolate also matched published ranges and means (Adams & Nguyen 2002; Yoshida 2003). It could therefore not be excluded that this isolate belonged to species *S. feltiae* based on the length of its IJs. Banding patterns for isolate 22-4 matched those obtained for some of the variants of Irish *S.feltiae* strain 4cfmo closely with regards to enzymes *Dde* I and *Hinf* I. Results for *Rsa* I and *Alu* I, however, did not provide a match. Taking into account the high intrastrain and intraspecific genetic variability for *S. feltiae* it is possible that isolate 22-4 represents yet another genetic variant of this species (Reid & Hominick 1992; Hominick et al. 1997; Spiridonov et al. 2004).

When considering the length of the infective juveniles and the global distribution of *Steinernema* species, another possible candidate for the isolate 22-4 was *S. kraussei*. This species has been found in Britain and its IJ length matches that of isolate 22-4 (Gwynn & Richardson 1996; Stock et al. 2000; Adams & Nguyen 2002; Yoshida 2003). Unfortunately, published RFLP banding patterns for British strains of *S. kraussei* were only available for three of the restriction enzymes used (*Alu* I, *Dde* I, *Hinf* I) (Hominick et al. 1997; Reid et al. 1997). Banding patterns of isolate 22-4 and *S. kraussei* for these three enzymes matched those published. Moreover, *in silico* digestion of the ITS sequence published by Spiridonov et al. (2004) for the UK isolate of *S. kraussei* matched the results for isolate 22-4 closely. *Steinernema* spp. isolate 22-4 can therefore be tentatively assigned to *S. kraussei*. This would represent the first record of this species in Ireland (Hominick 2002; Christine Griffin, personal communication).

Since restriction banding patterns for the ITS region were identical for *Steinernema* spp. isolates 6-4 and 22-7 and the length of their IJs was not significantly different for any of the first three weeks of emergence (Table 3.2, Chapter III), it can be safely assumed that these two isolates were of the same species. Based on the observations that, on average, the IJs of these isolates were significantly longer than those of *S. carpocapsae,* the spicule morphology did not match that of *S. carpocapsae* and banding patterns for the four restriction enzymes used did not match either those published for *S. carpocapsae* isolates or those found in the present study, it can be stated with some certainty that these isolates did not represent pockets of EPN that were persisting on this site after being applied to tree stumps (Hominick et al. 1997; Nguyen et al. 2001; Adams & Nguyen 2002;). The mean length and length range of IJs for both of these unidentified strains did fall within the range that was found by Dillon (2003) for IJs of *S. feltiae* that were isolated from clearfell site soil samples and that included strain 4cfmo (approximately 770 µm to 780 µm mean IJ length). However, RFLP results clearly indicated that the isolates did not belong to *S. feltiae* or *S. kraussei* (Hominick et al. 1997; Nguyen et al. 2001; Yoshida 2003; Spiridonov et al. 2004; Stock et al. 2004).

Based on IJ length measurements and biogeography of EPN species, it was thought that these isolates could represent populations of *S. affine*, an EPN species found in Ireland before, though not in a woodland environment (Griffin et al. 1991; Dillon 2003). Infective juveniles, however, not only lacked the refractile tip at their posterior end, a diagnostic morphological characteristic for this species, but RFLP results did not match those found for an *in silico* digest of a published sequence of a British isolate of the species, nor did it match those published in RFLP studies to date (Hominick et al. 1997; Adams & Nguyen 2002; Spiridonov et al. 2004).

It seems highly unlikely that isolates 6-4 and 22-7 represent one of the other species with IJs in a similar size range, either due to differences in spicule morphology (e.g. *S. ceratophorum,* mean IJ

length 706 µm, has a very slender, curved spicule as it was not seen in males of either isolate) or geographic distribution (e.g. *S. monticorum*, mean IJ length 706 µm, has so far only been isolated in Korea) (Nguyen & Adams 2002; Hominick 2002). RFLP patterns did not match those published for any of these species either (Hominick et al. 1997). It must therefore be concluded that these isolates either represent a morphologically and genetically distinct population of a known species such as *S. affine,* or that they constitute a new species within the genus *Steinernema*.

It has been noted that EPN diversity based in Ireland on records available so far seems to be comparatively low (Blackshaw 1988; Griffin et al. 1991; Dillon et al. 2008a). Nematode fauna in Ireland in general is depauperate compared with Britain and Europe (Keith et al. 2009). It can be assumed, however, that the extent of sampling and species characterisation, identification and classification to date has not been exhaustive and that some species still await discovery even in Ireland. Taking this into account, it is possible that isolates 6-4 and 27-1 represent such a new *Steinernema* sp. (Hominick et al. 1996; Nasmith et al. 1996; Hominick et al. 1997; Nadler et al. 2006). Several new species have been characterized in the past decade alone, one (*S. sylvaticum*) isolated from a coniferous woodland environment in Northern Germany (Hazir et al. 2003; Stock et al. 2003; Stock et al. 2004; Sturhan et al. 2005). Final word on the identity of isolates 27-1 and 6-4 must be reserved for when a complete sequence of their ITS region and more extensive morphological data is available for taxonomic placement.

CHAPTER V

Assessing the risk of entomopathogenic nematodes to non-target wood decomposers on clearfell sites

5.1 Introduction

Saproxylic beetles depend on dead or dying wood for their development. They play an important role in the decomposition of such wood and the associated recycling and release of nutrients in forest ecosystems. The larvae of most of these beetles feed and develop to the adult stage within woody debris such as dead standing or fallen trees (Speight 1989; Grove 2002). In many European forests, saproxylic beetles can represent a major component in forest invertebrate communities (Grove 2002). Their diversity is usually greatest in mature forest stands where they facilitate the rapid breakdown of both standing and fallen deadwood (Grove et al. 2002; Jacobs et al. 2007; Dollin et al. 2008; Müller et al. 2008), but can be just as high on young clearfell sites (Sippola et al. 2002). Due to the intensive nature of plantation forestry and its focus on monocultures of coniferous tree species and the resulting isolation of the remaining old-growth forests, many saproxylic beetle species have gone regionally extinct in parts of Western and Central Europe and a high percentage of the remainder are listed as endangered (Speight 1989; Grove 2002; Adlbauer 2003; Buse et al. 2007). The presence of saproxylic beetles has knock-on biodiversity effects by making deadwood more accessible to wood-decomposing fungi and bacteria. Rotting wood can also provide a series of additional benefits, including soil stabilisation, colonisation opportunities for fungi, plants and insects and water storage (Harmon et al. 2004).

Most of the studies that have dealt with the ecological significance and functions of saproxylic beetles have focused on successional and mature forest stands and the effects the management of such stands has on the local saproxylic community. These studies indicate that the removal of deadwood from mature and growing stands of trees reduces species diversity and abundance of associated insects and other organisms (Økland et al. 1996; Siitonen et al. 2000; Siitonen 2001; Similä et al. 2003; Jacobs et al. 2007; Dollin et al. 2008). Investigations of saproxylic beetle communities on clearfell sites indicate that logs and other woody debris that is left behind after felling can be an essential factor in retaining rare species (Kaila et al. 1997; Martikainen 2001; Sippola et al. 2002; Jonsell et al. 2007). In mature stands, species diversity and abundance of saproxylic beetles has been shown to be significantly related to the size of individual deadwood habitats available

(branches < small logs < large logs) (Økland et al. 1996; Grove 2002). However, small pieces of woody debris (1 - 15 cm diameter) can also harbour large numbers and a great range of saproxylic beetles, including species that are endangered (Jonsell et al. 2007). Due to the importance of saproxylic beetles, especially in relation to sustainable forestry management, it has been suggested that woody debris, logs and standing wood be left behind on clearfell sites to provide suitable habitats for this group of beneficial insects (Kaila 1997; Grove 2002).

With more than 25,000 species described worldwide, one of the most important families representing saproxylic beetles is that of the cerambycids (Coleoptera: Cerambycidae) (Twinn & Harding 1999). Representatives of this family are often conspicuous due to their relatively large size and the long antennae most species feature, no doubt the inspiration for the common name 'longhorn beetles' that has been bestowed upon them (Duffy 1953; Bílý & Mehl 1989; Twinn & Harding 1999). Since the larvae of a number of cerambycids feed and develop within live trees, approximately 20 % of species are recognized as pest insects in various European countries (Twinn & Harding 1999). One of the acute examples of a cerambycid pest species on live trees is that of the Asian longhorn beetle (*Anoplophora glabripennis*), a wood-boring pest that was introduced into the USA from China within the last two decades and that is feared to cause severe damage in hardwood trees if it cannot be eradicated (Haak 2003; Fallon et al. 2004). The beetle has not yet invaded Europe, but risk assessment of its potential as a pest across the continent has been conducted in preparation of such an event (MacLeod et al. 2002).

On clearfell sites, two distinct habitats for saproxylic cerambycids can be distinguished. Tree stumps are left behind after felling and represent the main volume of deadwood on clearfell sites. On Irish coniferous clearfell sites two years after felling, only one cerambycid species, *Asemum striatum,* was caught in emergence traps erected over tree stumps (Griffin et al. 2008). This species is uncommon, but widely distributed in Britain, where it has also been found to be mainly associated with freshly cut stumps and logs (Duffy 1953; Twinn & Harding 1999). As stump decomposition progresses over three to four years after trees have been felled, they are colonized by another cerambycid species commonly found on Irish clearfell sites, *Rhagium bifasciatum* (Fabricius, 1775) (author's observation). After clearfelling, this species is mainly found in decomposing logs, branches and other woody debris left behind on clearfell sites. It is a widely distributed across Europe and is considered an endangered species in some localities (Becker 1950; Steiner 1999; Tozlu 2001; Kuś & Kuś 2004), while it is regarded as a potential pest on Oak trees in others (Duffy 1953). *Rhagium bifasciatum* is common in the UK, where it is reported to favour moist, decomposing pine logs and stumps as its larval habitat, though the species is remarkably polyphagous (Duffy 1953; Twinn & Harding 1999). Reports of the species from across Ireland go back over 100 years, indicating that it is well

established and widely distributed on the island (Johnson 1909; Nicholson 1915; Janson & Wyse 1923). The immature stages (five to six larval instars are reported) take two to three years to develop. The adult ecloses within the pupation chamber that is usually located close to the surface of the log (author's observation). Development and eclosion seem to be poorly synchronised and adults that eclose late in the summer (late August and September) will usually spend the winter in their pupation chamber to emerge in the spring (April and early May) of the following year (Duffy 1953; Bílý & Mehl 1989; Twinn & Harding 1999; author's observation).

Plate 5.1: Late instar larva of *R. bifasciatum* within a decomposing log (top), first instar larva (bottom) and adult beetle (right) (not to scale).

In addition to their direct benefits as primary biodegraders, saproxylic beetles are host to a wide range of parasitoid wasps (Hilszczanski et al. 2005; Hedgren 2007). Some of these parasitoids are generalists that also parasitize *H. abietis* larvae (Kenis et al. 2004). Saproxylic beetles also increase the prevalence of predatory beetles on clearfell sites (Johansson et al. 2007). Birds and other vertebrate predators that could be attracted by high densities of invertebrate prey on clearfell sites are often generalists that may target the adult stages of *H. abietis* as well. In general, the greater the biodiversity and abundance of beneficial saproxylic beetles, the more likely it becomes that low-impact, sustainbable forestry management will be achieved (Grove 2002).

The saproxylic beetle community also represents a large pool of non-target hosts available for infection with EPN after these have been inundatively applied as biocontrol agents against *H. abietis*. This has the potential to exacerbate one of several adverse effects associated with inundative biological control. Infective juveniles may be dispersed by phoresis when nematodes are carried on or within a within a non-target host insect, thus facilitating the spread of EPN to areas outside of that originally treated (Downes & Griffin 1996; Kruitbos et al. 2009). Phoretic transport by beetles in the field has been reported to range from 50 m to 150 m from the point of application in individual studies, but may be even greater in some instances (Parkman, 1993; Lacey 1995). The nematodes may also recycle within non-target hosts, thereby releasing new generations of IJs into

the environment and thus increasing the likelihood of EPN persistence and possible long-term establishment on treated sites. These risks are especially relevant with regards to exotic EPN species being introduced for biological control. All of these factors have been identified as key aspects with regards to evaluating the risk posed by inundatively applied biological control agents and should therefore be examined closely when considering EPN as a potential alternative to conventional control methods in any given setting (Gaugler 1988; Smits 1996; van Lenteren et al. 2003; Georgis et al. 2006).

The **aims** of this chapter were to:

- expose the developing stages of the saproxylic non-target longhorn beetle *Rhagium bifasciatum* to different concentrations of EPN in its natural microhabitat (i.e. within segments of decomposing logs) to gauge the potential impact of EPN on *R. bifasciatum* under optimal conditions (laboratory) and those they would encounter when applied in the field. It was predicted that infection of would be lower in a field setting due to additional abiotic and biotic stressors acting on nematodes (Gaugler 1988). Each EPN concentration tested represented a risk scenario associated with large-scale application of EPN to tree stumps to control the large pine weevil (*H. abietis*) and infection was predicted to be greatest at high concentrations similar to those applied around tree stumps to control the pine weevil. Based on the reported performance of *S. carpocapsae* in cryptic wood habitats (e.g. Lindegren et al. 1982; Brixey et al. 2006; Dillon et al. 2006 & 2007) it was expected that this species would be similarly effective at penetrating deadwood and infecting insects within it, despite the reputation of the two species as an ambusher and a cruiser, respectively (Lewis et al. 2006).

- record the number of IJs emerging from *R. bifasciatum* cadavers to allow predictions on the potential this host had for EPN recycling (a factor linked to persistence and spread of EPN). Due to the wide host range of EPN, both *S. carpocapsae* and *H. downesi* were expected to produce viable IJs within *R. bifasciatum* (Peters 1996).

- assess *R. bifasciatum* infection in decomposing logs collected on clearfell sites on which tree stumps had been sprayed with *S. carpocapsae* to control the large pine weevil. This would provide direct information on the impact this biological control measure was having on saproxylic non-target insects following large-scale field application. Impacts were expected to be low, as generally reported for inundative application of EPN previously (Bathon 1996) and restricted to the immediate vicinity of treated tree stumps due to the targeted nature of EPN application (Dowens & Griffin1996; Aoife Dillon, personal communication).

5.2 Materials and Methods

5.2.1 Measurements and characteristics of decomposing logs used

Whenever decomposing logs were collected in the field to assess saproxylic beetle community or infection of *R. bifasciatum* as well as at the end of each exposure experiment in which such logs were exposed to EPN, several measurements were taken for each log. The diameter of each log was measured three times at each end of the log at aspects 120° apart. The length of each log was measured once between the tip at each end to the closest cm (except in Exposure Experiments, where segments were cut to 50 cm length before experiments). The mean of all six diameter measurements and the length of the log were used to calculate the volume of the log ($V = 2 * \pi * (^{d}/_{2})^{2} * l$, where l was the length of the log and d was the mean diameter of the log). All logs used in Exposure Experiments conducted in the laboratory or the field were weighed to the closest 10 g at the time of destructive sampling. Logs were also assigned to nominal or ordinal categories for three additional parameters that were assessed visually prior to destructive sampling of the log (wood decay, porosity and soil contact). Details on how these parameters were determined at what categories they included are given In Appendix A.2.

5.2.2 Assessment of saproxylic beetle community in woody debris on clearfell sites

To examine the composition of saproxylic beetle fauna in woody debris (excluding stumps) on Irish clearfell sites while at the same time recording infection of such beetles with EPN, decomposing logs were collected from two sites on which all stumps had been treated with *S. carpocapsae* eight weeks earlier. The sites were located at Lackenrea and Ballymacshaneboy (for site details see section 2.5) and were sampled in September of 2007. Logs collected were all greater than 1 m in length and betweeb 5 cm - 15 cm in diameter. Only those logs which looked likely to harbour saproxylic beetles based on their appearance were collected. Such logs were typically in advanced stages of decomposition, as evidenced by peeled and shed bark, the outer portion consisting of soft, spongy wood, often with emergence holes scattered across the surface and occasionally also by frass produced by saproxylic insects visible along the outside of logs or at either end. On each site, ten such logs were selected from strips cleared of most logs, branches and brush (cleared strips, or CS) and Lop and Top piles running in between these strips where brash, branches and other wood debris had been piled up after clearfelling (LT, see Fig 5.1). Logs were wrapped in black plastic bags for transport back to the laboratory, where they were stored at 4°C and sampled in random order over the course of the following week. For sampling, each log was cut into two 50 cm segments. Any excess wood left over after cutting was discarded. One segment was placed upright in a large plastic bin (height 80 cm, diameter 50 cm), with a separate bin being used for each of the four site/area

combinations (i.e. log segments from logs collected in the CS at Lackenrea were placed in one bin etc.). Once all ten segments had been placed in the respective bin it was filled with compost (Wastland Horticulture; Dungannon, Ireland) to a height of 45 cm, leaving the top of each segment exposed. The compost was moistened with 2 L of tap water. A ventilation hole was cut into the lid (10 cm by 5 cm) and covered with a layer of wire mesh (pore size 2 mm). The bins were incubated at 20°C for two and a half years with 2 L of water being added every two weeks. Beetles that had emerged from the logs were collected from bins and identified every other month. The other segment of each log was used for destructive sampling to record presence and abundance of wood decomposing insects. Logs were then taken apart with the aid of a chisel and forceps. Logs that were dry and showed little decomposition (decay category 1, see Appendix A.2) were cut into small sections (each approximately 5 cm in length) and the sections were checked for any saproxylic beetles within frass galleries.

All saproxylic beetle larvae, pupae and adults were recorded and those that constituted a major component of the overall community (> 5 %) were identified to species level if possible. The insects were placed in 24 well plates (one individual per well) and incubated at 20°C for one week to record any delayed mortality due to EPN infection. In addition, wood from each of the sampled logs was set aside for baiting with waxmoth larvae (*G. mellonella*) to check for presence of EPN in or on the logs. Three wood samples of approximately 10 g each were taken from different areas of the sampled log. For dry logs that showed little decomposition (decay category 1, see Appendix A.2), sawdust produced by cutting off segments of wood was used. Wood samples were collected in a 9 cm Petri dish each and baited with ten *G. mellonella* larvae before being wrapped in Parafilm® and incubated for three days at 20°C. Six dishes were included in each stack in random order (i.e. samples from different logs in one stack) and dishes with wood samples but without bait insects were placed on each end of a stack. Stacks of Petri dishes were arranged in random order on a plastic tray for incubation. After incubation, bait insects were removed, infection with EPN was recorded based on cadaver appearance and surviving bait insects were transferred to a 9 cm Petri dish (wrapped in Parafilm® and stacked as above with empty Petri dishes at each end of a stack) and incubated for an additional seven days at 20°C to record delayed mortality. This procedure was performed twice on each sample. After it had been determined that *R. bifasciatum* was the main saproxylic insect present in deadwood on Irish clearfell sites (see 5.3.1), all sampling efforts and experiments conducted subsequently were focussed on this species.

5.2.3 Exposure of decomposing logs containing *R. bifasciatum* to EPN: laboratory

In this set of three experiments, IJs of *S. carpocapsae* and *H. downesi* were applied to decomposing logs thought to contain *R. bifasciatum* individuals that were collected in the field. The aim of these

experiments was to determine whether these EPN species infect *R. bifasciatum* sheltered within logs when applied directly to the latter. The selection of IJ concentrations applied to logs in these experiments was based on those recommended for pine weevil control. The high concentration in the experiments (1.8 million IJs) represented roughly half of the concentration being applied to tree stumps (3.5 million IJs). Logs treated with this concentration were intended to simulate a 'worst case scenario' in which a large amount of EPN suspension is sprayed on a piece of deadwood during treatment of tree stumps. The low concentration (18,000 IJs) simulated a small amount of nematode suspension hitting a log, maybe as the result of leakage from a spray nozzle.

a) Laboratory Exposure Experiment I – April 2008 (*S. carpocapsae*)

Logs used for this experiment were collected at Glendalough (see 2.5) and stored at 4°C for one month prior to use. In preparation for the experiment, each log was cut into 50 cm segments. A total of 15 log segments were assigned randomly to each treatment (control and two nematode treatments), with the only restriction that no treatment received more than one segment from the same log. Each segment was placed in an opaque plastic container with a lid (measurements 60 cm by 39 cm, 15.5 cm high; Plate 5.2) on a layer of 6 L of evenly spread compost (resulting depth of compost was approximately three to four cm; Westland Horticulture). The compost was moistened with 1 L of tap water. A shallow trench (approximately 1 cm deep) was created in the compost along the centre of the container and the segment was placed in this trench oriented in such a way that the side of the log that had been facing the soil in the field was facing the compost in the container. The compost was gently padded down along the side of the log segment to ensure that roughly 30 % of the log was in contact with the compost. Treatments were a high concentration of 1.8 million IJs of *S. carpocapsae* or a low concentration of 18,000 IJs, the control group received only tap water.

Plate 5.2: Photographs of log segment in plastic container just prior to EPN application for laboratory exposure experiment (left) and stack of containers with log segments prior to them being transferred to incubation rooms (right).

Nematodes used for the inundative application were cultured in bulk as described in Chapter II (2.2). Nematodes were applied suspended in 250 ml of tap water, with 125 ml being applied evenly to the top of the log along its length. The remaining 125 ml were split into two portions of 62.5 ml which were applied evenly to the compost along each side of the log. Control logs were treated correspondingly with tap water. To confirm that the applied IJs were infective, *R. bifasciatum* larvae from laboratory storage were exposed to 500 IJs. Nematodes were applied in 1 ml of tap water in a 5 cm Petri dish containing 5 g of decomposing wood. The dishes were wrapped with Parafilm® and the larvae checked for infection after five days incubation. Five replicates of ten waxmoth larvae each were also exposed to a concentration of 10 or 100 IJs, respectively, in a 9 cm Petri dish lined with a single layer of filter paper (Whatman no.1). Application volume was 1 ml of tap water and infection was scored after 5 days of incubation at 20°C. Containers with logs were incubated in three different climate rooms, each set to 20°C. Each room received five logs from each treatment that were chosen at random. In each room, containers were selected randomly to be stacked in pairs. The climate rooms were kept dark. Destructive sampling took place over a period of three days for each treatment and logs were sampled in random order, though the same number of logs from each treatment was sampled on each day.

Each of the log segments was destructively sampled with the aid of a saw, chisel and forceps and any *R. bifasciatum* individuals (larval, pupal or adult stage) recovered were checked for EPN infection based on cadaver appearance. Infection was confirmed by dissection of the cadavers. Individuals that were judged to have died of causes other than EPN (bacterial infection, fungal infection etc.) were either excluded from the data set immediately based on appearance (e.g. sporulation bodies on the cadaver indicating fungal infection), or were incubated in a 24 well plate at 20°C for five days and then dissected to confirm cause of death. All implements and tools used to destructively sample logs were wiped clean with 70 % ethanol in between logs to prevent cross – contamination. All insects found alive were incubated in 24 well plates for an additional week at 20°C to record delayed mortality. Several measurements and location parameters were recorded for all *R. bifasciatum* individuals that were recovered. Any longhorn beetle larvae found were measured in length to the closest 1 mm (pupae and adults were not measured for length) and their position within the log was recorded in two ways. First, it was determined whether the insect was located in the half of the log that had been facing toward the compost during the experiment, or if it was in the central part of the log (the latter was considered to be the cylinder of wood occupying the central 25 % of each log) (Fig 5.2). Second, the depth within the log at which the insect was located was noted by measuring the shortest distance from the insect to the surface of the log. Thus, wherever possible, each insect was assigned to a log region with respect to the compost (facing, facing away or centre) and a distance to the outer surface of the log (Fig 5.1).

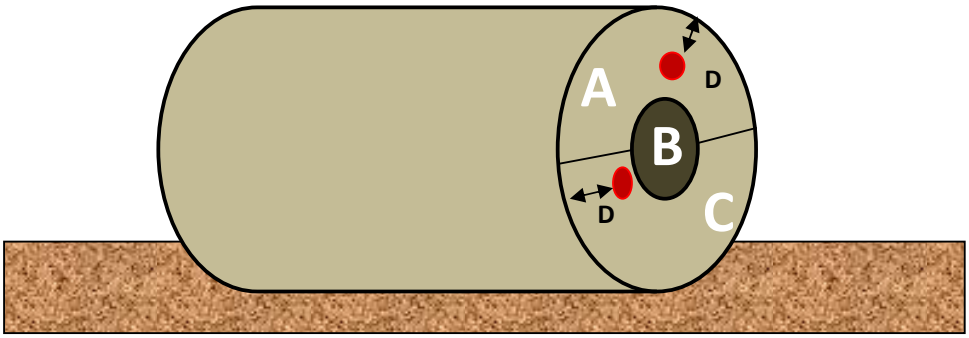


Fig 5.1: Schematic view of measurements taken for *R. bifasciatum* individuals found in logs sampled throughout this study. **A** = portion of the log facing away from the compost, **B** = central cylinder comprising approximately 25 % of log volume, **C** = portion of the log facing the compost. **D** = depth in wood, which was measured from each individual (red dot) to the surface of the log along the shortest direct line.

To screen for EPN presence inside the log segments, wood samples were taken during sampling. Portions of 10 g of wood were collected in 9 cm Petri dishes from three sections of each log segment (left, middle and right section of log, each encompassing approximately 33 % of the total segment length) and from three regions within each section: from the surface of the log to a depth of approximately 10 % of log radius ('Surface'), from between 10 % to 45 % log radius ('Outer') and from the centre of the log (> 45 % log radius, 'Inner') (Fig 5.2). This procedure yielded nine 10 g wood samples from each log segment (a 'Surface', 'Outer' and 'Inner' region sample each for the left, middle and right sections of the log segment). These samples were baited with five *G. mellonella* larvae each and incubated as described for wood samples from logs collected at Lackenrea and Ballymacshaneboy (5.2.2), except that this procedure was repeated until no further infection of bait insects was recorded. At each baiting, any surviving *G. mellonella* larvae from a dish were placed in the wells of a 24 well plate and incubated for an additional 5 days, after which time any cases of delayed mortality were recorded.

The number of IJs that were present in each of the 10 g wood samples at the end of the experiment was estimated based on standard curves that related the number of dead bait insects to the number of IJs in a wood sample. Two standard curves each were generated for *S. carpocapsae* and *H. downesi* by spiking 10 g wood samples with a range of IJ concentrations (5 IJs to 7200 IJs). The methods and results for these are given in Appendix (A.3).

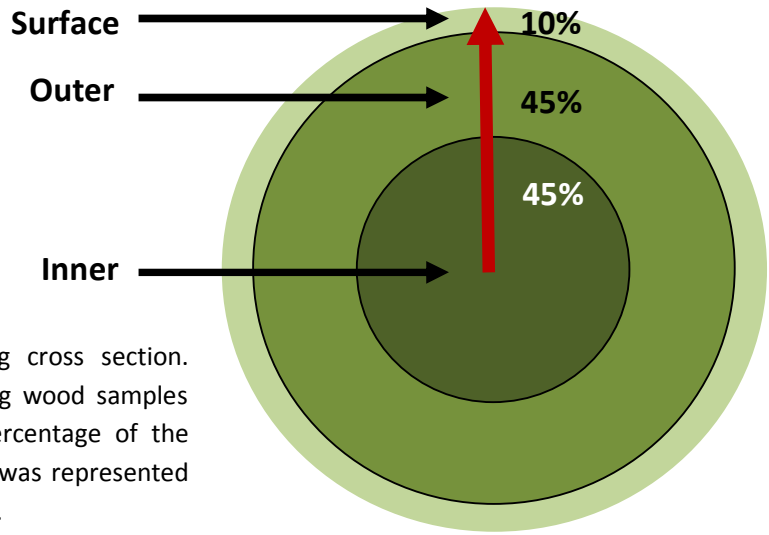


Fig 5.2: Schematic view of a log cross section. Regions from which individual 10 g wood samples were taken are indicated. The percentage of the radius (red) of a log segment that was represented by each sampled region is indicated.

b) Laboratory Exposure Experiment II – March 2009 (*H. downesi*)

Logs for this experiment were collected at Featherbed from an area in which stumps had not been treated with EPN (see section 2.5). No control was included in this experiment since no control mortality had occurred in the first experiment and infected *R. bifasciatum* individuals could easily be distinguished from healthy ones based on cadaver colouration. Fifteen log segments each were included in treatments receiving 1.8 million IJs of *H. downesi* per log or 18,000 IJs per log. All other experimental conditions and protocols were identical to those described for Laboratory Exposure Experiment I (5.2.3a).

c) Laboratory Exposure Experiment III – May 2009 (*S. carpocapsae* + *H. downesi*)

This experiment was carried out to allow a direct comparison of the effects of the two EPN species included in the previous two experiments with respect to *R. bifasciatum*. All methods used were the same as those described for Laboratory Exposure Experiment II (5.2.3b), except that only seven logs were included in each treatment. Treatments were 18,000 IJs of either *H. downesi* or *S. carpocapsae*. Incubation of logs was carried out in only one climate room.

5.2.4 Exposure of decomposing logs containing *R. bifasciatum* to EPN: field

These experiments were designed to replicate the experiments conducted in the laboratory (5.2.3) in a field situation. Two field experiments were carried out: In Field Exposure Experiment I, 50 cm log at Featherbed were treated with three different concentrations of *S. carpocapsae* (1.8 million IJs, 18,000 IJs and an intermediate dose of 180,000 IJs). In Field Exposure Experiment II, 50 cm log segments on an untreated site at Kildalkey were exposed to *S. carpocapsae or H. downesi* at two concentrations each (1.8 million IJs and 18,000 IJs).

a) Field Exposure Experiment I – August 2008 (*S. carpocapsae*)

This experiment was conducted in late August 2008 at Featherbed (stumps on site treated with EPN eight weeks previously, see 2.5). Logs with a diameter between 5 and 15 cm were selected and cut into 50 cm segments on site one week before the experiment. Only logs that were judged to contain *R. bifasciatum* were selected (based on decomposition of wood, moisture content etc.) and logs situated within 1 m distance from an EPN treated tree stump were excluded. Logs segments were moved no more than 5 m from the location they were found before being treated with EPN and were spaced at least 10 m apart. The log segments were distributed across the entire site and randomly assigned to treatments. To replicate conditions of the laboratory exposure experiments, a shallow trench was created in the soil for each log segment so that approximately 30 % of the log was in contact with it, making sure that the original orientation of the log to the soil was maintained.

Three treatments encompassing ten 50 cm log segments each were included and were treated with IJ concentrations of 1.8 million, 180,000 and 18,000, respectively (*S. carpocapsae*). Nematodes for this experiment were cultured according to the bulk method described in section 2.2 and transported to the field in 5 L plastic bottles. IJs were applied to the logs in the same fashion described for the laboratory experiments described above. Logs were collected from the field after 14 days and transported to the lab, each in a separate plastic bag. The wood baiting protocol was as described above for laboratory experiments, but in addition to the wood samples taken from each region, one sample of frass was collected from each section of the log segment (left, middle and right). Logs were stored at 4°C after being returned to the laboratory and sampled in random order over the subsequent three day period (an equal number of logs from each treatment were sampled each day).

b) Field Exposure Experiment II – September 2009 (*S. carpocapsae* + *H. downesi*)

The second experiment was carried out at Kildalkey in early September of 2009. In this experiment, log segments were exposed to *S. carpocapsae* or *H. downesi*. Log segments (50 cm each) for this experiment were cut from logs collected on three sites (Kildalkey: 23 segments, Featherbed: 8 segments, Clonkeen: 5 segments). These segments were randomly assigned to one of four treatments, with no treatment receiving more than one segment from any given log. Two treatments with ten segments each being treated with a concentration of 1.8 million IJs of *S. carpocapsae* or *H. downesi* and two treatments with eight segments each being treated with a concentration of 18,000 IJs of *S. carpocapsae* or *H. downesi* were included in this experiment. Logs for this experiment were arranged in randomized blocks within a 10 m by 100 m area, with each block containing a segment from each treatment and segments within each block being randomly arranged. All

segments within a block were at least 1 m apart and blocks were at least 3 m apart. All other protocols were identical to those described for Field Exposure Experiment I (5.2.4a).

5.2.5 Assessing the emergence of IJs from *R. bifasciatum* that were infected with EPN within decomposing logs in Laboratory Exposure Experiments

Reproduction in non-target hosts may lead to prolonged persistence and increase the impact of EPN used for biological control. It was therefore assessed to what extent *S. carpocapsae* and *H. downesi* are able to reproduce in *R. bifasciatum.* In total, 52 *R. bifasciatum* larvae infected inside logs to which a high concentration of *S. carpocapsae* had been applied in Laboratory Exposure Experiment I (5.2.3a) were placed on White traps. The same was done for 33 larvae and seven adults from high concentration logs as well as 20 larvae and 14 adults from low concentration logs treated with *H. downesi* from Laboratory Exposure Experiment II (5.2.3b). All of these cadavers were incubated at 20°C for five days before being placed on individual White traps prepared in 9 cm Petri dishes according to the protocol outlined in Kay and Stock (1997).

For comparison, 20 *G. mellonella* larvae and 20 *R. bifasciatum* larvae were weighed and each exposed to 500 IJs of *S. carpocapsae* applied in 500 µl of tap water suspension to 5 cm Petri dishes lined with two pieces of filter paper (Whatman No 1). Ten adult *R. bifasciatum* was weighed and then infected in 3.5 cm diameter Petri dishes that contained no filter paper. The IJ suspension was instead pipetted onto a small wad of cotton wool that was placed in each dish. The dishes were wrapped with Parafilm® and incubated for five days at 20° C. Infected insects were incubated for an additional five days before being placed on individual White traps.

White traps were checked for emergence every two days and the day of first emergence was recorded. Seven days after first emergence and each week thereafter, IJs were removed from the White trap for a total time period of eight weeks. Emerging IJs were counted as described in section 2.4.2a. After eight weeks in the White trap, each cadaver was dissected (where possible) and the number of live and dead nematodes remaining within it was determined (2.4.2).

5.2.6 Field sampling of logs for *Rhagium bifasciatum* on treated sites

In addition to the logs collected on the clearfell sites at Lackenrea and Ballymacshaneboy in September of 2007, logs were also collected from sites at Kilworth, Deerpark, Featherbed and Raheenkyle (Table 5.1). Tree stumps on all of these sites had been inundatively treated with EPN species *S. carpocapsae* (3.5 million IJs per stump) no more than one year prior. Some stumps at Kilworth had been treated with *H. downesi* instead of *S. carpocapsae*.

In addition to taking length and diameter measurements (5.2.1), the distance from the log to the nearest tree stump that had been treated with EPN was measured for each log that was collected on these sites and an effort was made to collect roughly equal numbers of logs situated 'close' to a treated stump (distance < 50 cm) and logs situated 'away' from treated stumps (distance > 50 cm) on each site. Distance was measured from the point of the log closest to a treated stump. The side of the log that was facing the soil was also marked. The length of each log as well as the area of the log in contact with the soil (as percentage of total log surface area) was recorded. Logs were placed in a plastic bag for transportation to the laboratory and stored at 4°C and sampled over the course of a week in random order (see below). The only exceptions were logs at Deerpark, which were sampled on-site.

Table 5.1: List of clearfell sites (see section 2.5) at which logs were collected or sampled to record infection of *R. bifasciatum* with EPN. The time elapsed since application of EPN to tree stumps on each site as well as the applied EPN species are given.

Site	*Time since EPN application*	*EPN species applied*	*Number of logs sampled*
Kilworth	4 weeks	*S.carpocapsae*	12
		H.downesi	4
Featherbed	4 weeks	*S.carpocapsae*	15
	1 year	*S.carpocapsae*	15
Raheenkyle	4 weeks	*S.carpocapsae*	15
Deerpark	1 year	*S.carpocapsae*	16
Lackenrea	8 weeks	*S.carpocapsae*	20
Ballymacshaneboy	8 weeks	*S.carpocapsae*	20

Ten samples of soil were taken from the area directly under each log. These were pooled in a resealable plastic bag for transport to the laboratory, resulting in a bulk sample of approximately 100 g. Soil samples were stored at 4°C for one week and then transferred into a 100 ml plastic tub (8.5 cm diameter; Econo by Huhtamaki). Ten waxmoth larvae were placed in each tub. Tubs were then closed with a perforated lid and arranged in random order on a plastic tray in stacks of three (four by three grid layout). They were then incubated for seven days at 20°C after which time the insects were replaced and infection was recorded (surviving insects were incubated in Petri dishes as described above in section 5.2.2). The baiting was repeated once. No soil samples were collected for logs from Lackenrea or Ballymacshaneboy.

Collected logs from each site were transported to the laboratory in plastic bags, stored at 4°C and sampled over the course of a week. At Deerpark all sampling took place on-site. The number of infected *R. bifasciatum* larvae, pupae and adults was recorded in the field and insects were transported to the laboratory where infection was confirmed by dissection. Live insects were incubated as described above to record delayed mortality.

Apart from the fact that full logs that were usually longer than 50 cm were sampled after collection on field sites, all sampling protocols and measurements for *R. bifasciatum* individuals found within logs were identical to those described for Laboratory Exposure Experiments (5.2.3), as was the wood sampling protocol, except that in addition to samples baited in Petri dishes, a bulk sample of 750 g of the remains of each log after sampling (a mixture of large and small wood pieces, frass and sawdust) was collected in a resealable plastic bag. Ten *G. mellonella* larvae were placed in the bag before it was sealed and then incubated at 20°C for five days, after which time the number of infected bait insects was recorded. Surviving insects were incubated in 5 cm diameter Petri dish to record delayed mortality. Bags were laid side by side arranged in random order on plastic trays for incubation (separate trays for each site).

Such 750 g bulk wood samples were collected from eleven of the 16 logs sampled at Deerpark and they were baited as described above. Enough wood was collected from two additional logs to allow baiting of three wood samples in 9 cm diameter Petri dishes and bait them as described for Laboratory Exposure Experiment I (5.2.3a). Three 10 g subsamples were also taken from the eleven bulk wood samples collected, also to be baited as described for Laboratory Exposure Experiment I (5.2.3a).

5.2.7 Estimation of deadwood available to saproxylic beetles on clearfell sites

Deadwood and woody debris left behind on a clearfell site after the removal of trees includes small and large branches as well as large and small sections of logs (complete logs are rarely left behind). Besides the tree stumps, this debris is the main substrate colonized by the larvae of saproxylic beetles and various stages of other wood-associated insects. Most of this smaller debris is gathered up into 'Lop and Top' (LT) piles that run in lines parallel to stump rows. Typically, these LT piles are between 20 cm and 1 m high and are separated by three to five rows of stumps that have been cleared of most woody debris ('Cleared strips' or CS). A photograph showing the typical arrangement of LT and CS on a clearfell site on which stumps were treated with EPN is presented in Fig 5.3.

To estimate how much woody debris available for colonisation by saproxylic beetles was present on clearfell sites, three sites were surveyed.

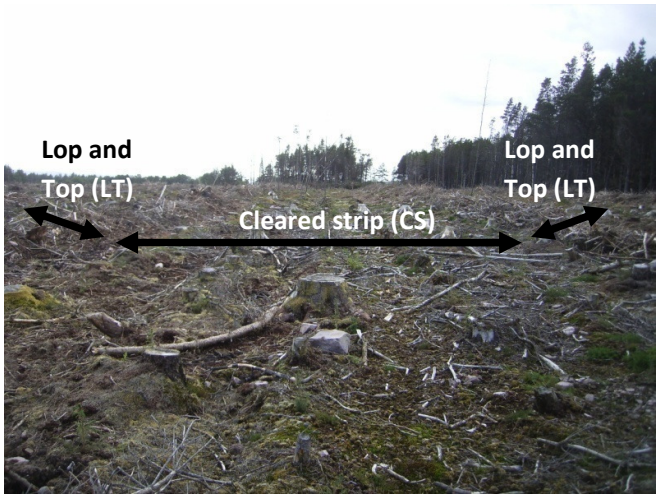


Fig 5.3: Distribution of woody debris on a clearfell site (Lackenrea, September 2007).

a) Lackenrea and Ballymacshaneboy

On these two sites, woody debris in CS and LT areas was estimated by pacing a felled area (Lackenrea: full site, 400 m by 400 m; Ballymacshaneboy: 25 % of site, 160 m by 160 m) and counting the number of LT and CS that ran across the site. On each site, at 20 randomly chosen locations within CS, the width of the CS was measured to the closest 50 cm. The amount of suitable woody debris in CS was estimated from measurements of length and diameter of any logs or branches lying across a straight line spanning the CS at this point. At a further 20 random locations, the height of a LT pile was measured to the closest 5 cm from ground level. At the point this measurement was taken, the proportion of logs and branches within each LT pile with a diameter > 5 cm was estimated and expressed as the proportion of the total amount of wood in the pile (five categories distinguished: 0 %, 25 %, 50 %, 75 %, 100 %).

b) Featherbed

On this clearfell site, a single CS was walked from one end of the site to the other and along the way any piece of wood with a diameter > 5 cm positioned within the CS was recorded and assigned one of four categories, depending on its distance to the closest stump treated with EPN: Adjacent (touching stump), close (less than 50 cm to stump), distant (between 50 cm and 1 m from stump), away (> 1 m from stump). It was also recorded whether the respective log was likely to harbour saproxylic beetle larvae (yes or no) based on a visual inspection.

5.3 Results

5.3.1 Assessment of saproxylic beetle community in woody debris on clearfell sites

a) Incubated decomposing log segments from Lackenrea and Ballymacshaneboy

While a large number of mostly small (< 5 mm) dipterans was found in the containers with incubated log segments, adult specimens of only one wood-associated coleopteran species was recorded over the two and a half years of incubation. These were identified as *Rhagium bifasciatum* (Coleoptera: Cerambycidae; Fabricius, 1775) (Table 5.2). In containers with log segments from Lackenrea, six beetles were found, two less than in containers with log segments from Ballymacshaneboy. For Lackenrea, all but one of the beetles was recorded in the Lop and Top (LT) container, whereas the cleared strip (CS) container produced all but one of the beetles for Ballymacshaneboy. The overall number of beetles was low for both sites, with no more than roughly one beetle emerging per 10 L of wood. There was no significant difference in the mean volume of logs between any of the treatments (One-way ANOVA, DF = 3, F = 2.34, P = 0.09, N = 10 each). All beetles were found within the first 18 months of incubation (incubation lasted 30 months in total).

Table 5.2: Number of adult *R. bifasciatum* beetles found in incubation containers with log segments from cleared strips (**CS**) and lop and top piles (**LT**) at Lackenrea and Ballymacshaneboy. Numbers in brackets give the total volume of wood (in L) in each container.

	Number of adult *R. bifasciatum* in emergence containers (and total volume of deadwood in container [L])	
Collection area	Lackenrea	Ballymacshaneboy
Cleared strip (CS)	1 *(151)*	7 *(87)*
Lop and top (LT)	5 *(123)*	1 *(99)*

b) Destructively sampled decomposing logs (all sites)

Only two types of saproxylic beetles were found in destructively sampled logs from Lackenrea and Ballymacshaneboy. The majority of beetle larvae found were of the same species as the adult specimens recovered from the incubation containers, namely *R. bifasciatum*. All developmental stages of this species were easily distinguishable from any other insects found within logs. No other longhorn beetle species were found in logs, though one other cerambycid species, *Asemum striatum* (Linnaeus, 1758), was found in tree stumps on some sites (Fig 5.4). The larvae of these two longhorn beetle species can be distinguished readily based on the morphology of the prothorax, which is noticeably wider in relation to the head capsule in *A. striatum* compared with *R. bifasciatum* (Duffy, 1957; see Fig 5.4).

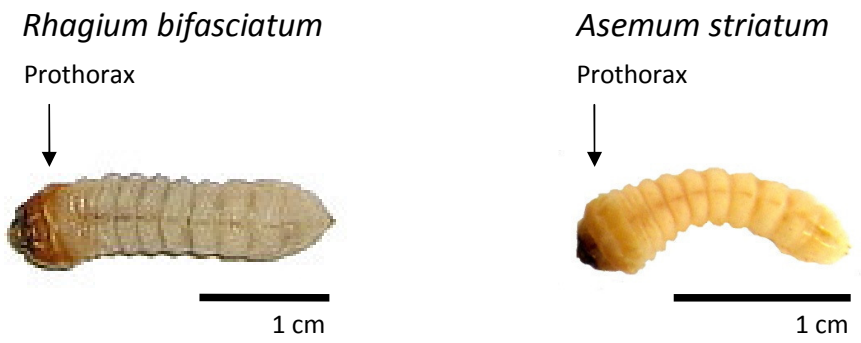


Fig 5.4: Photographs of mid to late instar longhorn beetle larvae indicating prothorax.

The only other saproxylic beetles that were found during subsequent sampling of woody debris were click beetles (Coleoptera: Elateridae). Adult specimens were identified as *Ampedus pomonae* (Stephens, 1830) and *Athous haemorrhoidales* (Fabricius, 1801). Larval stages were not identified to species. The abundance of click beetle larvae and adults was, however, much lower than that of longhorn beetle larvae. Moreover, elaterid species were only found in logs from some of the sites sampled. The first set of 40 destructively sampled log segments (50 cm in length each) collected at Lackenrea and Ballymacshaneboy in 2007 contained no click beetles, but did yield 97 individuals of *R. bifasciatum*. Of the 60 log segments (50 cm in length each) used for Exposure Experiment I (see Table 5.3.2), only 14 log segments contained any click beetles, resulting in a total of 23 click beetles (adults and larvae). By contrast, 49 of those log segments contained longhorn beetle larvae and/or adults, for a total of 191 individuals. It therefore was decided to focus on *R. bifasciatum* as the most common non-target wood-associated beetle species in all subsequent experiments and sampling of logs.

Larvae and cocoons of two parasitoids were found in frass galleries and on cadavers of *R. bifasciatum*. The first and more common of the two was *Dolichomitus tuberculatus* (Hymenoptera: Ichneumnidea; Geoffroy 1785), a solitary generalist predator. The second was the gregarious parasitoid *Histeromerus mystacinus* (Hymenoptera: Braconidae; Wesmael 1838). Multiple specimens of *D. tuberculatus* were found at Lackenrea, Featherbed, Glendalough and Deerpark. A single clutch of cocoons of *H. mystacinus* was found only in one log collected at Lackenrea.

5.3.2 Exposure Experiments – General results: IJ virulence, field temperatures

a) Virulence of EPN used for Exposure Experiments

No significant difference was detected between Exposure Experiments with regards to the virulence of the EPN that were applied to the log segments, indicating that this variable was controlled for (data not shown).

b) Temperatures recorded during Field Exposure Experiments

On the day of EPN application, air temperature was 17.5°C at Featherbed (Exp. I) and 16°C at Kildalkey (Exp. II). IJs were applied between 2 p.m. and 5 p.m. on each site. Conditions were overcast but dry on both occasions, though a strong rain shower that lasted approximately 30 minutes began at Kildalkey as the last two blocks of logs were being treated with IJ suspension. Weather data for the daily maximum and minimum temperature as well as daily rainfall was obtained from the Irish Meteriological Service (Met Ēireann) for the two weather stations situated closest to the respective field sites (Casement station for Featherbed [Exp. I] and Derrygreenagh for Kildalkey [Exp. II]). Location information and daily maximum and minimum air temperature as well as daily rainfall data for each station are included in the Appendix (see A.4). No statistical difference in the mean maximum temperature was found between experiments (t-test, T = 1.88, DF = 25, P = 0.072), but there was a highly significant difference between the means of the minimum daily temperature (t-test, T = 7.25, DF = 27, P < 0.001). Daily rainfall did not differ significantly between experiments (M.-W. U-test, W = 243, P = 0.647).

5.3.3 Infection of *R. bifasciatum* in log segments treated with EPN in the laboratory and the field

Rhagium bifasciatum individuals within segments of decomposing logs were infected by EPN in Exposure Experiments conducted in the laboratory and in the field. However, infection generally occurred more frequently in laboratory experiments, irrespective of the EPN concentration (high concentration of 1.8 million IJs or low concentration of 18,000 IJs) that was applied (Table 5.3). In the laboratory, the mean percentage of infected individuals within logs was 86.4 % (*S. carpocapsae*) and 79.8 % (*H. downesi*) at high EPN concentrations. In high concentration treatments in the field, the mean infection rate per log segment was approximately 20 % lower than in the laboratory, however, irrespective of the EPN species applied (Table 5.3).

In low concentration treatments, mean infection rates were as high as approximately 58 % when *H. downesi* was applied to log segments in the laboratory. In the field, log segments treated with a low concentration of IJs contained very few infected individuals, with mean percentages of infected individuals at or below 10 % in all cases (Table 5.3). In the only medium concentration treatment (180,000 IJs of *S. carpocapsae* in Field Exposue Experiment I), the mean percentage of infected individuals was approximately 37 %, a value that was intermediate between high concentration (67 %) and the low concentration (10 %) in that experiment. For detailed listing of log segment parameters and the number of live and infected *R. bifasciatum* individuals in all log segments of each experiment see Appendix A.8.

Table 5.3: Mean percentage of *R. bifasciatum* infected by EPN and mean *R. bifasciatum* density per litre of log volume for each treatment in each of the **Exposure Experiments** conducted in the laboratory (**LAB**) and the field (**FIELD**) ± standard deviation. Only logs that contained *R. bifasciatum* were included (N). SC = *S. carpocapsae*, HD = *H. downesi.* High concentration = 1.8 million IJs, medium concentration = 180,000 IJs, low concentration = 18,000 IJs.

Exposure Experiment	*EPN species*	*EPN concentration*	*N*	*Mean density of* R. bifasciatum	***Mean percentage of* R. bifasciatum *infected by EPN***
LAB I	**SC**	**High**	14	**1.65** ± 1.63	**86.4 ± 27.39**
		Low	11	**0.34** ± 0.34	**12.3 ± 18.87**
		Control	14	**1.11** ± 0.94	**0 ± 0.0**
LAB II	**HD**	**High**	14	**1.01** ± 0.82	**79.8 ± 27.66**
		Low	15	**0.98** ± 0.84	**62.6 ± 28.47**
LAB III	**SC**	**Low**	7	**1.33** ± 1.00	**42.5 ± 40.97**
	HD	**Low**	7	**1.14** ± 1.06	**31.9 ± 32.46**
FIELD I	**SC**	**High**	10	**2.40** ± 1.36	**67.2 ± 19.84**
		Medium	10	**3.07** ± 1.42	**36.5 ± 13.29**
		Low	10	**2.25** ± 1.76	**10.1 ± 15.69**
FIELD II	**SC**	**High**	10	**1.39** ± 0.56	**62.4 ± 27.85**
		Low	8	**0.74** ± 0.66	**5.6 ± 9.97**
	HD	**High**	8	**0.57** ± 0.35	**66.8 ± 14.62**
		Low	7	**0.22** ± 0.30	**0 ± 0**

In order to compare the overall effects of each of the nematode species used (*S. carpocapsae* or *H. downesi*), the setting in which the experiments were conducted (laboratory or field) and the concentration at which EPN were applied to log segments, data from all experiments were included in a General Linear Model (Table 5.4). The response variable was the percentage of *R. bifasciatum* individuals that was recorded for each log, while the variables listed above (EPN species, concentration and setting) were included as predictors. It was not possible to model for interactions of these variables due to the limited number of replicates (N ranged from 15 in Laboratory Exposure Experiments I and II to 7 in Exposure Experiment III). Logs that did not contain any *R. bifasciatum* individuals were excluded (see Appendix A.8). The control treatment from Laboratory Exposure Experiment I (no infection) and the medium concentration treatment (180,000 IJs) from Field Exposure Experiment I (only replicated once) were not included in the model.

Residuals for the model did not satisfactorily match a normal distribution (most likely due to the fact that no infection at all occurred in the low concentration *H. downesi* treatment in Field Exposure

Experiment II). However, since the results of the model are in line with the results for individual comparisons made within each experiment (Table 5.5), they are presented here (Table 5.4).

EPN species did not have a significant effect on the infection rate in a log segment (P = 0.675), but the setting of experiments (field or laboratory) and the concentration at which EPN were applied both had a highly significant effect (P < 0.001). The percentage of infected *R. bifasciatum* individuals was significantly higher in the laboratory compared with the field (Tukey's test, T = -4.058, P < 0.001) and also was significantly higher when a high concentration of IJs was used as opposed to a low concentration (Tukey's test, T = 7.675, P < 0.001).

Comparison of the total number of infected and live *R. bifasciatum* larvae among treatments within each of the five Exposure Experiments gave similar results (pupae and adults were excluded from this analysis to control for developmental stage; Table 5.5). Infection of *R. bifasciatum* was significantly affected by EPN concentration in the laboratory and the field (infection rates were lower in low dose treatments, see Table 5.3). In those experiments that included treatments with both *S. carpocapsae* and *H. downesi* (i.e. Laboratory Exposure Experiment III and Field Exposure Experiment II), there was no significant effect of EPN species on infection (Table 5.5). When infection was compared between field and laboratory (Laboratory Exposure Experiment I for *S. carpocapsae* and Laboratory Exposure Experiment II for *H. downesi* vs. Field Exposure Experiment II), a significant difference between the two settings was found for three of the four EPN/concentration combinations (*S. carpocapsae* high dose: χ^2 = 68.965, P < 0.001; low dose: P = 0.178; *H. downesi*: high dose: χ^2 = 5.456, P = 0.020; low dose: χ^2 = 24.326, P < 0.001; DF = 1 for all).

Elaterids were only found in significant numbers in log segments used in Laboratory Exposure Experiment I. At a high concentration (1.8 million IJs of *S. carpocapsae* per log), five out of seven (71.4 %) click beetle larvae or adults were infected and one out of three (33.3 %) were infected in the low concentration (18,000 IJs). None of the five click beetles from control logs were infected.

Table 5.4: Results of a General Linear Model in which the percentage of *R. bifasciatum* individuals infected with EPN per log segment was modelled against the EPN concentration applied (high or low), EPN species (*S. carpocapsae* or *H. downesi*) and the setting (laboratory or field). Test variables and P values are given for each predictor. Overall N = 130, for N of individual data sets see Table 5.3. Note: Residuals for the model did not satisfactorily match a normal distribution.

Response variable	***Predictor***	***DF***	***F***	***P***
Infection rate per log	EPN species	1	0.18	**0.675**
	Concentration	1	58.90	**< 0.001**
	Setting	1	16.47	**< 0.001**

Table 5.5: Results of statistical comparison of the percentage of *R. bifasciatum* larvae infected with EPN between treatments within each Exposure Experiment. In those cases where an experiment included two EPN species, comparisons by species and by EPN concentration were carried out. χ^2 – test used for all comparisons except Fisher's exact test used where P – value is labelled with asterisk. For N see Tables in Appendix A.8.

Exposure Experiment	*Infection of* R. bifasciatum *larvae compared by*	χ^2	**DF**	**P**
Lab I	EPN concentration	50.098	1	**< 0.001**
Lab II	EPN concentration	23.482	1	**< 0.001**
Lab III	EPN species	0.007	1	**0.933**
Field I	EPN concentration	135.096	2	**< 0.001**
Field II	EPN concentration (*S. carpocapsae*)	55.118	1	**< 0.001**
Field II	EPN concentration (*H. downesi)*	43.157	1	**< 0.001**
Field II	EPN species (High dose)	2.517	1	**0.113**
Field II	EPN species (low dose)	N/A	N/A	**0.160***

5.3.4 Exposure Experiments: Density of *R. bifasciatum* in logs and its effect on EPN infection rates

The density of *R. bifasciatum* per litre of log segment was as high as approximately six insects per litre of wood in some log segments, but generally was in a range below two individuals per litre (Appendix A.8). *R. bifasciatum* were not found in all logs, however. Since the density of individuals within a log segment might have had an influence on the rate of infection by nematodes, it was tested whether there was a significant difference in the density of insects in the log segments among treatments across all experiments. A One-way ANOVA on this data revealed a highly significant difference in the density among treatments ($F = 3.82$, $DF = 12$, $P < 0.001$, for N see Table 5.3; control for Laboratory Exposure Experiment I not included). However, Tukey's test ($\alpha = 0.05$) showed that in only one case was there a significant difference between two sets of log segments used in the same experiment. This was in Laboratory Exposure Experiment I, where the mean density of *R. bifasciatum* individuals was significantly lower in the low concentration treatment receiving 18,000 IJ of *S. carpocapsae* (0.34 individuals per litre) compared with the high concentration treatment in which 1.8 million IJs of this species were applied (mean of 1.54 individuals per litre).

Other significant differences were detected by Tukey's test between low concentration log segments in Laboratory Exposure Experiment I (*S. carpocapsae*, and *H. downesi*), as well as the high and low concentration treatments in Field Exposure Experiment I and the high concentration in Field Exposure Experiment II (*S. carpocapsae* only). Linear regression of the infection rate per log segment against the density of *R. bifasciatum* in logs was conducted for each treatment in each experiment where possible (i.e. where residuals conformed to a normal distribution) and no significant association was found in any case (see Appendix A.9 for test results).

5.3.5 Infection *R. bifasciatum* by experiment and comparison of life stage susceptibility to EPN

In most of the experiments in which *R. bifasciatum* at different developmental stages (larval, pupal or adult) were found, there was no statistical difference in the rate of infection among these groups (Table 5.6). In Laboratory Exposure Experiment II, the percentage of infected adult beetles was higher than that of larvae (95 % and 50 %, respectively), a difference that was highly significant ($P < 0.001$). Infection among pupae was higher in Field Exposure Experiment II compared with larvae, a significant difference in both cases ($P < 0.05$).

Table 5.6: Infection of larval, pupal and adult stages of *R. bifasciatum* in Laboratory (**LAB**) and Field (**FIELD**) Exposure Experiments. Statistical test results are for comparison of infection for larvae, pupae and adults in each treatment. P – values marked with an asterisk only apply to data marked in the same way within that treatment.

			Percenatge of infected* R. bifasciatum *(infected/total)			
Exposure Experiment	*EPN species*	*EPN concentration*	***Larvae***	***Pupae***	***Adults***	*Test result (χ^2 or Fisher's exact test)*
LAB I	**SC**	**High**	**89.4** *67/75*	0	**100** *18/18*	**P = 0.347**
		Low	**19.3** *6/31*	0	0	N/A
		Control	**0** *0/55*	0	**0** *0/12*	N/A
LAB II	**HD**	**High**	**85.9*** *67/78*	**85.7** *6/7*	**88.5*** *23/26*	***P = 1**
		Low	**50.0*** *41/82*	**100** *4/0*	**90.0*** *18/20*	χ^2 = 10.550, DF = 1, ***P = 0.001**
LAB III	**SC**	**Low**	**32.8** *19/58*	**25.0** *1/4*	**0.0** *0/1*	N/A
	HD	**Low**	**32.0** *16/50*	0	**50.0** *1/2*	N/A
FIELD I	**SC**	**High**	**75.2** *85/113*	**50.9** *28/55*	0	χ^2 = 9.930, DF = 1, **P = 0.002**
		Medium	**34.8** *62/178*	**24.1** *7/29*	**37.5** *3/8*	χ^2 = 0.051, DF = 2, **P = 0.975**
		Low	**1.69*** *2/118*	**33.3*** *5/15*	**14.3** *2/14*	***P < 0.001**
FIELD II	**SC**	**High**	**66.9** *85/127*	**79.2** *19/24*	**54.5** *12/22*	χ^2 = 3.153, DF = 2, **P = 0.207**
		Low	**8.5** *5/59*	0	**25.0** *3/12*	**P = 0.127**
	HD	**High**	**70.3*** *52/74*	**50.0** *1/2*	**75.0*** *9/12*	***P = 1**
		Low	**0** *0/31*	0	**0** *0/12*	N/A

5.3.6 Effect of *R. bifasciatum* larval length and depth within wood on infection with EPN

Where possible (N ≥ 8), the length and depth within the wood at which live and infected larvae were found were compared (Fig 5.5). Only in one treatment (Laboratory Exposure Experiment I, high concentration of *H. downesi*) was a significant difference found in the length of live and infected larvae (M.-W. U-test; W = 1163.0; P = 0.023; for N see Fig 5.5). No significant difference was found in the depth within the wood at which live and infected larvae were found (For test results see Appendix A.19).

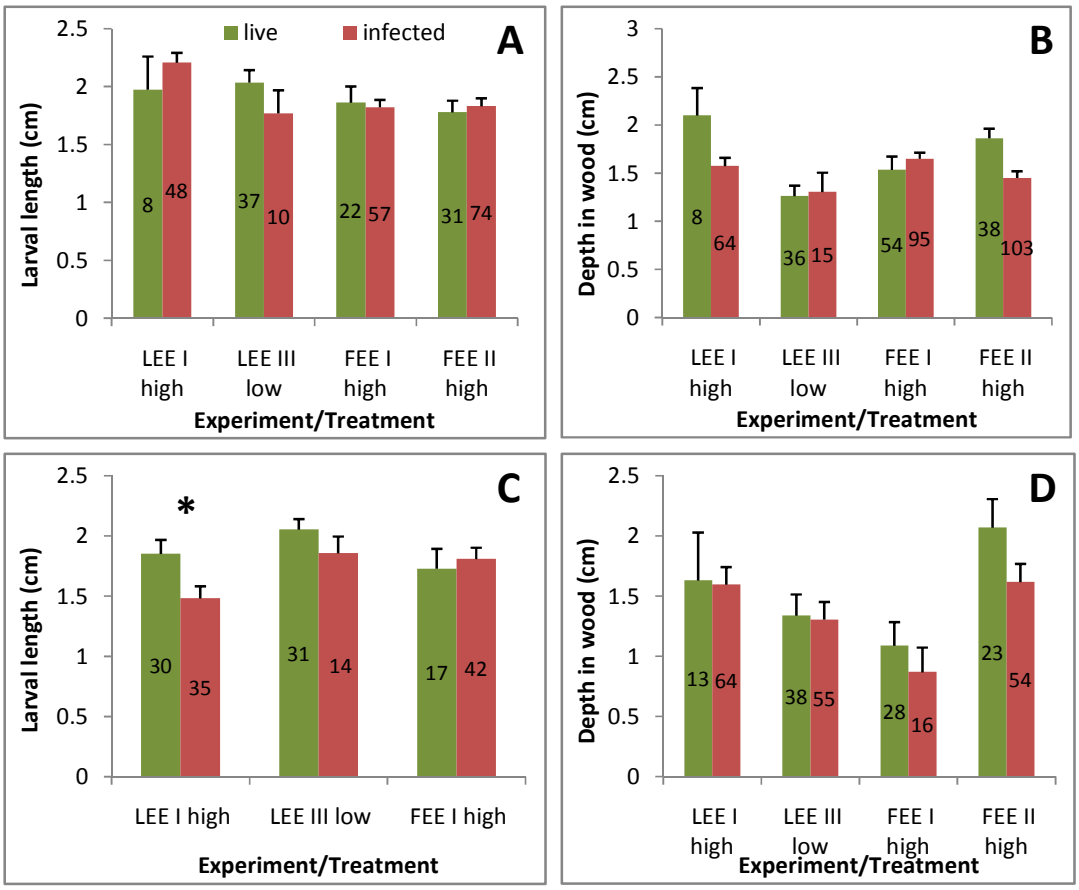


Fig 5.5: Comparisons of mean length and depth in wood of live and infected larvae from Laboratory and Filed Exposure Experiments (LEE and FEE, respectively). **A & B:** *S. carpocapsae* treatments **C & D:** *H. downesi* treatments. Numbers in bars give N, error bars represent standard errors. Pairs of bars marked with an asterisk are significantly different from each other (M.-W. U-test; α = 0.05). Legend in graph A, only treatments with N ≥ 8 measurements available for live and dead larvae included.

5.3.7 Distribution of infected *R. bifasciatum* individuals in log segments in relation to compost or soil

In none of the cases in which statistical analysis was possible was the percentage of infected *R. bifasciatum* individuals (larvae, pupae and adults combined) significantly affected by their location within log segments in relation to the compost or soil (i.e. the centre of log segments, the side of that

was facing the compost or soil during the experiment or the side that was facing away from it) (Table 5.7). Significance was approached in one instance (the *H. downesi* low concentration treatment in Laboratory Exposure Experiment III, P = 0.052).

Table 5.7: Number and percentage of infected *R. bifasciatum* individuals (larvae, pupae and adult stages of *R. bifasciatum* combined) in Laboratory (**LAB**) and Field (**FIELD**) Exposure Experiments. Statistical test results are for comparison of infection for larvae, pupae and adults in each treatment. P – values marked with an asterisk only apply to data marked in the same way within that treatment. Data sets in which infection rates were so low as to prohibit statistical analysis are not included.

			Number of infected* R. bifasciatum*/total (percentage)* *In each log segment region			
Exposure Experiment	*EPN species*	*EPN concentration*	***Facing compost or soil***	***Centre***	***Facing away from compost or soil***	*Test result (χ^2 or Fisher's exact test)*
LAB I	**SC**	**High**	14/18* ***(78.8)***	17/18 ***(94.4)***	27/30* ***(90.0)***	***P = 0.400**
LAB II	**HD**	**High**	29/30* ***(96.7)***	48/57 ***(84.2)***	15/18* ***(83.3)***	***P = 0.141**
		Low	15/38* ***(39.5)***	2/8 ***(25.0)***	21/48* ***(43.8)***	χ^2 = 0.159, DF = 2, ***P = 0.690**
LAB III	**SC**	**Low**	5/25* ***(20.0)***	4/8 ***(50.0)***	6/20* ***(30.0)***	***P = 0.500**
	HD	**Low**	9/30* ***(30.0)***	0/5 ***(0.0)***	8/13* ***(61.5)***	χ^2 = 3.774, DF = 1, ***P = 0.052**
FIELD I	**SC**	**High**	17/57 ***(29.8)***	28/75 ***(37.3)***	9/22 ***(40.9)***	χ^2 = 1.187, DF = 1, **P = 0.552**
		Medium	61/92 ***(66.3)***	5/15 ***(33.3)***	57/85 ***(69.5)***	χ^2 = 0.011, DF = 1, **P = 0.994**
FIELD II	**SC**	**High**	8/28* ***(28.6)***	3/5 ***(60.0)***	12/46* ***(26.1)***	*χ^2 = 0.054, DF = 1, **P = 0.815**
	HD	**High**	12/59* ***(20.3)***	6/9 ***(66.7)***	22/74* ***(29.7)***	χ^2 = 1.521, DF = 1, **P = 0.217**

5.3.8 Presence and distribution of EPN in logs at the end of Exposure Experiments

a) Number and distribution of IJs in wood samples from different regions of log segments

EPN were detected in almost all of the wood samples taken from log segments that were treated with a high concentration of IJs (1.8 million), both in Laboratory and Field Exposure Experiments and regardless of EPN species (*S. carpocapsae* or *H. downesi*). In low concentration treatments (18,000 IJs), recovery of IJs from wood samples was much lower, with approximately 20 to 40 % of wood samples scoring positive when experiments were carried out in the laboratory and less than 10 % in the field. The number of IJs per 10 g wood sample was estimated from standard curves (see Appendix A.3).

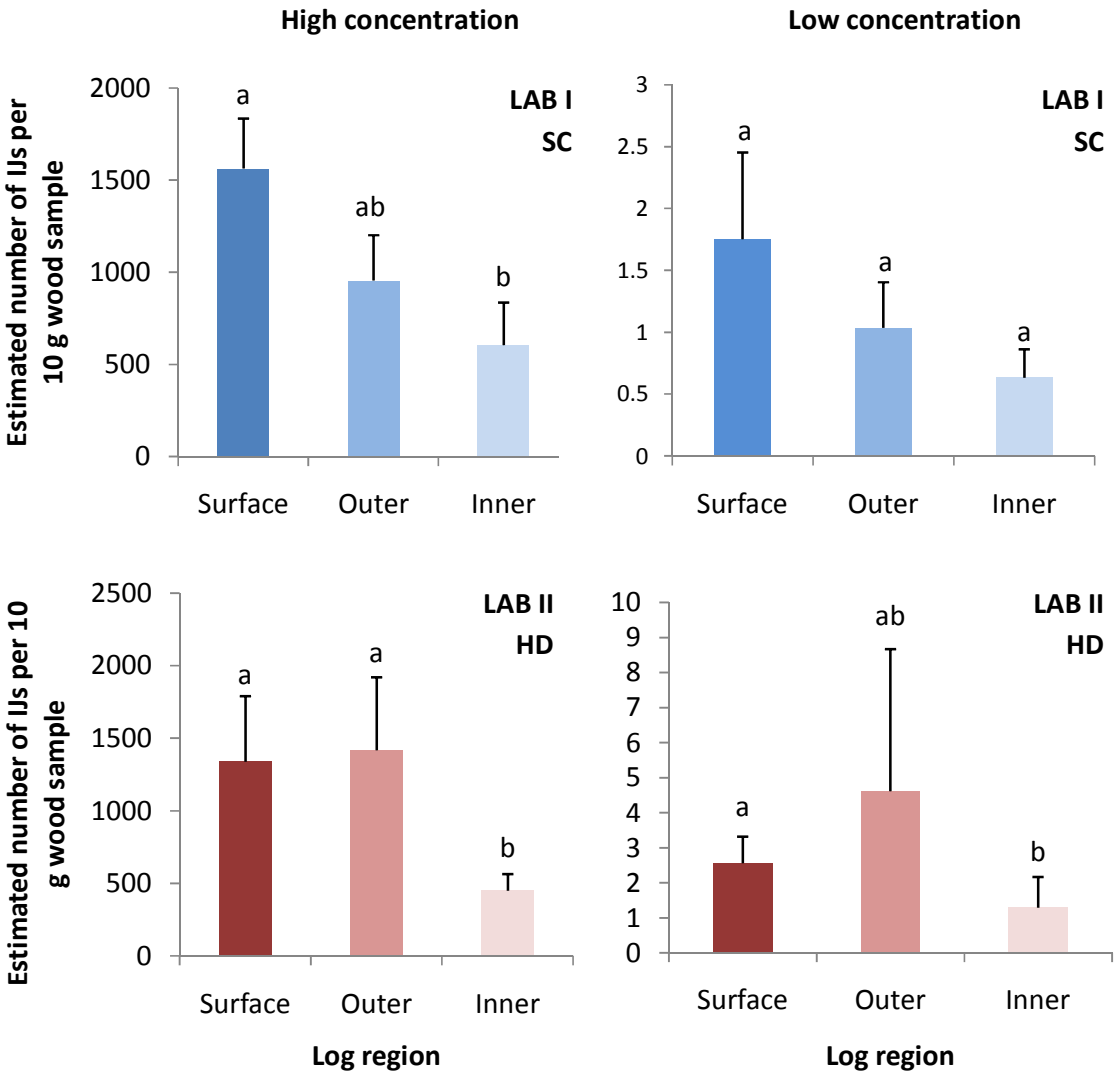


Fig 5.6: Mean estimated number of IJs (*S. carpocapsae* in blue or *H. downesi* in red) per 10 g wood sample from each of the three regions of logs in high concentration (1.8 million IJs, left) and low concentration (18,000 IJs, right) treatments of Laboratory Exposure Experiments I and II. Error bars represent standard error (N = 15 per bar). **LAB** I = Laboratory Exposure Experiment I, **LAB** II = Laboratory Exposure Experiment II. Within each graph, means of bars labelled with the same letters are not significantly different from each other (High concentration: Tukey's test, α = 0.05; low concentration: M.-W. U-test, α = 0.017).

In Laboratory Exposure Experiments I, the mean estimated number of *S. carpocapsae* IJs in wood samples from both high and low IJ concentration treatments was highest in the surface region of log segments and was lowest in the inner region (Fig 5.6). This drop was significant for the high concentration treatment (One-way ANOVA, F = 7.57, DF = 2, P = 0.002, N = 15 per region, followed by Tukey's test [α = 0.05]), but not the low concentration (K. - W. test, DF = 2, H = 1.74, P = 0.418, N = 15

for each region). The estimated number of IJs in log segments from both *H. downesi* treatments in Laboratory Exposure Experiment II was also significantly different among log regions (High concentration: One-way ANOVA; F = 3.97, DF = 2, P = 0.026, N = 15 for each region; low concentration: K. - W. test, H = 6.41, DF = 2, P = 0.041; N =15) (Fig 5.6). Tukey's test showed that there was a significantly lower number of *H. downesi* IJs present in the inner region of high concentration log segments than in the surface region ($\alpha = 0.05$), and M.-W. U-test also showed a significant difference between these two regions in the low dose (W = 280.0, P = 0.031; N = 15 each).

Though there was no significant difference in mean IJ numbers between the surface and outer regions or the outer and inner regions in any of the treatments from these experiments, a different trend in IJ distribution across log segment regions was observed between them. In Laboratory Exposure Experiment I (*S. carpocapsae*), IJ numbers were lower in the outer region than in the surface region, whereas in Laboratory Exposure Experiment II (*H. downesi*), the mean number of IJs was slightly higher in the outer region than in the surface region (Fig 5.6).

In Laboratory Exposure Experiment III, the estimated number of IJs in wood samples was consistently low averaging only about two to three IJs for most positive samples (data not shown). There was no significant difference in the mean number of IJs per sample among any of the log segment regions, regardless which of the two EPN species had been applied (K.-W.- test; H = 2.17, DF = 5, P = 0.825; N = 14). The estimated number of IJs per wood sample was similar between *S. carpocapsae* and *H. downesi*, with approximately 1,400 to 1,600 IJs being detected in samples from the surface region of log segments from the high concentration treatments. On average, however, IJ estimates for the outer region of log segments was higher for *H. downesi* in this treatment than it was for *S. carpocapsae* (approximately 1,500 vs. 1,000, respectively), and the opposite relationship was observed in the inner region (500 vs. 600, respectively).

IJs of *S. carpocapsae* were detected in all individual 10 g wood and frass samples taken from high concentration log segments in Field Exposure Experiment I, save for one frass sample. One surface sample from a high concentration log with an estimated IJ number of approximately 58,000 was excluded from analysis as an outlier. As in the Laboratory Exposure Experiment I (see section 5.3.8a) the mean estimated number of IJs per wood sample was highest in the surface region of high concentration logs (roughly 2,000 IJs). Estimated IJ numbers were lower in the outer and inner regions as well as in frass samples (means of 300 to 500 IJs) (Fig 5.7). There was a significant difference in the number of IJs per wood sample among log regions in this treatment (One-way ANOVA; F = 15.77, DF = 3, P < 0.001; N = 10 each). Tukey's post-hoc test ($\alpha = 0.05$) showed that estimated IJ numbers were significantly higher in the surface region compared with all others, including the frass samples.

A trend similar to that observed for the high concentration treatment was also observed for the medium concentration treatment, though the mean estimated number of IJs in the outer region was almost as high in the surface region (roughly 70 IJs) and dropped to between 10 and 20 IJs in the inner region and frass samples (Fig 5.7). There was a significant difference among log regions in this treatment (One-way ANOVA, F = 3.32, DF = 3, P = 0.031; N = 10 each).Using Tukey's post-hoc test (α = 0.05) it was, however, not possible to detect which samples were responsible for this effect.

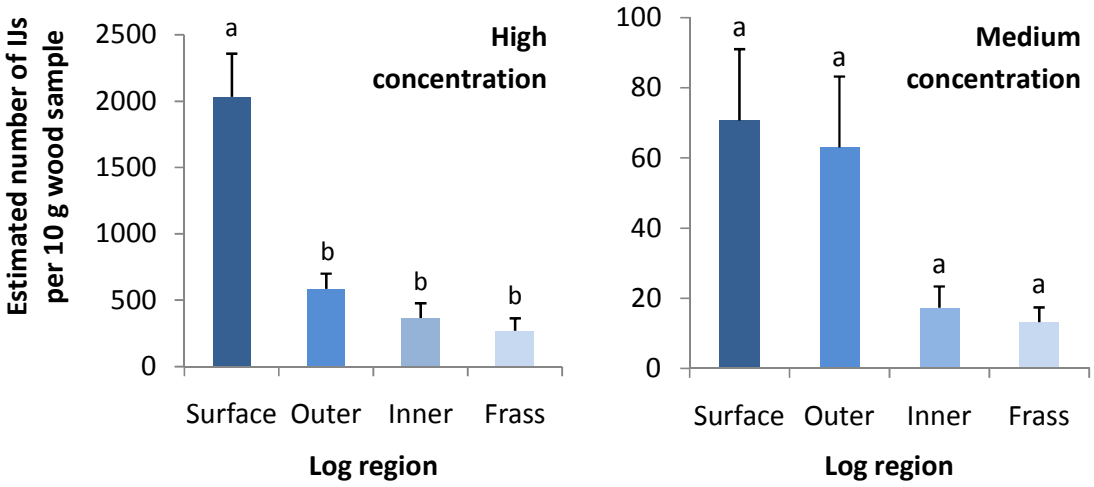


Fig 5.7: Mean estimated number of *S. carpocapsae* IJs per 10 g wood sample from each of the three regions of logs in high concentration (1.8 million IJs, left) and medium concentration (180,000 IJs, right) treatments of Field Exposure Experiment I. Estimated number of IJs per 10 g frass sample are also given. Error bars represent standard error (N = 10 per bar). Within each graph, means of bars labelled with the same letters are not significantly different from each other (Tukey's test, α = 0.05).

In logs that were treated with a high concentration of *S. carpocpasae* in Field Exposure Experiment II the mean estimated IJ numbers per wood sample dropped from roughly 700 IJs in the surface region to about 400 IJs in the innermost region. For *H. downesi* logs in the high concentration, estimated IJ numbers were highest in the inner log region on average (approximately 350 IJs) and similar in the outer and surface regions (about 150 IJs for each) (Fig 5.8). A One-way ANOVA that that all six data sets found a significant difference among the regions (F = 4.04, DF = 5, P = 0.003, N = 15) and Tukey's post-hoc test (α = 0.05) showed that the mean estimated number of IJs in the surface region of logs receiving 1.8 million IJs of *S. carpocapsae* was significantly higher than that found in the surface and outer regions of logs receiving the same concentration of *H. downesi*. No other significant differences were detected. Only four wood samples (out of 72) collected from low concentration *H. downesi* logs scored positive for EPN and 27 samples from low concentration *S. carpocapsae* logs did so (72 in total). Since only a single *G. mellonella* larva was infected in almost all of these samples (data not shown) no statistical comparison of data was carried out.

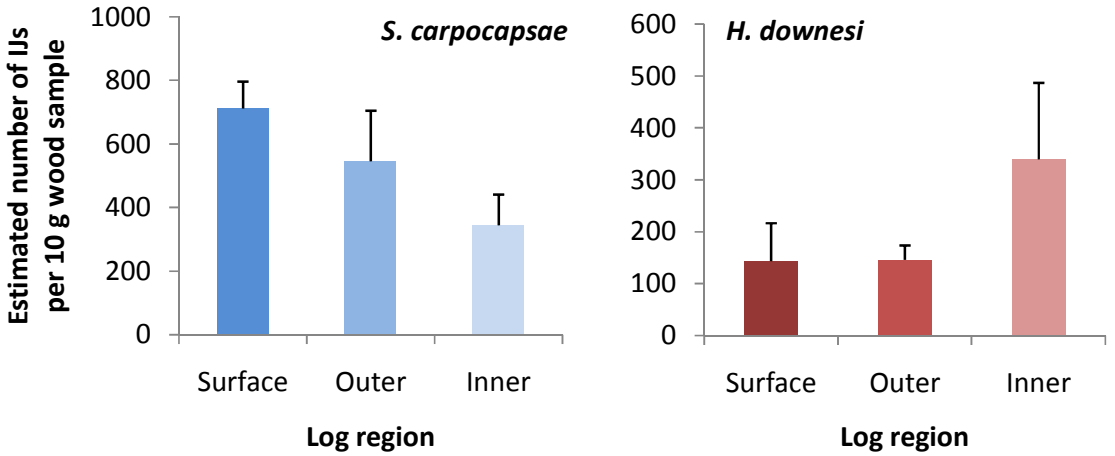


Fig 5.8: Mean estimated number of IJs (*S. carpocapsae* in blue and *H. downesi* in red) per 10 g wood sample from each of three regions of logs in the high concentration (1.8 million IJs) treatments of Field Exposure Experiment II. Error bars represent standard error (N = 10 per bar). There was no significant difference among log regions for either EPN species.

b) Overall effect of EPN species, EPN concentration and setting of Exposure Experiments on the number and distribution of IJs within log segments

To see whether the number of IJs in wood samples was affected by the log region from which samples were taken, the experimental conditions or the EPN species applied, the mean estimated number of IJs in wood samples from each region (surface, outer and inner; N = 3 for each mean) of high concentration logs were incorporated into a General Linear Model in which the setting of the experiment (Field or Laboratory), the EPN species (*S. carpocapsae* or *H. downesi*) and the region of the log (surface, inner or outer) where used as interacting fixed variables. Data from the low dose were not used as they included a large number of zero values.

The results indicated that all of these variables had a highly significant effect on EPN numbers within logs and that there was a significant interaction between experimental setting and EPN species as well as log region and EPN species, but not between setting and region ($P < 0.005$) (Table 5.8). Tukey's post-hoc test indicated that IJ numbers in wood samples from both the surface and outer region of log segments was significantly higher than in the inner region (surface vs. inner: $T = 2.482$, $P = 0.037$; outer vs. inner: $T = 4.143$, $P = 0.001$). There was no significant difference between the surface and outer regions, however ($T = 1.661$, $P = 0.223$). Numbers of IJs in wood samples were significantly higher in laboratory experiments compared with field experiments ($T = 6.002$, $P < 0.001$) and *Steinernema carpocapsae* IJs were present in wood samples in significantly higher numbers than those of *H. downesi* ($T = 4.654$, $P < 0.001$).

Table 5.8: Results of a General Linear Model in which the mean estimated number of IJs per wood sample from each region (high concentration logs from Laboratory and Field Exposure Experiments only, three samples per region) was modelled against the predictive variables of experimental setting, log region (surface, outer, inner) and EPN species (*S. carpocapsae* or *H. downesi*). For log segment N see Table 5.2.

Predictor	DF	F	P
Setting (lab. or field)	1	36.02	**< 0.001**
Log region	2	8.69	**< 0.001**
EPN species	1	21.66	**< 0.001**
Setting*log region	2	1.75	**= 0.176**
Setting*EPN species	1	14.71	**< 0.001**
Log region*EPN species	2	5.66	**= 0.004**

5.3.9 Emergence of EPN IJs from infected *R. bifasciatum*

Steinernema carpocapsae IJs emerged from 36 of the 52 larvae infected in high concentration logs that were placed in White traps at the end of Laboratory Exposure Experiment I. Full data sets recording the weekly emergence of IJs in White traps over eight weeks were collected for 22 of these (14 had to be discarded due to fungal growth on hosts). IJs emerged from all 33 infected larvae from logs receiving a high concentration of *H. downesi* and all 20 larvae from *H. downesi* low concentration logs that were White trapped after infection in Laboratory Exposure Experiment II. Only three White traps had to be discarded in the high concentration group as fungal growth occurred much less frequently on *H. downesi* infected hosts. Success of emergence from *R. bifasciatum* larvae was significantly affected by EPN species ($\chi^2 = 5.466$, DF = 1, P = 0.019).

Full emergence data sets (eight weeks) were collected for six out of seven White trapped *R. bifasciatum* adults infected with *H. downesi* in logs treated with a high concentration of EPN and eleven adults (out of 14 that were White-trapped) from logs receiving a low concentration of this species (Laboratory Exposure Experiment II). IJs also emerged from 19 of the 20 *R. bifasciatum* larvae and all of the 10 adults which were infected with *S. carpocapsae* in Petri dishes in the laboratory as well as 18 of the 20 waxmoth larvae (*G. mellonella*) that were infected in the same way (no significant difference in the percentage of White traps showing emergence among these groups; Fisher's exact test, $\alpha = 0.05$). In *R. bifasciatum*, first emergence most often occurred from the mouth of the cadaver, with large aggregations of IJs grouped directly on or under the head capsule.

When comparing the length of *R. bifasciatum* larvae infected in logs from which emergence was monitored, it was found that the larvae that were taken from *H. downesi* low concentration logs were significantly shorter than the larvae from both *S. carpocapsae* and *H. downesi* high

concentration logs (One-way ANOVA, DF = 2, F = 9.57, P < 0.001 followed by Tukey's test with $\alpha = 0.05$; N = 17, 20 and 29, respectively). Since this may have affected the number of IJs that emerged from these cadavers, emergence data for these three host groups was corrected for host length (number of emerging IJs/host length in cm) and data analysis was conducted using this corrected data as well as the raw data. Since correction of data had no effect on the outcome of statistical tests (i.e. significance or no significance), these are not included here, but can be found in Appendix A.5.

a) Time to emergence

The time it took for *S. carpocapsae* IJs to emerge varied considerably within each of the two sets of *R. bifasciatum* larvae (those from treated logs and those infected directly in the laboratory). A median time of only four days to first emergence was recorded for *S. carpocapsae* that had infected *R. bifasciatum* larvae in log segments. This was the lowest median value among all of the different host/EPN combinations (Fig 5.9). First emergence from such larvae, however, took up to 71 days in some cases. Compared with this, the median time to emergence was significantly shorter in *R. bifasciatum* hosts infected with *S. carpocapsae* in the laboratory (lower-tailed M.-W. U-test, W = 457.0, P < 0.001; for N see Fig 5.9). Even when infected with IJs in the laboratory, first emergence from *R. bifasciatum* larvae took 50 days in one instance. Among laboratory-infected hosts, IJs emerged from *G. mellonella* significantly sooner that they did from *R. bifasciatum* (lower-tailed M.-W. U-test, W = 193.5, P < 0.001; for N see Fig 5.9) and variation was much among *G. mellonella* hosts compared with *R. bifasciatum* (6 to 23 days, median: 13 days). The number of days that passed before IJs emerged from *R. bifasciatum* adults was very similar to that found for *G. mellonella* (14 days median, ranging from 48 days to 9 days) and was not significantly different (M.-W. U-test; W = 245.0, P = 0.454), but was significantly greater than that recorded for *R. bifasciatum* larvae (lower-tailed M.-W. U-test; W = 82.5, P = 0.001).

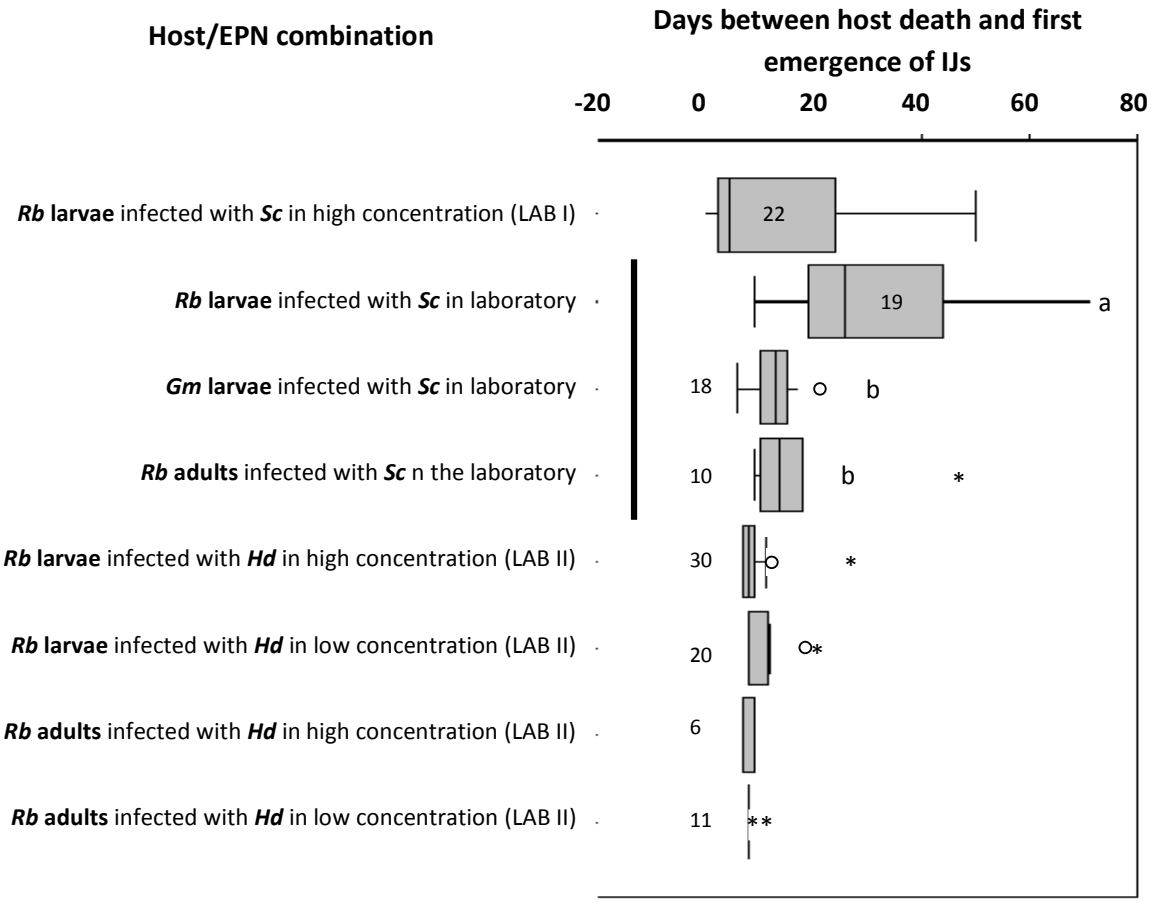


Fig 5.9: Box and whisker plot giving the median number of days between death of host insects and first IJ emergence from *R. bifasciatum* and *G. mellonella hosts*. **LAB** = Laboratory Exposure Experiment, *Sc = S. carpocapsae*, *Hd = H. downesi*, *Rb = R. bifasciatum* and *Gm = G. mellonella*. Boxes represent upper and lower quartiles and lines in boxes give the median. Asterisks and circles above boxes indicate outliers. Boxes that are connected by a black bar are significantly different from each other if they do not share the same letter (M.-W. U-test, $P < 0.05$). Numbers in or beside boxes give N.

The median number of days it took *H. downesi* IJs to emerge from *R. bifasciatum* hosts retrieved from Exposure Experiment II logs was higher than that found for larvae infected with *S. carpocapsae* (a median of 7 days versus 4 days), but the variability was much lower for *H. downesi* (emergence was recorded within 7 to 27 days). No significant difference in the median time to first emergence was found between the two groups ($W = 513.5$, $P = 0.194$, see Fig 5.9 for N). The median time to first emergence was very similar for all groups of *R. bifasciatum* individuals infected with *H. downesi* and no significant difference was found among high concentration and low concentration groups of larval and adult stage hosts (M.-W. U-tests, *R. bifasciatum* larvae high concentration versus low concentration: $W = 674.5$, $P = 0.068$; *R. bifasciatum* adults high concentration versus low concentration: $W = 427.5$, $P = 0.107$; for N see Fig 5.9).

5.3.10 Weekly emergence of IJs from EPN infected *R. bifasciatum*

a) *Steinernema carpocapsae* emergence trend

The mean number of IJs emerging from *R. bifasciatum* followed a very similar trend among host groups, regardless of whether the larvae had been infected within logs to which EPN had been applied or if they were infected in the laboratory (Fig 5.10). Emergence for both of these groups of hosts was highest in the first week (approximately 15,000 for hosts found in logs and 12,000 for hosts infected in the laboratory) and decreased to roughly 2,000 IJs (infected in logs) and 1300 IJs (infected in laboratory) by the eighth week of emergence, representing a decrease in emergence of approximately 90 % in both cases. By contrast, *G. mellonella* produced close to 125,000 IJs in the first week of emergence, but by the eighth week this number had fallen to an average of only 148 IJs per cadaver, representing a drop in emergence of over 99 %. Mean emergence from adult *R. bifasciatum* infected in the laboratory in week one of emergence was only about 28,000 IJs, which then fell to 578 IJs (2 %) on average by the eighth week.

To see whether or not the emergence trends among hosts infected with *S. carpocapsae* were significantly different from each other, the slope and intercept of the linear regression trend line for the base ten logarithm +1 of each data set (*R. bifasciatum* larvae, adults and *G. mellonella*) were compared amongst each other using a General Linear Model in which host group and the week of emergence were crossed predictors and the number of emerging IJs each week was the response (this data was log base 10 transformed) (Table 5.20).

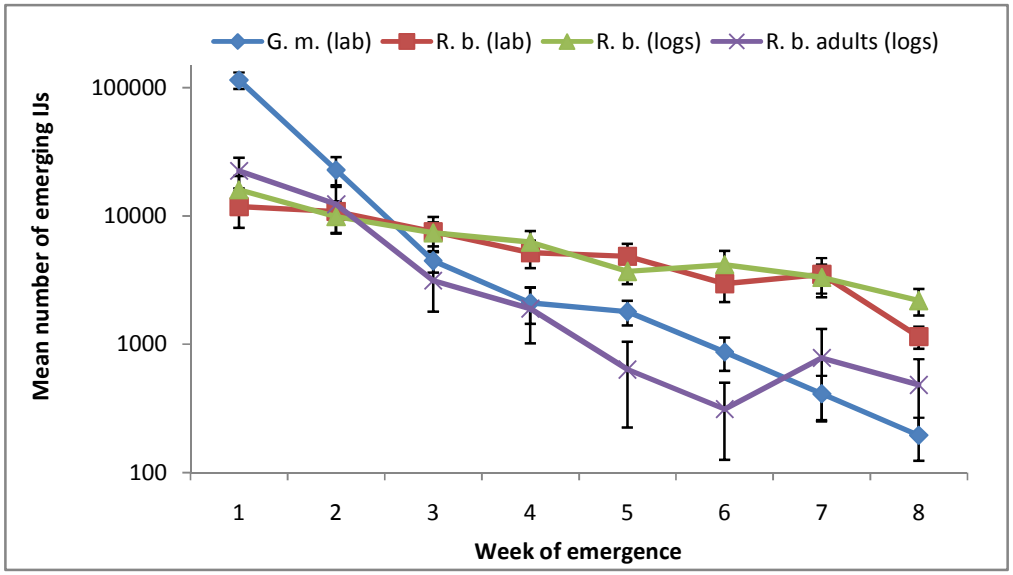


Fig 5.10: Mean number of ***S. carpocapsae*** IJs emerging per week after first emergence. N = 22 for *R. bifasciatum* larvae (R.b.) from logs, N = 19 for *R. bifasciatum* larvae infected in laboratory, N = 10 for *R. bifasciatum* adults infected in laboratory and N = 18 for *G. mellonella* (G.m.) infected in laboratory. Error bars represent standard error.

Table 5.9: Results of General Linear Models comparing the slope and intercept of linear regression trend lines fitted to the log base 10 of the weekly ***S. carpocapsae*** emergence (+1) for each group of hosts. N = 176 for *R. bifasciatum* from logs, N = 152 for *R. bifasciatum* infected in laboratory, N = 80 for *R. bifasciatum* adults infected in laboratory and N = 144 for *G. mellonella* larvae infected in

Comparison	***Factor***	***DF***	***F***	***P***
***R. bifasciatum* larvae** (laboratory) vs. ***R. bifasciatum* larvae** (logs)	Slope	7	0.68	**0.688**
	Intercept	1	0.09	**0.760**
***R. bifasciatum* larvae** (laboratory) vs. ***G. mellonella* larvae** (laboratory)	Slope	7	12.24	**< 0.001**
	Intercept	1	21.0	**< 0.001**
***R. bifasciatum* larvae** (laboratory) vs. ***R. bifasciatum* adults** (laboratory)	Slope	7	5.14	**< 0.001**
	Intercept	1	47.95	**<0.001**
***G. mellonella* larvae** (laboratory) vs. ***R. bifasciatum* adults** (laboratory)	Slope	7	1.76	**0.097**
	Intercept	1	5.85	**0.016**

There was no significant difference in the emergence trends of *R. bifasciatum* larvae that were infected with *S. carpocapsae* in logs or in a Petri dish (P = 0.688 for slope and P = 0.760 for Intercept). However, highly significant differences in trends were found between *R. bifasciatum* and *G. mellonella* larvae infected in the laboratory (P < 0.001 for slope and intercept). There was no significant difference in the emergence trends for *G. mellonella* larvae and *R. bifasciatum* adults infected in the laboratory (P = 0.097 for slope), though initial emergence was higher for *G. mellonella* (P = 0.016 for intercept) (Table 5.9).

b) *Heterorhabditis downesi* emergence trend

In White traps with larvae from *H. downesi* high concentration logs, the mean emergence per week dropped from a mean of roughly 109,000 IJs in week one to 189 IJs in week eight, a drop of over 99.9% (Fig 5.11). The drop was even more pronounced for larvae from low concentration logs (38,500 IJs to 2 IJs). IJ emergence had essentially ceased in the low concentration group after four weeks and fell to a mean of around 100 IJs per week in the high concentration group. While emerging numbers of IJs also decreased over time for *R. bifasciatum* adults, the trend was less consistent (Fig 5.11). The mean number of IJs emerging from high concentration adults was higher in week eight than it was in week four. Mean emergence dropped close to or below 100 IJs in both of these groups when it had been as high as 61,000 IJs for high concentration adults and 76,000 for low concentration adults in week one of emergence. In both cases this was a drop of more than 99%. The slopes and intercepts of linear regression fits to the emergence data for *H. downesi* were compared using General Linear models as described above (5.3.10a; Table 5.10). There was no significant

difference in the emergence trends among the different host groups (P > 0.05 for all slopes), though significance was approached in two instances (high concentration adults vs. Low concentration adults and high concentration larvae vs. low concentration larvae; Table 5.21). However, the number of IJs emerging each week as reflected by the trend line intercept was significantly different among all groups, with high concentration larvae producing a significantly higher number of IJs each week compared with the other host groups (Fig 5.13, Table 5.21).

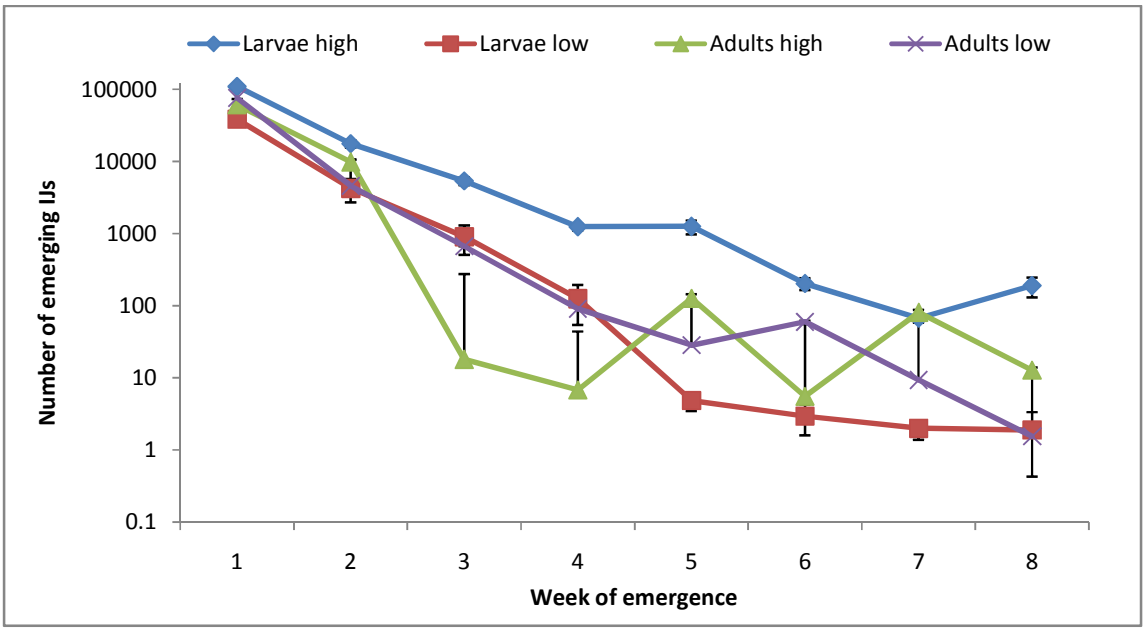


Fig 5.11: Mean number of ***H. downesi*** IJs emerging from *R. bifasciatum* cadavers per week after first emergence. N = 30 for larvae from high concentration logs (Larvae high), N = 20 for larvae from low concentration logs (Larvae low), N = 6 for adults from high concentration logs (Adults high), N = 11 for adults from low concentration logs (Adults low). Error bars represent standard errors, only upper error bars shown for adults due to logarithmic scale.

Table 5.10: Results of General Linear Models comparing the slope and intercept of linear regression trend lines fitted to the log base 10 of the weekly ***H. downesi*** emergence (+1). N = 240 for larvae from high concentration logs, N = 160 for larvae from low concentration logs, N = 48 for adults from high concentration logs, N = 88 for adults from low concentration logs.

Comparison	*Factor*	*DF*	*F*	*P*
High concentration larvae vs. **low concentration larvae**	Slope	7	1.41	**0.201**
	Intercept	1	121.45	**< 0.001**
High concentration adults vs. **low concentration adults**	Slope	7	1.83	**0.083**
	Intercept	1	4.16	**0.044**
High concentration larvae vs. **high concentration adults**	Slope	7	2.18	**0.057**
	Intercept	1	35.43	**< 0.001**
Low concentration larvae vs. **low concentration adults**	Slope	7	0.83	**0.560**
	Intercept	1	21.36	**< 0.001**

c) Comparison of *S. carpocapsae* and *H. downesi* emergence from hosts

Since only one group of hosts was represented for both EPN species investigated (*R. bifasciatum* larvae retrieved from high concentration logs), these two were compared with see whether the emergence pattern for *S. carpocapsae* and *H. downesi* differed significantly from each other (Fig 5.12).

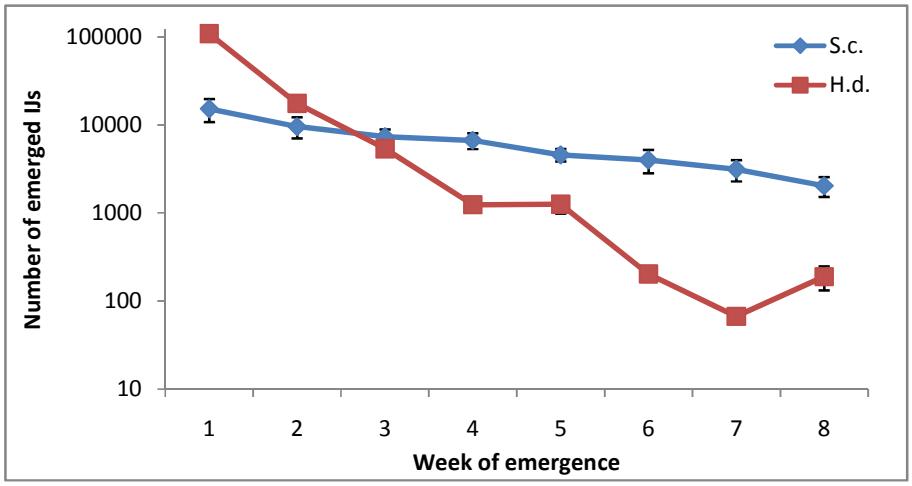


Fig 5.12: Mean number of ***S. carpocapsae*** and ***H. downesi*** IJs emerging from *R. bifasciatum* larvae (high concentration logs from Exposure Experiment I and II only) per week after first emergence. S.c. = *S. carpocapsae*, H.d. = *H. downesi*. N = 22 for *S. carpocapsae*, N = 30 for *H. downesi*. Error bars represent standard error.

Slope and intercept of the linear fit lines for the log base ten + 1 of these two data sets revealed a significant difference between their slopes ($F = 23.58$, $DF = 7$, $P < 0.001$, $N = 176$ for *S. carpocapsae* and $N = 240$ for *H. downesi*) and their intercepts ($F = 114.35$, $DF = 1$, $P < 0.001$, $N = 176$ for *S. carpocapsae* and $N = 240$ for *H. downesi*), indicating that there was a highly significant difference in the rate of decrease of mean IJ emergence over the eight weeks between the two species as well as in the initial emergence rate. As is reflected in Fig 5.12, initial emergence from larvae infected with *H. downesi* was higher than in *S. carpocapsae* but then decreased at a greater pace. Emergence from *S. carpocapsae* started out with lower numbers of IJs, but by the fourth week of emergence, more IJs were emerging from cadavers it had infected compared with those hosting *H. downesi*.

5.3.11 Total number of IJs emerging from *R. bifasciatum* cadavers

The total number of IJs that emerged over the eight week period was highest for *G. mellonella* larvae infected in the laboratory (mean: approximately 150,000 IJs), with *R. bifasciatum* larvae infected by *H. downesi* in Laboratory Exposure Experiment II yielding almost as many IJs (mean: approximately 140,000 IJs). Total emergence was approximately 40,000 to 50,000 IJs in all other host groups

infected with *S. carpocapsae* and between 70,000 and 80,000 in the other host groups infected with *H. downesi* (Fig 5.13). The lowest total emergence was recorded for larvae infected in logs receiving a low concentration (18,000 IJs) of *H. downesi* (approximately 40,000 IJs). There was a significant difference in total emergence among the groups (One-way ANOVA, $F = 7.19$, $DF = 7$, $P < 0.001$, for N see Fig 5.13). Tukey's test ($\alpha = 0.05$) showed that total emergence was significantly higher for *G. mellonella* infected in the laboratory compared with all other hosts from which *S. carpocapsae* emerged. It also showed that total emergence from *R. bifasciatum* larvae infected with *H. downesi* in high concentration logs from Exposure Experiment II was significantly higher than *S. carpocapsae* emergence *R. bifasciatum* as well as well as *H. downesi* emergence from larvae infected within low concentration logs (Fig 5.13).

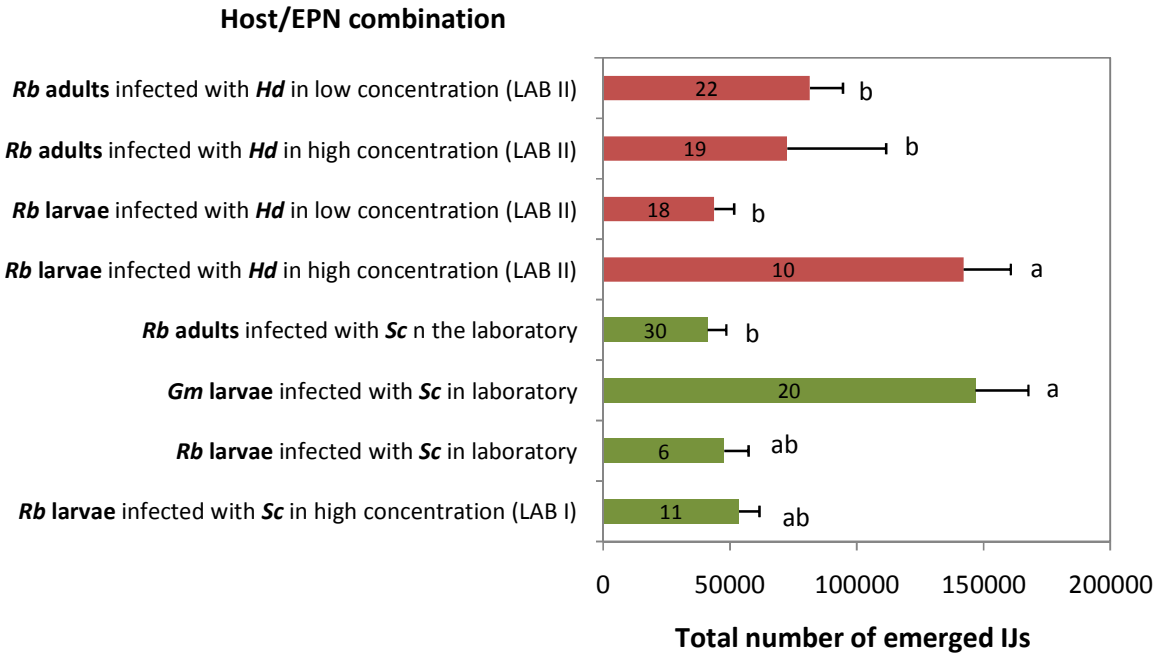


Fig 5.13: Mean total number of IJs emerged from hosts over the entire 8 week period of emergence. Error bars represent standard errors and numbers in bars give N. LAB = Laboratory Exposure Experiment, *Sc = S. carpocapsae*, *Hd = H. downesi*, *Rb = R. bifasciatum* and *Gm = G. mellonella*. Bars that are labelled with the same letter are not significantly differently from each other (One-way ANOVA and Tukey's test [$\alpha = 0.05$]).

5.3.12 Emergence from laboratory infected hosts corrected for host weight

To correct for effects of host biomass that was available to nematodes for consumption, the total number of IJs emerging from hosts was divided by the wet weight of the host as determined previous to infection. This was only possible for *R. bifasciatum* larvae and adults and *G. mellonella* that were

infected in the laboratory (Fig 5.14). Mean total emergence per gram of host wet weight was similar for *R. bifasciatum* adults and *G. mellonella* (450 IJs/mg for waxmoth larvae, 400 IJs/mg for *R. bifasciatum* adults). It was significantly lower for *R. bifasciatum* larvae (One-way ANOVA, $F = 11.30$, $DF = 2$, $P < 0.001$, followed by Tukey's test [$\alpha = 0.05$], for N see Fig 5.14).

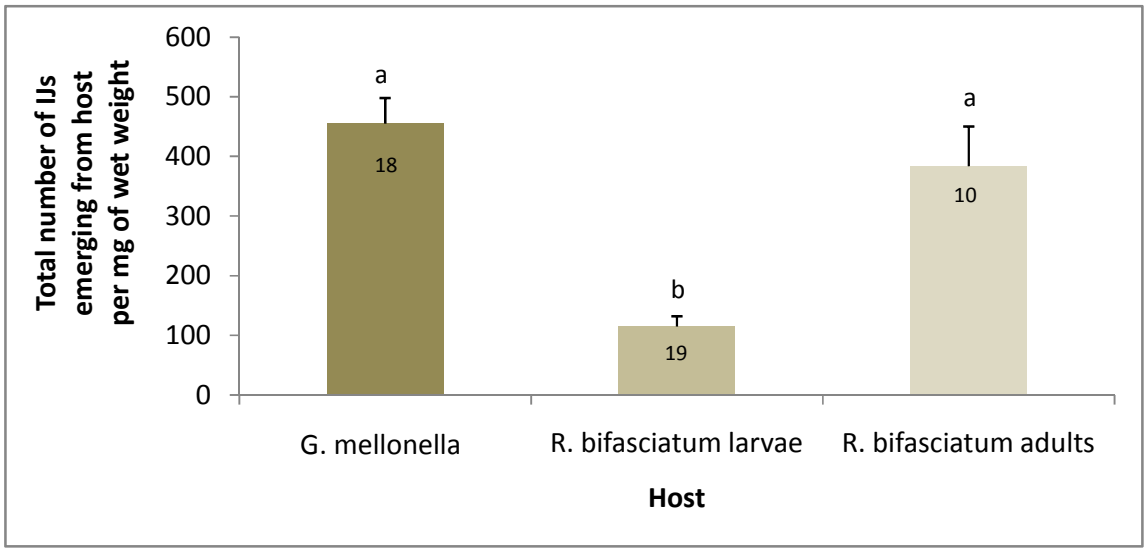


Fig 5.14: Mean number of IJs emerging (per mg of wet weight) over the entire 8 week period of emergence for laboratory infected *R. bifasciatum* and *G. mellonella*. Error bars represent standard errors and numbers in bars give N. Bars that are not labelled with the same letter are significantly differently from each other (One-way ANOVA followed by Tukey's test [$\alpha = 0.05$]).

5.3.13 IJ numbers in hosts after eight weeks of emergence

Hosts were dissected after the eight week emergence period if possible (in some cases, hosts had deteriorated to such a degree that there was no substantial amount of body mass left to dissect). A considerable number of *S. carpocapsae* IJs was found in *R. bifasciatum* cadavers upon dissection (Fig 5.15). There was a significant difference in the median number of IJs remaining in hosts among the host groups (K.-W.-test, $H = 18.69$, $DF = 3$, $P < 0.001$, see Fig 5.15 for N). In *R. bifasciatum* larvae, approximately 15,000 IJs (mean of larvae infected in logs) and 23,000 IJs (mean of larvae infected in the laboratory) were found in cadavers after eight weeks. This difference was not significant (M.-W. U-test, $W = 351.0$, $P = 0.390$, see Fig 5.15 for N). This meant that roughly 21 % and 45 %, respectively, of IJs that had developed in these two groups of *R. bifasciatum* larvae were still located inside host eight weeks after emergence had commenced.

In *R. bifasciatum* adults that were infected in the laboratory, these figures were much lower and significantly different (approximately 300 IJs on average which was equivalent to 2 % of total IJ production; M.-W. U-test, $W = 116.0$, $P = 0.0126$, see Fig 5.15 for N). The median number of IJs

remaining in both *R. bifasciatum* larvae and adults that were infected in the laboratory was significantly different from those remaining within *G. mellonella* larvae (M.-W. U-test, W = 190.0, P < 0.001 and W = 102.0, P = 0.562 compared with *R. bifasciatum* larvae and adults, respectively, for N see Fig 5.15).

The mean number of *H. downesi* IJs that were left behind in hosts ranged from 2,000 (*R. bifasciatum* adults from low concentration logs) to 50 (*R. bifasciatum* larvae from low concentration logs). There was a significant difference in the median number of IJs within hosts among these groups (K.-W.-test, H = 16.96, DF = 3, P = 0.001, see Fig 5.15 for N). Only one post-hoc comparison was carried out among these groups, namely between the high and low concentration *R. bifasciatum* larvae (M.-W. U-test, W = 773.5. P = 0.007, for N see Fig 5.15).

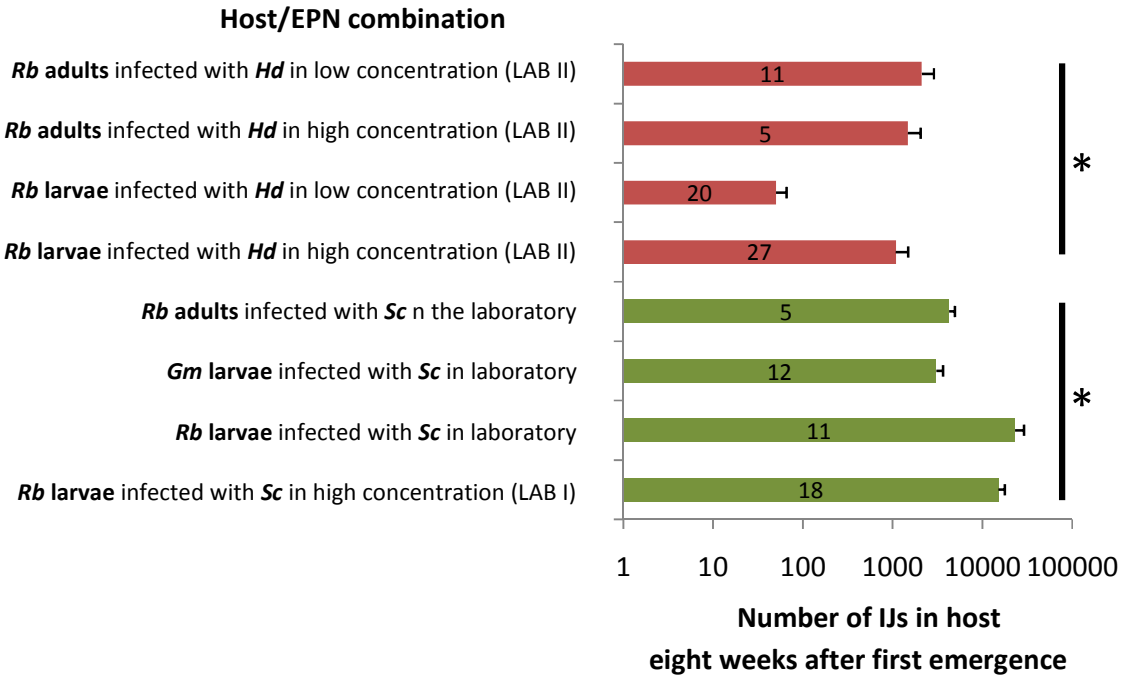


Fig 5.15: Mean number of IJs found in dissected hosts after the 8 week period of emergence. LAB = Laboratory Exposure Experiment, *Sc = S. carpocapsae*, *Hd = H. downesi*, *Rb = R. bifasciatum* and *Gm = G. mellonella*. Error bars represent standard errors and numbers in bars give N. Lines with an asterisk beside a group of bars indicates a significant difference among bars within that group (K.-W.-test; $\alpha = 0.05$).

Finally, a comparison of the median number of IJs remaining in *R. bifasciatum* larval hosts recovered from high concentration logs (*S. carpocapsae* and *H. downesi*) indicated that the number of IJs within a host after eight weeks of emergence was significantly higher for *S. carpocapsae* (lower-tailed M.-W. U-test, W = 403.0, P < 0.001, for N see Fig 5.15).

The proportion of IJs within hosts that were alive after eight weeks was generally much higher in *R. bifasciatum* hosts infected with *S. carpocapsae* compared with all others (Fig 5.16). No live IJs were found in all but four of the *R. bifasciatum* larvae that had been infected with *H. downesi* (therefore statistical analysis was not possible).

On average, approximately three quarters of the *S. carpocapsae* IJs found in *R. bifasciatum* larvae were still alive upon dissection, regardless of whether they were infected in a Petri dish or in a log (73 % and 75 %, respectively). There was no significant difference in the median number of live IJs between these two groups of hosts (M.-W. U-test, W = 480.0, P = 0.654, for N see Fig 5.16). The number of live IJs in *R. bifasciatum* larvae was also significantly higher than that in *G. mellonella* larvae when both were infected in the laboratory, regardless of whether the longhorn beetle was in its larval (lower-tailed M.-W. U-test, W = 104.0, P < 0.001, for N see Fig 5.16) or adult stage (lower-tailed M.-W. U-test, W = 78.0, P = 78.0, P = 0.002, for N see Fig 5.16). A comparison between the two stages of *R. bifasciatum* when it came to the median proportion of life IJs in a host showed no significant difference after Bonferroni-adjustment of the α-level, though significance was approached very closely (M.-W. U-test, W = 115.0, P = 0.0174, Bonferroni-adjusted α = 0.05; for N see Fig 5.16).

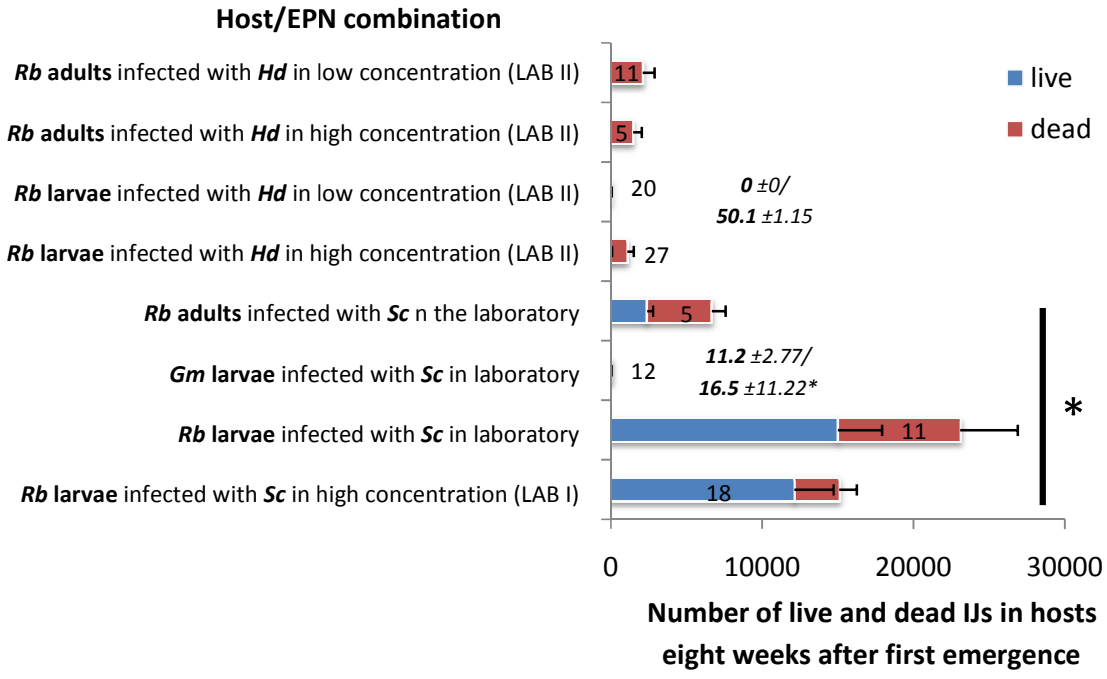


Fig 5.16: Mean number of IJs found in dissected hosts after the 8 week period of emergence (live or dead). Error bars represent standard errors and numbers in bars give N. Horizontal bars with an asterisk above a group of bars indicates a significant difference within that group (K.-W.-test). Values for groups which do not appear in the graph due to the y-axis scaling are given as the mean ± standard error in the appropriate position in the graph.

5.3.14 Infection of *R. bifasciatum* in decomposing logs on clearfell sites treated with EPN

A total of 1,997 *R. bifasciatum* individuals were found in the 113 decomposing logs that were sampled on clearfell sites on which tree stumps had been previously treated with *S. carpocapsae* IJs. A further 28 larvae and 17 pupae, all alive, were recorded in four logs that were collected in an area of the site at Kilworth in which stumps had been treated with *H. downesi* IJs. On four of the sites (Lackenrea, Ballymacshaneboy, Featherbed and Raheenkyle), all of which were sampled one month after EPN application, no infection of *R. bifasciatum* was recorded (Table 5.11). However, one *R. bifasciatum* larva infected with *S. carpocapsae* was found in a log from Featherbed one year after EPN application. This represented 0.7 % of all individuals in sampled logs on that site at the time.

At Kilworth and Deerpark, 7.3 % and 10.3 %, respectively, of all the *R. bifasciatum* individuals found in sampled logs were infected with *S. carpocapsae*. Infection had occurred in approximately 30 % of the sampled logs on both of these sites (Table 5.11). The site at Kilworth had been sampled only four weeks after EPN treatment of tree stumps to control *H. abietis*. At Deerpark, logs were sampled in the spring (March) eight months after *S. carpocapsae* had been applied to tree stumps on the site, indicating that EPN had persisted within logs for this period of time.

Data on log measurements and parameters as well as numbers of *R. bifasciatum* within individual logs for Featherbed, Raheenkyle, Deerpark and Kilworth are presented in Appendix A.8.

Table 5.11: Percentage of infected *R. bifasciatum* individuals (larvae, pupae and adults combined) in decomposing logs collected on clearfell sites after application of *S. carpocapsae* to tree stumps (Data for logs from area treated with *H. downesi* at Kilworth not included).

Site	*Time since EPN application*	*Number of logs sampled*	*Total* R. bifasciatum *individuals found*	*Percentage of* R. bifasciatum *infected with EPN*	*Percentage of logs sampled with at least one infected* R. bifasciatum
Lackenrea	1 month	20	70	**0 %**	**0 %**
Ballymacshaneboy	1 month	20	27	**0 %**	**0 %**
Deerpark	8 months	16	380	**10.3 %**	**31 %**
Featherbed	1 month	15	413	**0 %**	**0 %**
	12 months	15	238	**0.4 %**	**7 %**
Raheenkyle	1 month	15	349	**0 %**	**0 %**
Kilworth	1 month	12	520	**7.3 %**	**33 %**

5.3.15 Infection of *R. bifasciatum* in decomposing logs collected at Kilworth (4 weeks after EPN application)

a) Effect of distance to treated tree stump, developmental stage and depth in wood

In total, the logs collected at Kilworth four weeks after EPN application comprised 354.36 L of wood in which 565 *R. bifasciatum* individuals were found (1.59 individuals per litre of wood, logs from both *S. carpocapsae* and *H. downesi* treated areas included). Individuals infected by EPN were found in four of the 16 collected logs, all of them from cleared areas in which stumps had been treated with *S. carpocapsae* (Table 5.12). Within these logs, infection rates ranged from only 1.8 % in one log (a single infected larva to 55 live larvae) to approximately 26 % (20 infected larvae and 8 infected pupae out of a total of 108 individuals). Since EPN application on clearfell sites is targeted to tree stumps, it was predicted that infection of *R. bifasciatum* within decomposing logs would decrease with increasing distance to the nearest treated stump. This was investigated by regression analysis.

Table 5.12: Logs collected at **Kilworth** four weeks after EPN had been applied to site with distance measured from closest treated stump, percentage of infected *R. bifasciatum* (larvae and pupae) and the number of infected *G. mellonella* (out of ten) used to bait bulk wood samples (750 g) and bulk soil samples (100 g). S.c. = *Steinernema carpocapsae*, H.d. = *Heterorhabditis downesi*.

	EPN species applied to stumps	*Distance to stump (cm)*	*Dead* G. mellonella *(out of 10) in bulk **wood** sample*	*Dead* G. mellonella *(out of 10) in bulk **soil** sample*	***Number of infected* R. bifasciatum *individuals/total* (percentage)**
K1	*S.c.*	**97**	3	0	**0/80**
K2	*S.c.*	**37**	0	0	**0/28**
K3	*S.c.*	**52**	0	0	**0/40**
K4	*S.c.*	**22**	10	0	**28/108** *(25.9)*
K5	*S.c.*	**70**	0	0	**0/7**
K6	*S.c.*	**41**	0	0	**0/31**
K7	*S.c.*	**27**	8	0	**0/14**
K8	*S.c.*	**45**	0	7	**1/48** *(2.1)*
K9	*S.c.*	**56**	0	0	**0/3**
K10	*S.c.*	**15**	4	10	**8/66** *(12.1)*
K11	*S.c.*	**29**	6	1	**1/56** *(1.8)*
K12	*S.c.*	**58**	0	0	**0/39**
K13	*H.d.*	**54**	0	0	**0/8**
K14	*H.d.*	**62**	0	0	**0/3**
K15	*H.d.*	**27**	0	0	**0/17**
K16	*H.d.*	**358**	0	0	**0/17**

Both linear regression of the arcsin of the infection rate (response) against distance to stump (predictor) (Coef. = - 0.00179, P = 0.087) and binary logistic regression in which infection was treated

as binary data (any infection in a log) (Coef. = - 0.07367; P = 0.060) indicated that there was a negative relationship between infection of *R. bifasciatum* within logs and the distance between log and treated stump (Fig 5.17 and Table 5.13) though significance was approached in both cases.

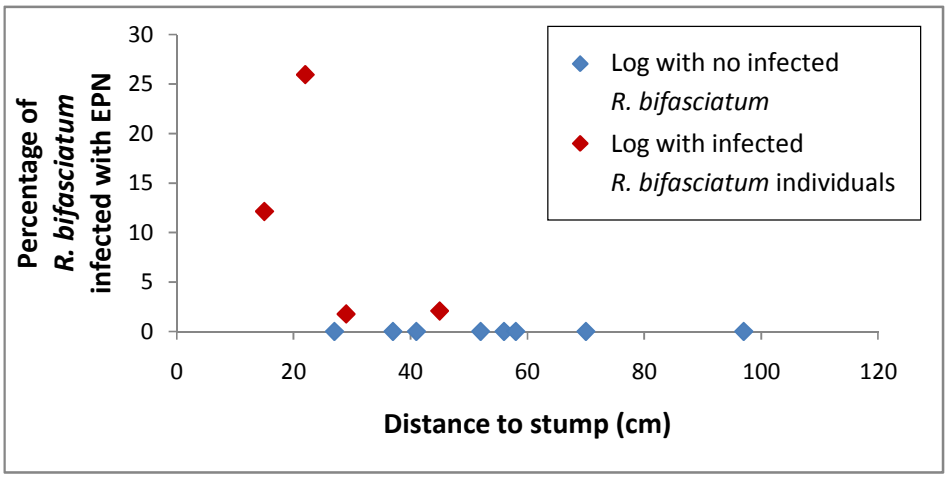


Fig 5.17: Infection rate of *R. bifasciatum* individuals (larvae and pupae combined) in logs collected at **Kilworth** in *S. carpocapsae* treated area plotted against distance to the nearest treated tree stump. N = 12 logs.

Table 5.13: Regression analysis results for **Kilworth** logs with the arcsin of infection rate regressed against the distance to closest treated stump. Only logs from *S. carpocapsae* treated areas were included. Link function with Pearson Goodness-of-Fit P-value is given for binary logistic regression. T given for linear regression, Z for binary logistic regression. N = 12.

Type	*Link function*	*Equation/ Coefficient*	R^2	*T or Z*	*DF*	*F*	*P*
Linear	-	y = 0.117 – 0.00179x	0.27	-1.90	1	3.60	**0.087**
Binary logistic	Normit (P = 0.584)	-0.07367	-	-1.88	-	-	**0.060**

The infection of *R. bifasciatum* individuals in the larval and pupal stage was compared statistically to see whether developmental stage affected susceptibility to EPN. No significant effect was detected, irrespective of whether individuals from all logs with any infection were combined (P = 0.715) or analysis was restricted to a single log (K4, see Table) to control for variation between logs (P = 0.395) (Table 5.14).

There was no significant difference in the length of live and infected *R. bifasciatum* larvae (M.-W. U-test; W = 16863.0, P = 0.644) or depth with logs at which they were located (M.-W. U-test; W = 12282.0, P = 0.746) (Fig 5.18). Individuals were, however, significantly more likely to be infected

by EPN in the side of log that was facing away from the soil (approximately 27 % infection rate versus 12 % in the side of logs facing the soil) and least likely to be infected in the centre (5 %) (Table 5.15).

Table 5.14: Results of χ^2-tests comparing infection rates of *R. bifasciatum* larvae and pupae in all logs from *S. carpocapsae* treated area of **Kilworth** site in which infection was recorded.

	***Infection of* R. bifasciatum *in logs collected in area treated with* S. carpocapsae**				
Logs included	***Larvae infected/total***	***Pupae infected/total***	***X^2***	***DF***	***P***
All logs with infected individuals	30/213	8/65	0.133	1	**0.715**
Log K4	18/80	8/26	0.725	1	**0.395**

Fig 5.18: Box and whisker plot presenting length and depth in wood of live and infected *R. bifasciatum* larvae from logs collected at **Kilworth** with any infection. Lines in boxes represent median, whiskers represent upper and lower quartiles. Black circles indicate outliers. Numbers below boxes give N.

Table 5.15: Number of infected *R. bifasciatum* individuals (larvae and pupae combined) found in three regions of a log with respect to its orientation in the field.

Number of infected* R. bifasciatum*/total* *(percentage)			
Facing soil	***Centre***	***Facing away from soil***	
16/129 ***(12.4)***	2/20 ***(10.0)***	18/67 ***(26.9)***	χ^2 = 7.346, DF = 2, **P = 0.025**

b) EPN presence in soil samples and wood samples for sampled logs and its association with *R. bifasciatum* infection

Bait insects (*G. mellonella*) were infected in bulk wood samples taken from five of the twelve logs collected in the area of the site in which stumps had been treated with *S. carpocapsae*. No nematodes were detected in wood samples from logs collected in areas of the site that were treated with *H. downesi* (Table 5.12). IJs were also detected in individual 10 g wood samples taken from the surface, outer and inner regions of some logs (Table 5.16; for the standard curve used for estimation see Appendix A.3).

Table 5.16: Estimated total number of *S. carpocapsae* IJs in the three 10 g wood samples taken from each region of **Kilworth** logs, as estimated from standard curve (see Appendix A.3). The percentage of infected *R. bifasciatum* individuals is given for each log. Totals per region are given in the bottom row. Each value given for a region is the total for three samples and thus N = 9 for totals across regions.

	Log region					
Log	*Surface*	*Outer*	*Inner*	*Total*	***Mean*** *± stdev*	*Percentage of* R. bifasciatum *infected in log*
K1	1	0	0	**1**	**0.33** *± 0.58*	**0.0**
K4	3	29	16	**49**	**16.00** *± 13.00*	**26.2**
K7	0	263	38	**301**	**100.33** *± 142.15*	**0.0**
K10	1	0	0	**1**	**0.33** *± 0.58*	**12.1**
K11	97	3	0	**101**	**33.33** *± 55.16*	**1.8**
Totals	**102**	**295**	**54**	**452**		

The highest estimated number of nematodes was found in samples from log K7, (301 in total) in which no infected *R. bifasciatum* individuals were found. EPN presence in logs as detected in 10 g wood samples matched results for the 750 g bulk wood samples (Table 5.12). A cross tabulation of logs in which *R. bifasciatum* infection coincided with EPN presence in wood samples was analyzed using Fisher's exact test, but no significant effect was detected (P = 0.222) (Table 5.17).

EPN were detected in soil samples taken from underneath three of the logs collected at Kilworth. The number of dead bait insects in these samples ranged from 1 to 10 out of a possible 10. All of the logs with soil samples containing EPN also contained infected *R. bifasciatum* individuals. analyzed using Fisher's exact test, a significant association between the detection of EPN in soils samples and any infection in the respective log was found (P = 0.005).

Table 5.17: Cross tabulation of any infection of *R. bifasciatum* in a log and EPN detection in any wood sample from a log collected at **Kilworth**. Only logs from *S. carpocapsae* treated area are included.

	EPN detected in wood samples	*No EPN detected in wood samples*	*Test results (Fisher's exact test)*
Infected *R. bifasciatum* in log	3	1	**P = 0.222**
No infected *R. bifasciatum* in log	2	6	

5.3.16 Infection of *R. bifasciatum* in decomposing logs collected at Deerpark (8 months after EPN application)

a) Effect of distance to treated tree stump

At Deerpark, logs were sampled eight months after *S. carpocapsae* IJs had been applied to tree stumps to control the large pine weevil. Sixteen logs were sampled on-site (Plate 5.3). Distance to the closest treated stump was measured for fourteen of these logs. Due to the constraints of working in the field, no measurements were taken regarding the position, length or depth in wood of *R. bifasciatum* individuals that were found (Table 5.18).

Table 5.18: Logs collected at **Deerpark** eight months after EPN had been applied to site with distance measured from closest treated stump, percentage of infected *R. bifasciatum* (larvae and pupae) and the number of infected *G. mellonella* (out of ten) used to bait bulk wood samples (750 g) and bulk soil samples (100 g).

	Distance to stump (cm)	*Dead* G. mellonella *(out of 10) in bulk **wood** sample*	*Dead* G. mellonella *(out of 10) in bulk **soil** sample*	***Number of infected* R. bifasciatum *individuals/total* (percentage)**
DP1	***N/A***	*N/A*	0	**0/13**
DP2	***N/A***	*N/A*	0	**0/17**
DP3	**58**	*N/A*	0	**0/12**
DP4	**12**	*N/A*	0	**10/35** *(29.4)*
DP5	**4**	*N/A*	0	**14/42** *(34.2)*
DP6	**60**	0	3	**0/8**
DP7	**295**	0	0	**0/3**
DP8	**268**	0	0	**0/20**
DP9	**358**	0	0	**0/17**
DP10	**14**	0	0	**0/10**
DP11	**74**	10	0	**0/31**
DP12	**57**	8	0	**3/23** *(13.0)*
DP13	**20**	10	0	**6/53** *(11.3)*
DP14	**75**	0	0	**0/23**
DP15	**49**	0	0	**0/61**
DP16	**170**	10	1	**6/12** *(50.00)*

Plate 5.3: Photographs showing the clearfell site at **Deerpark** (top left), log DP5 as it was found next to a treated stump (top right) and three *R. bifasciatum* larvae found in the log. The larva on the right was found alive, while the other two showed a colouration indicating infection with *S. carpocapsae*.

Infected individuals of *R. bifasciatum* were found in five of the sixteen logs that were sampled. Infection rates ranged from 50 % (6 out of 12 infected) to roughly 11 % (6 out of 47 infected) (Table 5.18). In those cases where log parameters were recorded, logs had little soil contact (no more than 20 % of log surface area in contact with soil for all such logs) and one log that contained infected *R. bifasciatum* had no soil contact at all (log DP13, see Appendix A.8). In total, 380 longhorn beetle larvae were recovered, 42 of which were infected with EPN (approximately 10 % overall infection rate). Only two adults were found, one of which was infected.

Aside from the log with the highest infection rate, all logs that contained infected individuals were found within 1 m distance from a treated stump (Fig 5.19). Regression analysis (linear or binary) did not produce normally distributed residuals and results were therefore not deemed reliable. Since all but two of the *R. bifasciatum* individuals found were in the larval stage, no investigation of a possible effect of developmental stage on infection with *S. carpocapsae* was possible.

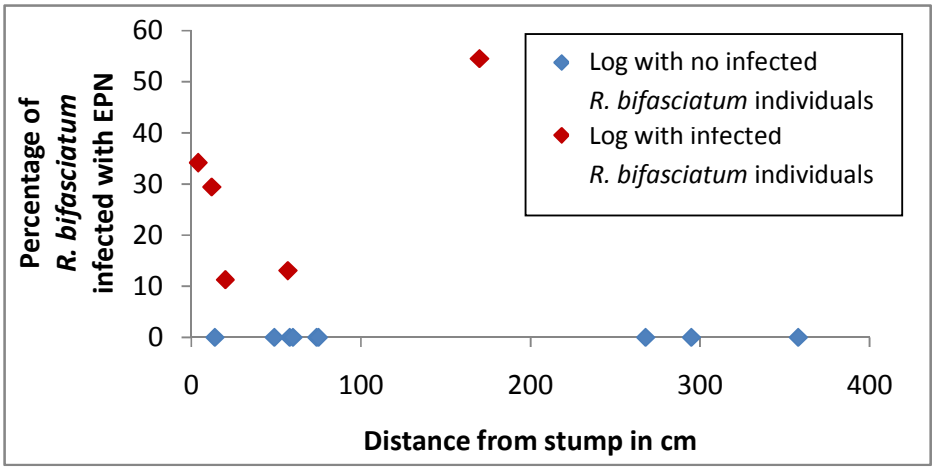


Fig 5.19: Percentage of *R. bifasciatum* individuals (larvae and adults) infected with EPN in logs sampled at Deerpark (in %, y-axis) plotted against distance to the nearest treated tree stump (in cm, x-axis). N = 14 logs.

b) EPN presence in soil samples and wood samples for sampled logs and its association with *R. bifasciatum* infection

EPN were detected in the bagged bulk wood samples from four of eleven logs for which data was available. One of these logs did not contain infected *R. bifasciatum* individuals. In wood samples from three of the four logs that contained infected *R. bifasciatum*, all ten *G. mellonella* larvae in the bag were infected. Three individual 10 g wood samples were baited for 13 of the sampled logs. These samples represented mixtures of wood from all regions of the log as they had not been separately taken from the surface, outer and inner regions of a log. The estimated number of *S. carpocapsae* IJs in some of the 10 g wood samples was very high, the highest being recorded for log DP16 (6551 IJs in sample 3) (Table 5.19). Consistently high estimates (> 500 IJs per sample) were obtained for all three samples from log DP5. However, considerable variation was found between samples from some logs (DP13, DP16). Only one log from which wood samples were positive for EPN contained no infected *R. bifasciatum* individuals (DP11). There was a significant association of EPN presence in the wood of a log and any *R. bifasciatum* within it being infected (P = 0.005) (Table 5.20).

EPN were detected in soil samples collected from under two of the logs at Deerpark and one of these logs had a high infection rate (50 %) whereas the other contained no infected longhorn beetle individuals (Table 5.18).

Table 5.19: Estimated number of *S. carpocapsae* IJs in individual 10 g wood samples taken during destructive sampling of **Deerpark** logs (for standard curve used for estimation see Appendix A.3).

	Sample					*Percentage of* R. bifasciatum *infected with EPN in log*
Log	*1*	*2*	*3*	***Total***	***Mean*** *± st dev*	
DP4	22	703	97	**823**	**274.0** *± 373.41*	**29.4**
DP5	605	4071	2572	**7249**	**2416.0** *± 1738.26*	**34.2**
DP11	3	8	3	**14**	**4.1** *± 2.89*	**0**
DP12	3	0	8	**11**	**3.7** *± 4.04*	**13.4**
DP13	121	6477	148	**6746**	**2248.7** *± 3661.87*	**11.3**
DP16	97	2126	6551	**8775**	**29.24.7** *± 3300.29*	**54.6**

Table 5.20: Cross tabulation of *R. bifasciatum* infection and EPN detection in any wood sample from logs at **Deerpark**. All 13 logs from which wood samples were taken are included.

	EPN detected in wood samples	*No EPN detected in wood samples*	*Test results (Fisher's exact test)*
Infected *R. bifasciatum* in log	5	0	**P = 0.005**
No infected *R. bifasciatum* in log	1	7	

Linear regression analysis of the mean number of infected bait insects from the three 10 g wood samples of each log (x) against the overall infection rate (y) revealed no significant connection between the two (T = 1.34, Coef. = 0.0062, R^2 = 0.37, DF = 1, F = 1.79, P = 0.273; N = 5, only logs with infected individuals included).

c) Combination of data from Kilworth and Deerpark

Due to the large number of differing variables between the two sites, including their geographic distance from each other, the difference in the previous crop (Sitka spruce and lodgepole pine at Kilworth and Sitka spruce and larch at Deerpark), the timing of sampling (4 weeks and 1 year after EPN application to site) etc. the results from the two sites could not be statistically compared with any confidence. In an effort to elucidate the relationship between the infection rates observed in logs from both sites and their distance to the closest treated tree stump, these data were combined for the two sites to increase the number of data points available for regression analysis. Since regression analysis using this complete data set resulted in residuals that were significantly different from a normal distribution and logs collected at a distance greater than 1 m from treated stumps were responsible for observations within the model that had large standardized residuals, two subsets of

the data were used for analysis: Logs in which at least one infected *R. bifasciatum* individual was found, or logs that were collected no more than 1 m distant from a treated stump. For this data, a highly significant connection was found between distance to a tree stump treated with EPN and the infection rate (arcsin of infection rate used; T = -3.58, Coef. = -0.00279, R^2 = 0.39, DF = 1, F = 12.83, P = 0.002, N = 21). This was also the case when presence of any infected individuals in a log was used a response variable in a binary logistic regression model (Normit link function with P = 0.438; Coef = -0.0454, Z = -2.62, P = 0.009). Moreover, the total number of dead bait insects in 10 g wood samples from a stump were significantly and negatively correlated to the distance of logs from stumps when logs were no more than 1 m distant from a treated stump (T = -3.27, Coef. = -0.261, R^2 = 0.36, DF = 1, F = 10.72, P = 0.004, N = 21).

5.3.17 *R. bifasciatum* in decomposing logs collected at Raheenkyle, Featherbed, Lackenrea and Ballymacshaneboy

At Raheenkyle, 234.75 L of wood were collected (four weeks after EPN application to the site) and a further 296.62 L and 301.69 L of wood were collected at Featherbed four weeks and one year after application of EPN, respectively (Appendix A.8). At Lackenrea and Ballymacshaneboy 261.34 and 184.49 L of wood, respectively, were collected. *R. bifasciatum* individuals found in the logs collected on these sites totalled 831 larvae, 161 pupae and 7 adults. None of the individuals collected at Raheenkyle or Featherbed four weeks after EPN application were infected, but a single infected larva was found in a log from Featherbed collected one year after EPN had been applied. This represented an infection rate of 7.7 % in this log (14 larvae in log) and an overall infection rate of only 0.7 % for that site and year. The log in question had been situated close to a treated stump (15 cm distance). No nematodes infected bait insects in the wood samples collected from the Lackenrea and Ballymacshaneboy log segments eight weeks after EPN application to these sites, nor was any infection of *R. bifasciatum* recorded.

Whether baited in bulk (750 g in plastic bag) or individually (10 g in Petri dish), no EPN were detected in any wood samples from Raheenkyle logs. The same was true for all wood samples collected at Featherbed four weeks after EPN application to the site. However, a single waxmoth larva was infected in the 10 g wood sample taken from the surface of a log one year after EPN application. This was a log that had been situated 16 cm from the closest treated stump. Nematodes were only detected in three soil samples from Featherbed, all of them taken under logs four weeks after EPN had been applied to the site. These logs were located 24 cm, 0 cm and 15 cm from the nearest treated stump. None of these logs contained infected *R. bifasciatum* or featured wood samples positive for EPN. Two soil samples from Raheenkyle scored positive for EPN. These logs had been situated close to treated tree stumps (22 cm and 0 cm, respectively) (see Appendix A.8).

5.3.18 Estimation of woody debris colonised by and available to saproxylic beetles on clearfell sites

a) **Survey of deadwood at Featherbed**

A cleared strip on this site was walked from end to end. Along this cleared strip, approximately five metres wide, 100 logs of a size suitable for colonisation with saproxylic beetles were found. Of these, 29 logs (29 %) were deemed to most likely harbour saproxylic beetles based on appearance of these logs. Data for these logs was grouped by whether or not they were adjacent to, close to (less than 50 cm distant from), distant from (between 50 cm and 1 m distant from) or away from (> 1 m from) a treated tree stump (Table 5.21). The majority of logs suitable for or already colonised by saproxylic beetles were found at distance greater than 50 cm from the closest treated tree stump (70 %).

Table 5.21: Number of decomposing logs adjacent to, close to (less than 50 cm distant from), distant from (between 50 cm and 1 m distant from), away from (> 1 m from) a treated tree stump encountered along a cleared strip on the **Featherbed** clearfell site. Logs are listed according to whether or not they appeared to contain *R. bifasciatum* individuals (+ or -).

	Number of logs situated			
Appearance of log	**Adjacent to stump**	**Close to stump**	**Distant from stump**	**Away from stump**
+ *R. bifasciatum*	1	6	11	11
- *R. bifasciatum*	3	17	18	23

In several areas at Featherbed, large logs were present on site in dense aggregations, representing either dead trees left behind after felling or trees fallen into the site from its edges after windthrow. Some of these logs were in stages of advanced decomposition (possibly they had been cut for thinning purposes before the site was clear cut and had been left on site or the windthrow had occurred before clearfelling). When these logs were sampled for *R. bifasciatum*, high densities of larvae were found (up to 50 within a 50 cm segment).

b) Survey of deadwood at Lackenrea and Ballymacshaneboy

Logs on this site were collected eight weeks after *S. carpocapsae* application to stumps on each site 70 larvae were found in logs from Lackenrea and 27 from logs collected at Ballymacshaneboy (Table 5.22). No pupal stages were found. None of the larvae or adults showed any signs of nematode infection and this did not change after they were incubated to allow for delayed mortality caused by EPN.

Table 5.22: Number of *R. bifasciatum* larvae and adults found in logs collected at **Lackenrea** and **Ballymacshaneboy**. Collection areas: CS = Cleared strip, LT = Lop and Top. Total wood volume sampled for each area is given. The mean number of individuals (adults and larvae combined) per 10 L of log volume is given as well. N = 10 for each site/area combination.

Site	*Area logs were collected*	*Total wood volume of logs (L)*	R. bifasciatum *recovered*	*Mean number of* R. bifasciatum *per 10 L of log volume)*
Lackenrea	CS	145.57	68	5.1
	LT	115.77	2	0.15
Ballymacshaneboy	CS	100.35	27	2.61
	LT	84.14	0	0

A General Linear Model in which the number of *R. bifasciatum* individuals found per 10 L of log volume was modelled against site and area (CS or LT) as predicting variables showed that site did not have a significant effect (F = 0.87, DF = 1, P = 0.357), but that the area in which logs had been found did (F = 7.15, DF = 1, P = 0.011). There was no significant interaction between the two variables (F = 0.69, DF = 1, P = 0.413). Of the 40 log segments that were sampled, no longhorn beetle larvae or adults were found in the seven logs that had been classified as belonging to category 1 (no decay), but 5 of the 21 logs in category 2 (little decay) and 6 of the 12 logs in category 3 (medium decay) contained at least one *R. bifasciatum* individual. Most individuals overall were found in logs of category 2 (83 individuals, 86% of the total) (criteria for assigning woody debris to decay categories see Appendix A.2).

No complete logs of fallen or cut trees were found on either of these two surveyed sites. Rough estimates of the total volume of wood debris available for colonisation by saproxylic insects in the area surveyed on each site were calculated from the measurements taken of CS and LT piles (Table 5.23). Standing deadwood was not taken into account, though approximately 10 small (< 20 m) dead trees were seen within the survey area at Ballymacshaneboy. No standing deadwood was present at Lackenrea, however. The majority of woody debris pieces found at both sites had a diameter of 10 cm or less. No complete logs or trees (standing or fallen) left behind after felling were found on either of the two sites surveyed.

Based on these measurements and the site dimensions and number of CS and LT per site, the total amount of deadwood in CS and LT on each of the two sites was roughly estimated (for formulas and procedure see Appendix A.6). The resulting estimates are given in Table 5.24.

Table 5.23: Measurements for deadwood in cleared strips (CS) and Lop and Top piles (LT) at **Lackenrea** and **Ballymacshaneboy** eight weeks after EPN application to these sites. All means are based on 20 measurements. Numbers in brackets reflect the total number of logs found in CS transects. All values ± standard deviation.

Site (area surveyed)	*Area*	*Mean width of CS or LT (m)*	*Mean height of LT piles (m)*	*Proportion suitable for saproxylic beetles (mean %)*	*Mean volume of wood found along CS (L)*	*Number of CS or LT on site*
Lackenrea	CS	**5.37** *± 1.9*	-	-	**13.57** *± 16.58 (25)*	20
(400 m x 400 m)	LT	**6.08** *± 1.24*	**0.15** *± 0.07*	**42.5** *± 16.42*	-	20
Ballymacshaneboy	CS	**6.96** *± 1.92*	-	-	**17.01** *± 27.84 (14)*	14
(160 m x 160 m)	LT	**4.73** *± 0.9*	**0.20** *± 0.08*	**42.5** *± 27.44*	-	14

Table 5.24: Woody debris available to saproxylic insects on **Lackenrea** and **Ballymacshaneboy** sites as estimated from measurements taken in September of 2007. * Values marked with an asterisk are corrected for irregularity in site shape (see Appendix A.6).

Site	*Area*	*Woody debris per 2 m section (in L)*	*Woody debris on site (in L)*	*Total volume of deadwood in m^3 per ha*
Lackenrea	CS	13.57	**27,140***	3.38*
	LT	387.60	**775,200***	96.90*
Ballymacshaneboy	CS	17.01	**19,051**	7.44
	LT	402.00	**450,240**	175.88

Based on the mean number of deadwood pieces found at each of the 20 locations surveyed within CS (1.25 at Lackenrea and 0.7 at Ballymacshaneboy), the site dimensions and the number of CS per site it was also possible to estimate the total number of deadwood pieces with a diameter of >5 cm within CS on each site (for detailed procedure and formula used see Appendix A.5). The resulting estimates were 2,500 such pieces in CS at Lackenrea and 784 in CS at Ballymacshaneboy. While these estimates include all deadwood that was available for colonisation by saproxylic beetles, based on observations made on site while searching for logs suitable for sampling as well as results from log incubation and destructive sampling (see Table 5.22) it was estimated that only approximately one in ten logs in CS areas were actually colonised. The estimated number of logs containing *R. bifasciatum* in CS was thus roughly 250 at Lackenrea and 78 for Ballymacshaneboy.

5.4 Discussion

Both *S. carpocapsae* and *H. downesi* infected *R. bifasciatum* larvae, pupae and adults within decomposing woody debris, which should not be surprising in light of the wide host range of these insect pathogens (Bathon 1996; van Lenteren et al. 2003). Click beetles also seemed to be infected at a similar rate judging upon the results gathered in Laboratory Exposure Experiment I. It can therefore be assumed that *R. bifasciatum* infection was representative of at least some of the other non-target insects commonly associated with woody debris on Irish clearfell sites. A laboratory assay investigating the pathogenicity of *S. carpocapsae* and *H. downesi* against *R. bifasciatum* in Petri dishes filled with 5 g of decomposing wood yielded mean LC_{50} values for beetle larvae at approximately 30 to 40 IJs per insect (14 days of exposure) (Khalil Alameen, unpublished data). Adult and pupal stages of *R. bifasciatum* were significantly more susceptible than the larval stage. These values are slightly higher than those reported for the Asian longhorn beetle *Anoplophora glabripennis*, for which a larval LD_{50} of 9 to 17 IJs of *S. carpocapsae* has been reported, depending on larval instar (Fallon et al. 2004). Nematodes are quick to kill *R. bifasciatum* larvae and adults, with LT_{50} values of three to six days for larvae, pupae and adults alike (concentration: 1000 IJs), longer than the two days it took *S. carpocapsae* took to kill *A. glabripennis* on filter paper at a concentration of 500 to 2,000 IJs (Solter et al. 2001; Khalil Alameen, unpublished data). The susceptibility of *R. bifasciatum* larvae to EPN appears to be similar to that of *H. abietis* larvae (Pye & Burman 1978).

The fact that *R. bifasciatum* individuals were infected in all regions of log segments and the high estimates for IJ numbers in many wood samples collected at the centre of logs that were treated with a high concentration of IJs suggest that both nematode species were able to move through the decomposing wood with relative ease. The behavioural ecology of EPN is complex and the age of IJs as well as the temperature, humidity and substrate texture all can influence nematode movement, activity and infectivity (Kaya 1993; Lewis et al. 2006). Interpretation of results regarding EPN efficacy, especially those recorded in the field, must therefore be approached with caution.

In the present study, infection rates recorded in Field Exposure Experiments were significantly lower than those recorded in the laboratory. The presence of EPN in wood samples from all regions of log segments was also significantly lower in the field experiments when compared with laboratory experiments. This was most probably the result of suboptimal temperatures and other abiotic or biotic stressors encountered by IJs in the field. Mean maximum temperatures in the field were about two to three degrees centigrade below the constant 20°C at which incubation was carried out in the laboratory. Air temperature may not accurately reflect soil or log temperature, however. UV radiation in sunlight is also known to rapidly kill or immobilize IJs, especially those of

heterorhabditids (Gaugler & Boush 1978; Gaugler et al. 1992). Many of the IJs applied in the field may have died before they were able to penetrate the wood and find shelter within, while IJs in laboratory experiments were not subject to this stressor. IJs on the surface of logs would also have been more exposed to UV radiation and thus may have been reduced in number quickly. When collecting logs in the field it was often noted that logs were noticeably warm to the touch on the side exposed to the sun.

Interestingly, a significant interaction was found between settings in which experiments were carried out (laboratory or field) and the EPN species that was applied to log segments (*S. carpocapsae* or *H. downesi*) with regards to the estimated number of IJs in wood samples at the end of an experiment. EPN species also interacted significantly with the log region (surface, outer or inner) in this regard. Compared with *H. downesi*, IJs of *S. carpocapsae* were found in significantly higher numbers in wood samples and numbers of *S. carpocapsae* in wood samples were higher than those of *H. downesi* for logs incubated in the field. This suggests that *S. carpocapsae* IJs either survived field conditions better than *H. downesi* IJs did, were less affected by field conditions with respect to their subsequent infectivity against the bait insects in the laboratory, or were more adept at penetrating logs. Temperatures that IJs of a particular species are not adapted to can severely affect their chances of survival (Smits 1996; Brown & Gaugler 1997). Steinernematids generally have a broader temperature range in which they are infective and mobile compared with heterorhabditids (Molyneux 1986). *Heterorhabditis downesi* comparatively fares poorly in standard pathogenicity assays at low temperatures (Griffin & Downes 1991; Adam Guy, personal communication). Differences in temperature adaptation between *S. carpocapsae* and *H. downesi* may have had an impact on their absence in wood samples.

Steinernema carpocapsae has been shown to be very resistant to dessication compared with other *Steinernema* spp., which may explain some of the higher persistence of this species in outer, more exposed layers of logs (Kung et al. 1992; Patel et al. 1997). For *H. downesi,* the number of IJs in the inner regions of logs exposed in the field was higher than that in the surface and outer regions, the reverse of what was observed in the laboratory. There was a statistically significant interaction between the region a wood sample was taken from (surface, outer or inner) and the EPN species that was applied to a log segment in connection with the estimated number of IJs in a given wood sample. Since there was no great difference between the number of IJs in the inner region of logs treated with *H. downesi* in the laboratory and the field (approximately 400 IJs in each case when a high concentration of IJs was applied), it is most likely that this change in EPN distribution was due to poor survival of *H. downesi* IJs on the surface and in the outer layer of logs rather than an increase in movement of IJs toward the core of the log. Though there were differences in IJ distribution between

the two EPN species, they both infected *R. bifasciatum* individuals at all depths within the log. The fact that infected insects within logs showed strong colouration and signs of progressed EPN infection and that emergence from insects infected in logs in the laboratory often occurred within days of removal from the log for both EPN species are an indication that some insects were infected within the first few days after application of IJs. The time to emergence recorded from longhorn beetles of all stages that were infected in log segments was, however, highly variable for *S. carpocapsae*, but not to the same extent in *H. downesi.* This could indicate that infection was more synchronized for *H. downesi,* occurring mainly early on in experiments, whereas infection of *R. bifasciatum* with *S. carpocapsae* was more protracted and occurred over the entire duration of the experiment. After this early bout of rapid IJ movement and infection, abiotic factors may have started to affect IJ distribution in the log differently for the two EPN species as discussed above. For example, daily minimum temperatures were particularly low in the second week of Field Exposure Experiment II and this may have disproportionately affected *H. downesi* IJs in the surface region of log segments. It is also conceivable that the differences in EPN estimates in log regions between species was due to different responses of the IJs to temperature stress, which then in turn would have affected baiting results (Saunders & Webster 1999). This should have been corrected to some degree by baiting at a constant temperature of 20°C, however.

The number of *S. carpocapsae* IJs in wood samples of treated log segments was significantly higher than that of *H. downesi* IJs. Not only that, *S. carpocapsae* IJs were also able to move into and through decomposing wood and infect insects within it at rates no different from *H. downesi.* These observations are somewhat surprising, seeing as the two species are thought to exhibit very different foraging strategies. *Steinernema carpocapsae* is commonly classified as an 'ambusher' (little dispersal and host seeking behaviour), while *H. downesi* is considered a 'cruiser' (dispersal and active host seeking) (Lewis 2002; Lewis et al. 2006). Published experimental data, however, suggests that the dispersal and movement rate of these EPN may be majorly dependent upon the substrate texture. *Steinernema carpocapsae* has been shown to move through peat soil as swiftly as heterorhabditid cruiser species (Kruitbos et al. 2010). Though IJs of both *S. carpocapsae* and *H. downesi* moved through columns of moistened coconut husk or frass produced by *R. bifasciatum* in laboratory assays, S*. carpocapsae* IJs performed better (author's unpublished data). Furthermore, *S. carpocapsae* has been used successfully in field trials against wood-boring pest beetles. It is thought that the nematode can find the host quickly and efficiently by following the frass galleries in the wood (Lindegren 1981; Fallon et al. 2004). Moore (1970) observed that given a sufficient moisture film for movement, IJs of *S. carpocapsae* strain DD-136 were able to penetrate pine bark to a minimum depth of 3-4 cm to infect *Dendroctonus frontalis* within an hour of application. *Steinernema carpocapsae* has also shown to be effective in controlling *H. abietis* in tree stumps,

suggesting that IJs of the species are capable of penetrating the bark of stumps and locating the pine weevil larvae within, often at considerable depths in the soil and along roots at a distance of 50 cm or more from the bole of the stump (Brixey et al. 2006; Dillon et al. 2006; Torr et al. 2007).

The porous structure of deadwood may have allowed some IJs to be carried directly into the inner regions of the log with the tap water suspension immediately upon application, accounting for some of the infections observed deeper within log segments. Infected *R. bifasciatum* individuals were, however, found in the inner regions of dry logs of low porosity (i.e. few holes, cracks, fissures or porous areas on surface of log segment), indicating that IJs were able to travel directly through the wood or along the frass galleries produced by longhorn beetle larvae. The latter seems more probable as wood density was high in most dry logs, especially toward the centre of the log, restricting any direct dispersal of IJs through wood (Portillo-Aguilar et al. 1999). The position of *R. bifasciatum* individuals within logs with respect to the soil or compost in experiments had no significant effect on the infection rate with the exception of *H. downesi* applied in the laboratory. This could mean one of two things: either IJs of both EPN species were mobile enough within logs to be able to move to the opposite side of a log after being poured on top (this would suggest that *H. downesi* was less successful at doing so in the lab) or IJs were migrating into the log from the soil or compost, to which half of the EPN concentration had been applied (again implicating *H. downesi* as being less successful at doing so). A conclusion on which of these two dispersal processes was more important in this context cannot be made based on the data available, though mobility of IJs in the logs seemed to be high enough to allow for traversal of the log diameter within the experimental period. Finally, differing infection rates recorded for the two EPN species in one area of the log compared with another could simply be a reflection of them responding differently to biotic and abiotic factors in the respective microhabitat (i.e. top or bottom half of the log) (Lewis 1992 & 2002; Grewal et al. 1997; Lewis et al. 2006).

Aside from their ability to move through decomposing wood, there is a question of how IJs were able to find *R. bifasciatum*, some of which were located at the end of frass galleries running through dense and dry areas of wood. IJs of cruiser species especially are known to show taxis toward host cues, such as volatiles, CO_2 or vibrations. Host cues are less important to ambushers (Lewis et al. 1992; Lewis et al. 1995 & 2006). In laboratory trials *S. carpocapsae* shows little to no attraction to chemical cues or host feces, but is attracted to artificially created vibrations (Lewis et al. 1992; Grewal et al. 1993; Torr et al. 2004). *Heterorhabditis megidis* was found to respond to vibrations as well, but heterorhabditid species in general are more responsive to chemical cues emitted by hosts, including host feces (Grewal et al. 1993; Boff et al. 2001; Torr et al. 2004). *Steinernema carpocapsae* IJs were not attracted to frass extracts of the Asian longhorn beetle and preconditioning of IJs in frass

extract actually had a suppressant effect on infection rates (Fallon et al. 2004). The presence of frass in Petri dishes with rotting wood did not increase infection of *R. bifasciatum* with *S. carpocapsae* or *H. downesi* in laboratory experiments, nor did frass extract have any discernable attractant effect on IJs of either species (McGann 2009). IJs of both EPN species that were applied to coconut husk columns wetted with longhorn beetle frass extract did not move through the columns any faster than those in columns in which the husk was wetted with tap water (author's unpublished data). Moreover, frass samples from logs to which *S. carpocapsae* were applied in the field contained fewer IJs than even the inner regions of logs. All of this suggest that chemical cues within the frass galleries or emitted by feeding *R. bifasciatum* larvae or inactive pupae and adults most probably did not play a significant role in guiding IJs of either species to their location. It may be the case that IJs simply travelled along the galleries by default as they presented a more permeable substrate compared with the denser wood within logs (Finney & Walker 1977; Lindegren 1981; Fallon et al. 2004). Vibrations created by feeding and moving larvae may have acted as additional attractants to IJs, especially once they were within galleries (Torr et al. 2004). This might also have applied to *H. downesi* IJs.

The developmental stage of *R. bifasciatum* (larval, pupal or adult) did not appear to affect susceptibility to EPN infection. Also, there was no clear association between the length of *R. bifasciatum* larvae and their susceptibility to EPN. Fallon et al. (2004) have reported that larval instar had a significant effect on infection of *A. glabripennis* larvae in laboratory assays. Admittedly, using the length of larvae as a variable to estimate developmental stage is not as accurate as would be desireable. Regression of length and head capsule measurements (the latter being a more commonly accepted indication of larval instar) of 242 *R. bifasciatum* larvae confirmed that there is a highly significant correlation between head capsule width and length of larvae (author's unpublished data). Since the application of EPN against pine weevil usually is recommended for early to mid summer (Brixey et al. 2006; Dillon et al. 2007), most *R. bifasciatum* adults that overwintered within logs should already have emerged at the time of application and therefore be safe from infection, thus providing little opportunity for phoretic dispersal of EPN (Duffy, 1953). Earlier application of EPN, e.g. in spring, may however increase this risk. Since the LT_{50} for adults has been found to be relatively short at roughly three to five days, the opportunity for phoretic dispersal over significant distances should be low even in this scenario (Khalil Alameen, unpublished data).

Though the initial infection of non-target insects caused by the EPN being applied as biocontrol agents may have a significant short-term effect on these populations, it is the reproduction and subsequent establishment and spread of EPN that poses a long-term risk. As the emergence data that was collected in the present study shows, both *S. carpocapsae* and *H. downesi* were able to reproduce and generate new IJs in *R. bifasciatum* hosts. When infected in log segments,

R. bifasciatum larvae infected with *H. downesi* nematodes were significantly more likely to show IJ emergence than those infected with *S. carpocapsae*, a possible consequence of these cadavers apparently being more resistant to fungal infection.

The number of IJs that emerged from cadavers was in the tens of thousands for both EPN species (means of 140,000 for *H. downesi* and 50,000 *S. carpocapsae*). Over the eight weeks of emergence, *R. bifasciatum* larvae infected with *H. downesi* within logs produced more IJs than *G. mellonella* hosts infected with the same species, while those infected with *S. carpocapsae* produced significantly lower numbers than both. Even if IJs remaining in *R. bifasciatum* larvae after those eight weeks are taken into consideration, the yield for *S. carpocapsae* in this host type was approximately 75,000 IJs, only about half the value recorded for *H. downesi*. Figures for *S. carpocapsae* emergence from larvae of saproxylic beetles that have been published range from below 10,000 per host (second and third instar larvae larvae of Asian longhorn beetle (*A. glabripennis*) exposed to concentrations of 10, 20 or 50 IJs) (Fallon et al. 2004) to approximately 50,000 IJs on average for *H. abietis* infected in treated tree stumps (Dillon 2003) and up to 150,000 in *A. glabripennis* of the third or fourth instar exposed to 2,000 IJs each (Solter et al. 2001). For *H. downesi*, mean emergence from *H. abietis* was 60,000 per larvae infected in tree stumps (Dillon 2003), while emergence of three *Heterorhabditis* species (*H. downesi* not among them) from third to fourth instar *A. glabripennis* larvae yielded over 300,000 IJs in some cases (Solter et al. 2001). Thus, EPN emergence recorded for *R. bifasciatum* in this study fell within the published ranges for saproxylic host larvae.

Increasing the dosage of IJs against larvae of the longhorn beetle *A. glabripennis* beyond 100 IJs did not lead to higher total IJ yields (Fallon et al. 2004) while in *G. mellonella,* IJ yield for *H. megidis* has been reported to peak at infection concentrations of 300 IJs (40,000 IJs emerging) (Boff et al. 2000). A reduction in IJ production in *G. mellonella* was observed for *S. glaseri* concomitantly with an increase in the number of invading IJs (Koppenhöfer & Kaya 1995). It is not possible to make any judgement on the absolute number of IJs invading *R. bifasciatum* in logs, though it is reasonable to assume that invasion rates in high concentration logs on average were higher than in low concentration logs. This could explain the difference in total yields for *R. bifasciatum* larvae infected by *H. downesi* in high concentration and low concentration log segments. In summary, the fact that *H. downesi* was able to produce much greater numbers of IJs in *R. bifasciatum* larvae than *S. carpocapsae* indicates that the former species was either more efficient at utilising the available resources or was simply better adapted to conditions in this host. Emergence trends of *S.carpocapsae* from *R. bifasciatum* larvae were significantly different from culturing host *G. mellonella*, regardless of whether the former were infected in logs or on filter paper. Mean weekly emergence from *R. bifasciatum* larvae infected with *S.carpocapsae* decreased in an almost linear

fashion and remained above 1,000 IJs for the duration of the 8 week monitoring period. *Heterorhabditis downesi* emergence had dropped to below 100 IJs within five weeks of first emergence and it dropped off significantly less sharply for larvae from high concentration logs compared with low concentration logs. The overall IJ yield from the former was significantly higher as well.

The observation that large numbers of *S. carpocapsae* IJs remained in *R. bifasciatum* cadavers but not *G. mellonella* cadavers after eight weeks of emergence is somewhat puzzling. Since this phenomenon was observed in larval cadavers infected in both logs and in Petri dishes lined with filter paper, experimental conditions cannot account for the lagging emergence of these IJs. San-Blas et al. (2008) have demonstrated that the accumulation of ammonia in *G. mellonella* cadavers is one factor that can trigger the emergence of *S. feltiae* IJs. If and how this applies to *S. carpocapsae* and *H. downesi* and how this process might lead to different emergence patterns for the two in *R. bifasciatum* is unclear, however. *Heterorhabditis downesi* has an oral tooth that it uses to penetrate the cuticle of prospective hosts (Bedding & Molyneux 1982; Dowds & Peters 2002). After eight weeks in a White trap, most of the *R. bifasciatum* larvae infected with *S. carpocapsae* were completely flaccid and showed signs of decomposition which should have made the cuticle more penetrable. Both *H. downesi* and *S. carpocapsae* appeared to preferentially begin emerging from the mouth of *R. bifasciatum*. This implies that a suitable avenue for leaving the host was available to both species from the beginning of emergence.

The ability of *S. carpocapsae* and *H. downesi* to reproduce in *R. bifasciatum* and to do so with comparatively high efficiency has implications for the risk of these EPN establishing, spreading and continuing to infect non-target hosts, particularly those within decomposing woody debris on clearfell sites (van Lenteren et al. 2003). First emergence of IJs took about two to three weeks on average from the time of infection for both EPN species (no significant difference) when infection was carried out in Petri dishes containing decomposing wood (author's unpublished data). This suggests that EPN could recycle two to three times if EPN application occurs early in summer. When taking into account that temperatures recorded in the field were considerably lower than in the laboratory during Exposure Experiments, this should significantly delay reproduction and subsequent emergence, thus reducing the extent of EPN multiplication in the field over the summer season (Dunphy & Webster 1986; Grewal et al. 1994; Brown & Gaugler 1997). Though previous studies have indicated that *S. carpocapsae* and most other *Steinernema* spp. are better suited to survive suboptimal temperatures than heterorhabditid species, both are known to be able to survive sub-zero temperatures and even freezing at least to some extent (Kung et al. 1991; Susurluk & Ehlers 2008; Sirjani et al. 2009). IJs, particularly those of *S. carpocapsae*, could therefore survive the winter

within the buffered confines of woody debris in the field, especially if they have an opportunity to multiply during the summer. IJs may also be able to overwinter within host cadavers, which could offer additional protection (Brown & Gaugler 1997). This appears a likely scenario, especially in light of considerable numbers of infective IJs staying within *R. bifasciatum* cadavers for two months or more as discussed above. Recycling is unlikely to occur during winter, as IJ movement and infectivity is severely reduce at temperatures below 15°C and EPN of most steinernematid and heterorhabditid species are not capable of developing beyond the IJ stage at temperatures below 10°C (Kaya 1993; Grewal et al. 1994b; Saunders & Webster 1999). Reduced air humidity in winter may also reduce IJ survival in woody debris in some areas of Europe where winter precipitation is low or air temperature drops significantly below the point of freezing (Kung et al. 1991; Kaya 1993; Brown & Gaugler 1997; Patel et al. 1997). In Ireland, temperatures are, however, generally not as low and precipitiation rates in winter are high. Also, the core of woody debris should be buffered against excessive dessication just as it is against temperature extremes.

Sampling of decaying logs on field sites treated with *S.carpocapsae* confirmed that recycling and persistence of this nematode was possible in a field setting. At Deerpark, the site at which the highest infection incidence of *R. bifasciatum* individuals was recorded, EPN had survived one winter following application. Based on the high numbers of IJs estimated to be in individual wood samples taken from logs (up to 8,000 in one case) there is a possibility that the nematodes had already recycled at least once in the spring that sampling occurred. Alternatively, IJs that had emerged from infected cadavers in the previous summer may have overwintered in the buffered environment of the log. *Rhagium bifasciatum* infection had already progressed in most individuals found at Deerpark and EPN of all developmental stages, including IJs, were found when the hosts were dissected in the laboratory. The same was true for infected insects taken from logs collected at Kilworth. EPN numbers in Kilworth wood samples were lower than in Deerpark samples, which may indicate that extensive recycling had not yet occurred in the four weeks that had passed since EPN application on that site. As was observed in Exposure Experiments, neither developmental stage nor depth at which individuals were located within logs seemed to have an effect on the likelihood of infection at Kilworth. A significant correlation between EPN presence in wood and infection of *R. bifasciatum* was only found for Deerpark logs. This fits with the hypothesis that recycling of EPN had occurred at Deerpark, whereas most of the IJs detected in samples from Kilworth were IJs that had survived since EPN application to the site and did therefore not originate from infected insects. *R. bifasciatum* individuals that were located on the side of the log facing away from the soil were more likely to be infected at Kilworth – another indication that it was the originally applied EPN that had probably been misdirected and sprayed across the surface of logs that had infected most *R. bifasciatum* individuals.

Most importantly, though results were not significant for each site individually, infection of *R. bifasciatum* decreased with increasing distance of sampled logs from treated tree stumps. A highly significant effect was found when including logs 1 m or closer to stumps. This suggests that increasing the accuracy of EPN application will significantly reduce the impact on non-target hosts on clearfell sites, though hitting logs lying directly adjacent to stumps would seem unavoidable. The survey conducted at Featherbed highlighted that about two thirds of logs in the cleared areas that potentially harboured saproxylic beetles were situated within 1 m distance of stumps. IJs migrating to logs from the point of application (stump) could account for a portion of the logs with infection, but studies have shown that the horizontal dispersal of *S. carpocapsae* IJs in soil is limited such that extensive invasion of logs found at distances greater than 50 cm is unlikely (Poinar & Hom 1986; Dillon et al. 2008a). Moreover, the maximum distance at which EPN were found around treated tree stumps at the time of log sampling at Kilworth was 40 cm from stumps and was low even at that distance (Chapter III).

EPN were detected in soil samples taken from under three of the four logs with infected longhorn beetle individuals, a significant correlation, but the number of infected *G. mellonella* larvae was low in two of these samples. No EPN were detected in soil samples from under one of the logs that contained a high proportion of infected individuals, but were found in soil samples collected under logs at Featherbed when the site was first sampled. None of the logs on the latter site contained infected insects, however. All of these data converges to support of the notion that it is spillage or inaccurately applied IJ suspension that accounts for the majority of intial *R. bifasciatum* infection within logs. EPN were also detected in some of the soil samples collected from under logs sampled at Deerpark. This suggests that IJs recycling within logs may be migrating into the soil below – a process that could contribute to on-site persistence of EPN in soil (Smits 1996). No infection of *R. bifasciatum* was found in logs on any other site, regardless of how close logs were situated to treated stumps. It is possible that local weather conditions on some of these sites reduced the likelihood of IJs surviving within logs – for example, the site at Raheenkyle was located on the side of Mount Leinster at a relatively high altitude which might be associated with cooler temperatures and increased rainfall that would wash off EPN applied to the surface of logs. One log at Featherbed contained a single infected larva one year after EPN application and EPN were detected in wood from another. This interesting find suggests that EPN can persist in woody debris at very low levels and in isolated patches and do not necessarily multiply enough to permeate entire logs, despite the relatively self-contained, buffered nature of this environment. Patchy, low-level persistence is commonly found after inundative application of EPN in the first one or two years after application (Klein 1992; Smits 1996; Dillon et al. 2008).

Overall, significant levels of infection of the wood-decomposer *R. bifasciatum* were only recorded when a high concentration of nematodes was applied (the 'worst case scenario'). Especially in the field, low concentration application of EPN had little to no adverse effect and EPN numbers in the wood were low. Even so, EPN are clearly able to recycle in non-target host *R. bifasciatum* and persist within decomposing logs for at least 8 months after application of EPN, as evidence by the results from field sampling at Deerpark. Taking into consideration the large amount of fine woody debris that is available to *R. bifasciatum* on Irish clearfell sites, the location of most of this debris at a safe distance from tree stumps as was found at Featherbed and the targeted nature of EPN application around tree stumps it can be concluded that risk to this species and probably also other saproxylic insects is relatively low. The non-target risks of EPN could be reduced further if spillage and inaccurate application of EPN is avoided. To this end, forestry personnel should be adequately trained and the equipment used to deliver nematodes should restrict leakage and spillage and allow precise application of the nematode suspension.

The vast majority (> 95 %) of deadwood that was left behind on the surveyed sites after felling was aggregated in 'Lop and Top' (LT) piles. When sampling the sites for logs containing saproxylic beetles, however, the cleared strips (CS) seemed to contain many more colonized logs and branches than the LT piles. This may simply be a result of sampling bias as the core and basal area of LT piles not accessible to sampling. Since wood moisture content and also wood decay can be expected to be greater in these areas of the LT piles, it is likely that logs suitable for and perhaps already colonised by saproxylic insects are present in greater numbers than reflected in the collected data. For example, Økland et al. (1996) report that the level of decay of woody debris was significantly associated with saproxylic beetle diversity in the mature forest in Norway they investigated. The overall estimated volume of woody debris in cleared strips on both surveyed sites (Lackenrea: 3.38 m^3/ha, Ballymacshaneboy: 7.44 m^3/ha) fell within the range of values published for mature coniferous stands in the UK and the temperate Southern region of Scandinavia. Fridman &Walheim (2000) report deadwood volumes of 4.9 m^3/ha in managed pine stands in the temperate region of Southern Sweden and 2.8 m^3/ha in spruce stands within the same region. In the Britain, the highest amount of deadwood conifer plantations was 21 m^3/ha, with half of the investigated sites containing less than 10 m^3/ha (Kirby et al. 1998). However, sampling and estimation techniques differed significantly between the present study and those cited and thus make comparisons among the resulting estimates problematic (Brown 2002). For example, Fridman & Walheim restricted their survey to snags and complete fallen logs (diameter > 10 cm), both of which were absent on two of the Irish sites that were surveyed (Lackenrea and Ballymacshaneboy), though some complete logs containing high numbers of *R. bifasciatum* were found at Featherbed. They did not include deadwood of a diameter < 10 cm. Such smaller pieces of woody debris (down to 5 cm of diameter as

was also the case in the present study) were included in the survey by Kirby et al. (1998), however, which may help account for a portion of the difference in deadwood volumes they report compared with Fridman & Walheim (2000). The survey protocol used at Lackenrea and Glendine was only suitable for producing rough estimates and allowed for large margins of error. None the less, the data collected showed that considerable amounts of smaller, more particulate deadwood are present on Irish clearfell sites after clearfelling, especially when Lop + Top piles are retained on site. The woody debris recorded on the surveyed sites was mostly small in diameter (< 10 cm) and should therefore be classified as 'fine' woody debris, as opposed to 'coarse' woody debris which generally is defined by a diameter > 10 cm (Brown 2002). Within standing forests, coarse woody debris is generally considered to be of major importance in terms of maintaining the diversity of saproxylic insects (Økland et al. 1996; Grove 2002). Økland et al. (1996) found that saproxylic beetle diversity in a Norwegian spruce-dominated forest increased significantly as the diameter of deadwood increased.

Management of standing forests and clearfelling can dramatically reduce the amount of coarse woody debris, as snags and fallen decomposing trees will be damaged and broken down by forestry machinery and any freshly cut logs will be removed immediately after felling (Fridman & Walheim 2000; Siitonen 2001). Standing deadwood is an especially valuable habitat on clearfell sites, as increasing height of stumps correlates with an increase in saproxylic beetle biodiversity (Jonsell & Weslien 2003; Abrahamsson & Lindbladh 2006). At Ballymacshaneboy and on other sites that were not included in the survey, standing deadwood was encountered and was found to be colonized by saproxylic beetles, including *R. bifasciatum* – but its volume was insignificant compared with the woody debris that lay scattered around the CS on these sites and was piled up in the LT. Not much data has been published on the ecological significance of fine woody debris on clearfell sites. However, a recent study by Jonsell et al. (2007) found that fine woody debris may be particularly important for the saproxylic fauna. They collected deadwood that ranged from only 1 cm to 15 cm in diameter and included spruce on clearfell sites in Southern Sweden and found it to harbour large numbers of saproxylic beetles, some of which were red-listed (Jonsell et al. 2007). *Rhagium bifasciatum* is known to specifically colonise moist and rotting wood in the early to intermediate stages of decomposition (Duffy 1953, Twinn & Harding 1999, Johansson et al. 2007; author's observation). The majority of *R. bifasciatum* on a clearfell site will therefore most likely be found not in the freshly cut branches and logs produced during felling activity (though these may be colonised in subsequent years), but in woody debris that predates felling and was produced in the mature stand. Since the majority of wood piled up in L + T strips would represent dry, freshly cut wood, the abundance of *R. bifasciatum* and other saproxylic beetles should be low in this area of the site and this prediction was borne out by the data. Overall, 75 of the 77 *R. bifasciatum* larvae that were found

in logs collected at Ballymacshaneboy and Lackenrea were from logs that had been situated in the CS.

As trials to control the pine weevil in Ireland continued in the summer of 2010, L+T piles were being removed from sites after felling to facilitate application of EPN to stumps situated below them (Aoife Dillon, personal communication). This practice, if expanded, will dramatically reduce the amount of wood available for saproxylic colonisation on sites, especially two to three years after felling at which point the L + T mounds will present a diverse habitat comprising a sheltered core and an outer layer of sun-exposed deadwood in addition to the deadwood originating from before felling that is in advanced stages of decomposition. Økland et al. (1996) isolated habitat diversity (i.e. the availability of deadwood at different stages of decay, varying diameter etc.) as one of the most significant predictive variables for saproxylic biodiversity in a mature spruce-dominated forest in Norway. The data published by Jonsell et al. (2007) suggest that this may also apply to coniferous clearfell sites. They found that on such sites, fine woody debris (diameter 1 – 4 cm) had high saproxylic beetle diversity, though not as high as wood of greater diameter (5 cm – 15 cm). Many of the beetle species they identified were significantly associated with deadwood of a particular diameter class. The authors also point out, however, that overall diversity was lowest for spruce clearfell sites (compared with aspen, birch and oak) and therefore believe that the removal of fine woody debris will be least problematic on such sites (Jonsell et al. 2007). Also, since the proportion of logs colonised by saproxylic beetles appeared to be highest in CS on Irish clearfell sites, removal of LT piles should have a disproportionately lower impact on the overall abundance of saproxylic beetles on-site immediately after felling.

With the considerable amount of fine woody debris that is present on Irish clearfell sites after felling, saproxylic beetles are of great importance as they help break down this deadwood and thus facilitate the release of nutrients while the replanted crop is growing. *R. bifasciatum* is susceptible to EPN and frequently is abundant on clearfell sites at the time of nematode application. However, as the results presented here indicate, large-scale application of EPN against the large pine weevil does not appear to pose a significant risk for this group of service-providing insects. While infection of *R. bifasciatum* was recorded on three of the six clearfell sites sampled, it was generally restricted to areas close to EPN treated tree stumps and live individuals were found even in logs that contained high numbers of IJs. Moreover, the more targeted and accurate the application of EPN to tree stumps is and the less accidental spillage of nematode suspension occurs, the lower the impact on *R. bifasciatum* and other saproxylic insects is expected to be. Correct training and management of personnel carrying out EPN application should therefore reduce infection of non-target hosts to a minimum.

CHAPTER VI

The effect of host condition and behaviour on parasitism rate and foraging strategy of *Bracon hylobii* (Hymenoptera: Braconidae)

6.1 Introduction

EPN species *S. carpocapsae* and *H. downes*i have proved to be very successful in infecting immature *H. abietis* in tree stumps and suppressing their emergence from them (Brixey et al. 2006; Dillon et al. 2006 & 2007). Their wide host range and their ability to recycle in the environment may, however, pose some risks to non-target organisms on clearfell sites (Smart 1995; McEvoy 1996; Smits 1996; Lacey et al. 2001; Dillon et al. 2008b). Some of these effects may be indirect – for instance, applying EPN against the pine weevil could cause intraguild competition and/or predation between the nematodes and other parasites and parasitoids of the target host (Georgis & Hague 1982; Polis et al. 1989; Shannag & Capinera 2000; Lacey et al. 2003; Everard et al. 2009). Processes that affect intraguild interactions and their outcomes can be very complex, especially if multiple parasites are involved or predation or competition occurs at multiple trophic levels (Polis et al. 1989; Polis & Holt 1992; Rosenheim et al. 1995; Holt & Polis 1997). In the case of the *H. abietis*, the number of associated parasitoids appears to be comparatively limited (Kenis et al. 2004).

The most common parasitoid of *H. abietis* larvae is the gregarious, idiobiont ectoparasitoid wasp *Bracon hylobii* (Hymenoptera: Braconidae; Ratzeburg 1848) (Wharton 1993; Henry, 1995; Kenis et al. 2004). It is widely distributed within the range of its host and has been reported in most European countries in which *H. abietis* is considered a forestry pest, including the UK and Ireland (Von Waldenfels 1975; Gerdin 1977; Henry & Day 2001; Hilszczanski et al. 2005; Dillon et al. 2008b). Once they have located a host, female wasps penetrate the bark covering it with their ovipositor. The wasp then injects paralysing venom. Paralysed hosts cease to move and feed, though they remain responsive to agitation (author's observation). In the laboratory, wasps may parasitize up to 17 hosts, laying an average of six to seven eggs on or beside each (Henry & Day 2001).

At optimal conditions (20°C), development from larva to cocoon takes approximately ten days. Eclosion of adult wasps occurs after another ten to twenty days (author's observation; Henry 1995; Henry & Day 2001). Parasitism of *H. abietis* on clearfell sites with a *B. hylobii* population usually

ranges between 20 % and 30 %, but can be as high as 90 % (Hanson 1943, Henry 1995; Dillon et al. 2008; author's observation). This shows that *B. hylobii* can exert a considerable amount of control on pine weevil at naturally occurring population densities. Efforts to augment natural populations and increase parasitism rates have been moderately successful, but use of *B. hylobii* as an inundative biological control agent is prohibitively labour intensive at the present time (Henry 1995, Henry 1999; Henry & Day 2001). EPN may interfere with local parasitoid populations after application, either by directly infecting the larvae or pupae of the parasitoid or by affecting larval development on the shared host (in this case *H. abietis*), due to the insect-pathogenic symbiotic bacteria EPN release after invading a host (Kaya 1978; Battisti 1994; Zaki et al. 1997). Conserving local populations of parasitoids to complement the efficacy of other, introduced biocontrol agents - including EPN – can, however, benefit the control of the pest insect (Murdoch et al. 1985; Lacey et al. 2003).

Little is known about the host-finding strategies employed by *B.hylobii*. The hosts are cryptic and usually sheltered by a layer of bark as well as soil (*H. abietis* larvae in tree stumps mainly remain below the soil horizon when feeding in stumps) (Leather et al. 1999), so direct visual cues that aid some parasitoids in locating a host cannot be a major component of the *B. hylobii* foraging strategy (Henry 1995). Two other host cues could be pivotal: volatiles released by the host (and/or its substrate, in this case the bark) and vibrations caused by the host as it moves within the bark. Numerous studies have showed that braconid parasitoids respond to volatiles specific to hosts and host plants and can be attracted by volatiles over great distances (Udayagiri & Jones 1992; Geervliet et al. 1994; Rutledge & Wiedenmann 1999; Reddy et al. 2002). It is thought that the more specific a parasitoid is with regards to its host, the more specific the volatiles it responds to (Hedlund et al. 1996; Röse et al. 1998). Short range host finding can also be aided by such olfactory attractants (Mills et al. 1991; Xiaoyi & Zhongqi 2008). *Bracon hylobii* females have been shown to move toward the source of volatiles released by *H. abietis* feeding on pine bark, but attraction to host or bark volatiles alone was weak. Experienced wasps showed a stronger response (Faccoli & Henry 2003).

Vibration and sound has been linked to parasitoid wasp behaviour, especially in cases where the host is cryptic (Meyhöfer et al. 1997; Meyhöfer & Casas 1999; Xiaoyi & Zhongqi 2008). Some insects are attracted from great distances by sound waves generated by members of the other sex and in parasitoid flies, acoustic signals created by the host insect (e.g. crickets) can help the fly in long-range host location and can even induce larvaposition in the absence of a host (Allen et al. 1999; Pollack 2000). In wasp parasioids, vibrations more commonly play a role in short-range host searching and detection (Meyhöfer & Casas 1999; Xiaoyi & Zhongqi 2008). Some studies have shown that wasps alter their searching patterns or behaviour when the host is creating vibrations in the substrate ('vibrokinesis'), while others have found that vibrations originating from host movement and feeding

help the wasp move toward the host in a directed fashion to pinpoint its location in the respective host microhabitat ('vobrotaxis') (Meyhöfer & Casas 1999). Some parasitoid wasps can cause vibrations of their own by 'drumming' their antenna on the substrate – they can then use the spread of vibrations to locate hosts within the substrate in a process analogous to echolocation as used by bats. This strategy has been observed mostly in parasitoids foraging on thin and flexible substrates, such as plant leaves (Meyhöfer et al. 1997). Laboratory experiments have shown that echolocation is more energy-consuming on denser substrates and its importance in host location decreases accordingly (Fischer et al. 2003). Efficacy of echolocation has also been shown to be lower for smaller females (Otten et al. 2001). Very few studies have provided any direct evidence of host movement being a necessary component in the location of cryptic hosts and fewer still have quantified the vibrations created by the host. Females of a number of parasitoid species associated with cryptic hosts appear to be unable of efficiently locating hosts that are dead, etherized or otherwise prevented from moving (Lawrence 1981; Cheah & Coaker 1992; Meyhöfer & Casas 1999). Non-random host searching and probing activity has also been elicited by application of artificial vibration in some parasitoid wasps, including braconids (Lawrence 1981; Meyhöfer et al. 1997; Meyhöfer & Casas 1999).

Experienced female wasps (i.e. wasps that have laid eggs successfully at least once before) are thought to become more effective in their foraging for hosts as they learn to associate novel cues with the host ('associative learning') or by becoming more responsive or sensitive to innate host-associated cues (Lewis & Tumlinson 1988; Du et al. 1997; Faccoli & Henry 2003; Meiners et al. 2003). The question of how *B. hylobii* is affected by EPN application on clearfell sites is an important one, as it not only addresses the possibility of non-target effects due to nematodes, but could also have relevance for the efficacy of *H. abietis* control with EPN. If competition or intraguild predation between the two parasitic organisms is low, then a complementary biocontrol effect of the two can be expected (provided *B. hylobii* are present on the site being treated) (Lacey et al. 2003; Everard et al. 2009).

The offspring of parasitoid wasps relies on the mother to find and choose suitable hosts for oviposition, as it is fully dependent on the host for development. This is especially true for idiobiont parasitoids, since the host will not develop any further once parasitism has occurred. The main foraging stages that lead to oviposition are host habitat location, host location and host acceptance. Wasps may follow a succession of hierarchical cues as they move closer to the host microhabitat and finally the host (Völkl 2000; Vinson 1976). Host suitability and quality then affects the development of wasp progeny (Vinson 1980). The instar and size of the larval host can have a significant effect on the developmental success of wasp progeny (Vinson & Iwantsch 1980). Moreover, infection or

predation of host larvae by other parasites (e.g. entomopathogenic bacteria, fungi or nematodes) can reduce host quality and lead to the loss of wasp progeny or the transmission of the host-associated pathogen within the parasitoid population (Vavre et al. 1999; Brodeur & Rosenheim 2000; Lacey et al. 2003). Though females of some parasitoids are reported to distinguish between pathogen infected hosts and healthy hosts, in many cases – sometimes depending on the progression of host infection - they do not appear to be able to do so (Sait et al, 1996; Hoch et al. 2000; Lord 2001; Lacey et al. 2003; Down et al. 2005; Everard et al. 2009; Mbata & Shapiro-Ilan 2010).

While the susceptibility of parasitoids to EPN in the laboratory has been established in several cases, investigation of non-target effects on parasitoids following inundative application of EPN are sparse (Bathon 1996). Battisti (1994) reports that emergence of two ichneumonid wasps from soil was suppressed by as much as 66 % when EPN were inundatively applied around spruce trees to control the web-spinning sawfly (*Cephalcia arvensis*). *Bracon hylobii* parasitism rate in tree stumps to which EPN species *S. carpocapsae* was applied to control against the large pine weevil was not affected by the nematodes (Dillon et al. 2008b). However, in the laboratory both larvae and eclosing adults of *B. hylobii* were found to be highly susceptible to EPN infection. Everard et al. (2009) were also able to demonstrate that *B. hylobii* wasps are less likely to parasitize *H. abietis* larvae infected by *H. downesi* when given a choice between an infected host and a healthy host. Investigations into the foraging behaviour of *B. hylobii* and how it may explain these observations should provide valuable data to help gauge the risk of intraguild predation occurring between the parasitoid and the nematodes when the latter are applied to tree stumps to control the large pine weevil.

The **aims** of this Chapter were to:

- assess whether there is a risk to *B. hylobii* populations on Irish clearfell sites following EPN application to tree stumps to control the large pine weevil. Eggs that are laid on EPN-infected *H. abietis* larvae do not develop. If wasps can detect EPN infection in live host larvae, it would be expected that they would parasitize such hosts significantly less frequently than healthy hosts. To compare parasitism of infected and healthy hosts, female *B. hylobii* were offered single *H. abietis* larvae that were covered by a small bark patch and that were either healthy or at various stages of infection with *S. carpocapsae* or *H. downesi.* Parasitism and behaviour of naïve and experienced wasps was compared to test whether experience confers an advantage to *B. hylobii* females in detecting host infection.

- investigate how wasps respond to infected *H. abietis* and other hosts, including hosts *B. hylobii* is not known to be associated with (*G. mellonella* and *R. bifasciatum*). It was predicted that if *B. hylobii* can detect EPN infection in hosts and determine host quality upon

encounter with it in general, wasps would be able to locate infected and unfamiliar hosts, but would be observed to reject them once this was achieved. To this end, wasps and hosts in some of the oviposition experiments were observed instantaneously in 5 minute intervals for two hours. However, if the failure of wasps to parasitize certain hosts was related to problems with locating them, wasp behaviour and parasitism should primarily be influenced by host movement and activity if the wasp relies on vibrational cues, or volatiles released by host and substrate (see next aim).

- study the behaviour of wasps in relation to host movement and host feeding activity. Female *B. hylobii* may use vibrational cues to locate their host (Hanry & Day 2001) and the movement and feeding activity of *H. abietis* larvae may be reduced by infection with EPN. As recommended by Meyhöfer et al. (1999), continuous behavioural observations were conducted on a large bark patch (20.25 cm^2) to test whether wasps responded to host movement. If *B. hylobii* uses vibrational cues, it would be expected that they respond to host movement with vibrotaxis and/or vibrokinesis. Also, dead hosts (killed either by EPN or by freezing) supplemented with artificially created vibrations were offered to wasps to test whether they would be attracted to such hosts and possibly even parasitize them.

Observational experiments in particular would give some valuable insights into the foraging strategy of *B. hylobii* as an example of a parasitoid that targets a cryptic host and allow some predictions about the risk of intraguild predation occurring between EPN and *B. hylobii* in the field.

6.2 Material and Methods

6.2.1 Source of *Bracon hylobii* wasps

The original *B. hylobii* culture maintained at NUI Maynooth consisted of wasps supplied by Dr Paddy Walsh (Galway, Mayo Institute of Technology; Ireland), but wasps reared from cocoons collected from tree stumps on clearfell sites at Glendine, Kildalkey and Clonava (see 2.5) were introduced to be used both for culturing and experiments throughout.

6.2.2 Standard arena used for wasp culturing (also used for oviposition trials with and without instantaneous observation)

The arena consisted of the bottom halves of two 9 cm Petri dishes taped together with masking tape to form a chamber 9 cm in diameter and 2 cm high. A hole approximately 1 cm in diameter was melted into the top centre of the chamber. Hosts were offered in perspex chambers. A single host insect was placed in one of two chambers on a perspex slide. Chambers were created by taping a piece of clear perspex (dimensions 7.5 cm long by 3 cm wide, 4 mm thick) with two 0.9 cm diameter holes drilled approximately 2.5 cm apart to a standard microscopy slide (Menzel GmbH, Braunschweig; Germany) with a strip of masking tape (see Fig 6.1). After each use, all perspex pieces were separated from tape and glass slides and washed thoroughly in 70 % ethanol and then by tap water to remove any wasp- or host-associated scents or contamination.

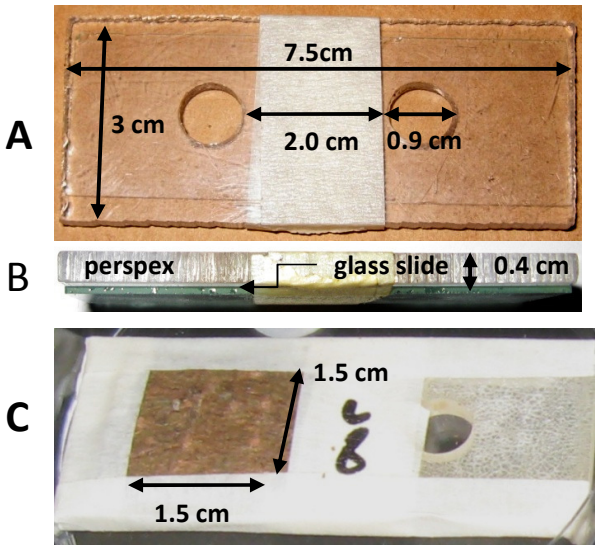


Fig 6.1: Perspex slide with two chambers in top view (**A**) and side view (**B**) with dimensions. (**C**) Prepared perspex slide with host in chamber under a 1.5 cm by 1.5 cm bark patch (left side of slide) as used in the standard arena setup for oviposition trials. (For culturing, both chambers contained a host).

A piece of fresh bark approximately 2 cm by 2 cm was stripped from a freshly cut Sitka spruce log (approximately 5 to 10 year old trees were used) with the aid of a scalpel and a chisel. Logs providing bark were cut on field sites, stored at 4°C and used for no more than two months. Approximately 1 - 2 mm of sapwood was carefully stripped from the underside of each bark patch, resulting in bark patches of approximately 1-2 mm thickness. The underside of each patch was painted with red food colouring (Goodall's; Dublin, Ireland) to facilitate the recording of the area of bark chewed by the host in the course of the trial. One bark patch each was then carefully fastened in place over each chamber containing a host by taping it to the slide along all four edges. A bark area of approximately 2.25 cm^2 (1.5 cm by 1.5 cm) that covered the host chamber was thus exposed to the wasp (Fig 6.1).

A 1.5 cm diameter piece of filter paper (Whatman No 1) saturated with a 50:50 honey/tap water solution was placed inside the arena, beside the perspex slide. Wasps were transferred into the arena via the hole in its top using a pooter and the hole was covered with a strip of masking tape. A wad of wetted tissue paper was placed over the tape to maintain moisture in the arena. Plate 6.1 shows an arena with host in place, just prior to introduction of the female wasp.

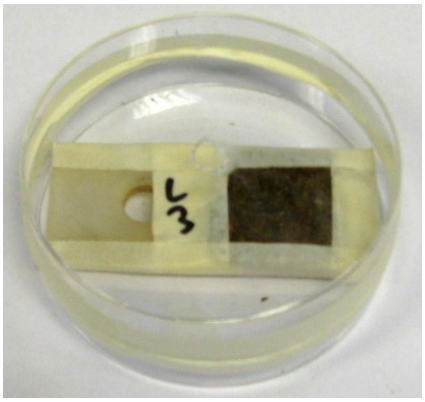


Plate 6.1: Completed arena (wasp not introduced yet) used in no-choice trials with *B. hylobii*. Arenas used for culturing wasps were identical, but had a host larvae in both of the chambers of a perspex slide.

6.2.3 Culturing of *B. hylobii* wasps

Female wasps were allowed to mate for at least five days before being used for culturing (Everard et al. 2009). Wasps were provided with two live pine weevil larvae (weight > 100 mg) in a standard arena as described in section 6.2.2. Wasps were allowed to oviposit for 24 to 48 h after which time hosts were removed. The bark patch was removed from each host and where eggs were present, the chamber was covered with a piece of filter paper, masking tape was wrapped around the perspex slide and ten to twenty slides were incubated together at 20°C in a plastic container with a lid (15 cm

diameter, 20 cm high). A layer of tissue paper (approximately 3 cm deep) that was moistened with tap water was added to the bottom of the container and the inverted base of a 9 cm diameter Petri dish was placed on top of this layer to prevent direct contact between slides and tissue paper. Slides were checked every week until wasp larvae had spun cocoons, at which point the cocoons were transferred to a 50 ml plastic tube. A hole was melted into the cap of each tube to allow ventilation and a layer of gauze was inserted between the cap and the end of the tube (Plate 6.4). Cocoons were stored at either 4°C to induce diapause for long-term storage, or allowed to develop and eclose at 20°C. Eclosing wasps were collected daily from plastic tubes with a pooter and transferred to a separate tube that was identical to the one used for storage of cocoons (Plate 6.2).

Plate 6.2: Plastic tube used for storage and incubation of *B. hylobii* cocoons.

6.2.4 Arena used for continuous observation of wasps and hosts

This arena was specifically used for continuous observations with the Observer™ software (Noldus Information Technologies; Wageningen, Netherlands). A hole 0.9 cm in diameter was drilled through the centre of a square of perspex 5.5 cm by 5.5 cm in dimension and 0.5 cm thick. This piece of perspex was then taped directly to the inside of the base of a 9 cm Petri dish. A host was placed into the chamber at the centre thus created and was covered by a patch of bark 4.5 cm by 4.5 cm in dimension that was prepared as described for the standard arena (Plate 6.3). In some trials, freeze-killed or EPN-killed hosts (*H. downesi*) were offered to wasps while also scratching the bark from below manually. In these instances, a small hole (0.3 cm in diameter) was melted through the centre of the Petri dish base of the arena beside the host, allowing access for the needle that was

used to scratch the bark. The needle used was a standard stainless steel dissection needle with a diameter of approximately 50 µm at the very tip. The arena was identical to the standard arena in all other aspects, though no filter paper with honey/water was included and the hole for wasp introduction was placed off-centre so as not to obstruct the view during observation.

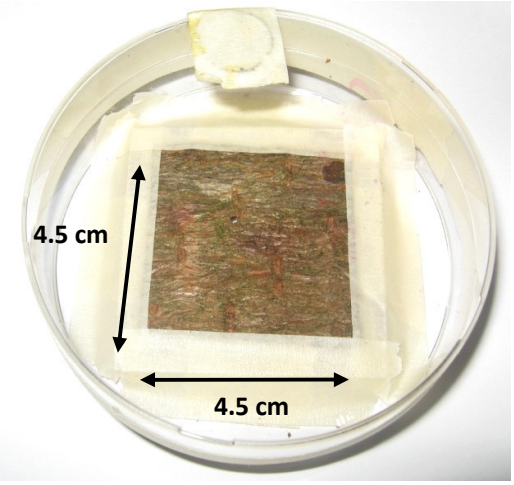


Plate 6.3: Completed arena without wasp used in no-choice continuous observation trials with the Observer™ software.

6.2.5 Arenas with added scratching or poking stimulus

Mechanical scratching and poking of the bark patch from below was intended to simulate feeding and/or movement of a *H. abietis* larva. The arenas used in this setup were similar to the standard arena, with the exception that a hole in their base allowed entry of a wire that would scratch or poke the underside of the bark patch that was covering the host (depending on the setup). Arenas receiving a scratching stimulus were only 3.5 cm in diameter and 1.5 cm high. In each of the two setups used to apply either a scratching or poking stimulus, several arenas were mounted on a platform that was designed in such a way as to allow a motorized articulation of the wires entering into each chamber. For the scratching stimulus, the platform accommodated ten arenas receiving no scratching (control) and ten receiving scratching, arranged side by side. The wires were articulated by a driveshaft connected to an electric motor (11 rpm = 22 scratches per minute). For the poking stimulus, six arenas were accommodated on the platform and the wires were moved up and down by a rotating plate placed under the platform to which each wire was connected (set to 10 rotations per minute = 10 pokes per minute). The scratching stimulus and poking stimuli were applied at alternating 15 minutes intervals for the first 12 h of the experiment and 30 minute alternating intervals for the remaining 12 h. The rationale for adjusting the timing of the application for these stimuli in this way was two-fold: first, the periodicity was adjusted to approximate the movement

and feeding of a live *H. abietis* larva in accordance with the observations of host behaviour made during oviposition trials (see 6.2.12); second, interspersing periods of applying the stimulus with periods of inactivity would not only reflect observations of live hosts, but would also allow wasps a period in which to lay eggs without being disturbed by the wire in the chamber. A complete description of the design of each platform and the changes made to arenas in comparison to the standard arena used for oviposition trials and culturing are given in the Appendix (A.10 and A.11).

6.2.6 Host insects

Pine weevil larvae (*H.abietis*) larvae and other hosts used were stored as described in section 2.1. When using *H. abietis*, only larvae weighing more than 150 mg were used in oviposition trials (observed and unobserved) and continuously observed trials. All host insects were weighed immediately before setting up trials. Unless otherwise stated, all hosts were incubated for two hours at room temperature after removal from 9°C storage before use in trials to allow acclimatisation.

a) Control

Live, untreated *H. abietis* larvae were used for control trials. In those cases where they were used in controls in parallel to trials with hosts exposed to nematodes, they were incubated at the same temperature and for the same time period as their counterparts and exposed to the same volume of tap water in which IJs were applied to hosts exposed to EPN. All other control hosts were incubated for 2 h at room temperature before trials.

b) Hosts infected with EPN

Infected: Hosts insects were exposed to a dose of 6000 IJs in 100 µl of tap water for 48 h prior to the experiment. Exposure was carried out in a 24 well plate plate, each chamber lined with three layers of filter paper (Whatman No 1). IJs of *S. carpocapsae* or *H. downesi* were used to infect *H. abietis* larvae. Larvae that were not dead after this exposure period were considered to be putatively infected with nematodes when going into the trials. All putatively infected hosts were washed three times in tap water to remove excess IJs before being introduced into a trial. Larvae were prodded with forceps and only those hosts that responded with movement were used in trials. Hosts found dead after 48 h of exposure to EPN were used as EPN-killed hosts (see next).

EPN killed: These hosts were *H. abietis* cadavers showing clear signs of nematode infection (based on cadaver colouration and consistency) that had been dead for 24 h to 48 h before trials they were used in commenced.

c) Freeze-killed

These were *H. abietis* larvae that had been chilled at -20°C until frozen solid (no less than 15 minutes) and then allowed to thaw for 2 hours before being used in the respective experiment.

d) Paralysed

Paralysed hosts were *H. abietis* larvae that had been parasitized by *B. hylobii* in the 24 h preceding the trial they were used in, either in an oviposition trial or culturing of wasps. These hosts were washed three times in tap water before use to remove eggs from first parasitism and any scent associated with the wasp previously parasitizing the host. That these hosts were alive before they were introduced into trials was confirmed by prodding them with forceps.

e) Prepupae

These were *H. abietis* larvae that were in the transitional stage between the final larval instar and the pupal instar (still in larval cuticle, no formation of pupal morphological structures such as wings or legs). Hosts in this stage were easily identifiable based in the morphology of the thoracic segments (segments directly posterior of head capsule) and a change in the angle of the head capsule with respect to the body axis (larval stage: 45°, prepupal stage: 90°).

f) *H. abietis* with glued mandibles

A drop of super glue (approximately 20 µl; B&Q super glue liquid; B&Q, Eastliegh, England) was carefully applied to the mandibles of a *H. abietis* larva and allowed to set for 15 minutes before the host was placed in its chamber and introduced into the trial arena.

g) Glue control

This host had a drop of super glue (approximately 20 µl) placed carefully on the back of its head capsule (Fig 6.2). The glue was allowed to set for 2 hours before introducing the host into the trial arena. Before use of these hosts, each was checked for the ability to freely articulate their mandibles by prodding them until they opened their mandibles in response.

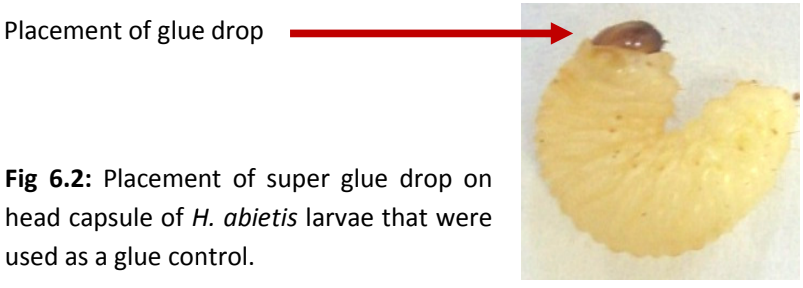


Fig 6.2: Placement of super glue drop on head capsule of *H. abietis* larvae that were used as a glue control.

h) Hosts other than *H. abietis*

Oviposition trials included three hosts not known to be associated with *B. hylobii* in the field. Two of these hosts, *R. bifasciatum* and *A. striatum*, are the saproxylic larval stage of longhorn beetles, whereas *G. mellonella* belong to a different order of insects (Lepidoptera), and their natural habitat are bee hives where they feed on wax. All of these hosts were weighed prior to use. Only *G. mellonella* larvae falling in a weight range identical to that of *H. abietis* larvae (150 mg to 300 mg) were used. Due to the limited availability of *R. bifasciatum* and *A. striatum* larvae, no restrictions were placed on their weight, though the majority of *R. bifasciatum* larvae used were within the same weight range delineated for *H. abietis*.

i) Empty:

An empty chamber without a host.

6.2.7 General conditions for *B. hylobii* oviposition trials

All trials conducted were of the no-choice type (i.e. only one host offered to each wasp at a time). The trials were conducted using standard arenas (see 6.2.2). All experiments and observations described in this chapter were carried out in a climate room set to 20°C and a 24 h light period. Arenas were placed on white plastic trays for the duration of trials and were arranged in random order. Trials described in this section were conducted over a one year period (June 2009 to June 2010) and results for all trials were pooled for analysis.

Trials were conducted according to the availability and the timing of emergence of *B. hylobii* females as well as the availability of *H. abietis* larvae of each type used (i.e. infected with EPN, pupating etc.). It was therefore not possible to include the same number of trials for each host type in each set of trials. However, a proportion of control trials with live *H. abietis* larvae were included each time a set of trials was conducted. Any trials in which the wasp died during the trial period or in which the host larvae escaped from its chamber during the trial were also excluded from analysis.

Hylobius abietis larvae that were exposed to EPN prior to a trial (*S. carpocapsae* or *H. downesi*) did not always die during the 24 h trial period. If they survived the trial, they were placed in a well of a 24 well plate and incubated at 20°C for five days after the trial ended. If the larvae died during this time (or if it had died during the trial period), it was considered as confirmation of EPN infection and the respective larva was classified as such for data analysis. If it did not die, however, it was considered to be a healthy, uninfected host and data collected for this host larva was then included in the control data set.

To get a measure of the amount of bark that was chewed by the host during the 24 h of the trial, after most of the trials the underside of bark patches was scanned (Epson Stylus DX3850; Epson) and saved as 800 dpi, 24-bit Bitmap files. The absolute area of chewed bark was then determined by importing the image to imaging software (GIMP 2.0, Sun Microsystems) and calculating the chewed area in pixels.

6.2.8 Age and experience of wasps

Both naïve and experienced wasps were used in trials with all host types (except prepupal hosts which were only offered to naïve wasps). Wasps were allowed to mate for five days before use and were not given access to host before trials. Naïve wasps were five days old when introduced into trials. Experienced wasps were seven days old and had successfully oviposited once in the 48 h preceding the experiment. After their first oviposition, experienced wasps were stored individually at 20°C in the arenas they had first laid eggs in (the perspex slide with the host was removed from the arena). They were supplied with fresh 50:50 honey/water solution and wet tissue paper during storage. In some cases, naïve wasps that laid eggs on infected hosts were retained and subsequently used as experienced wasps. In some of the earlier trials with EPN - infected *H. abietis* hosts, wasp were used in two trials (once as a naïve wasp and again when experienced). In those cases it was noted which *H. abietis* host the wasp had parasitized when naïve – a healthy host or an infected host. Wasps were not given access to males once they had oviposited.

6.2.9 *B. hylobii* oviposition trials

In these trials, the following host types were included: Live control (live *H. abietis*), *H. abietis* putatively infected with *S. carpocapsae* or *H. downesi and H. abietis* killed by EPN (*S. carpocapsae* or *H. downesi*). *H. abietis* killed by freezing and thawing, paralysed *H. abietis*, *G. mellonella*, *R. bifasciatum* and empty chambers. After the end of the 24 h trial period, the condition of host larvae (alive or dead) and the number of eggs laid by the wasp was recorded and the area of bark that had been chewed on the underside of the bark patch was also determined (see section 6.2.7).

6.2.10 Oviposition trials in which hosts were prevented from chewing on bark

To examine whether the chewing of bark by *H. abietis* larvae had an effect on the parasitism rate of *B. hylobii*, trials that included only three types of *H. abietis* host larvae were conducted separately from the other oviposition trials. Hosts included: control (live *H. abietis* larvae), glue control and glued mandibles. The latter host type was offered to wasps in two different contexts: a) host with glued mandibles covered by bark patch (as in glue control and control) or b) host with glued mandibles in a chamber to which approximately 25 mg of wood shavings were added and chambers

were covered with a bark patch that had been chewed upon previously (wood shavings and chewed bark patches were produced by allowing a live *H. abietis* larvae not used in experiment to feed for 2 to 6 h before the start of each trial). The number of eggs that was laid on each host as well as the area of chewed bark on the underside of the bark patch were determined at the end of the trial.

6.2.11 Oviposition trials with artificial scratching or poking

In this set of trials, wasps were either offered a live control host (*H. abietis*), a freeze-killed host (*H. abietis*) or a *H. abietis* larva that had been killed by infection with *H. downesi*. In each set of trials that was run on the scratching platform, an equal number of control arenas (no scratching) and arenas receiving scratching of the bark in the host chamber (scratching) were included for each of the host types used (i.e. if two freeze-killed hosts were included in the scratching arenas, two would be included among the control arenas with no scratching). At least two trials with live hosts were included each time this experiment was conducted.

For instance, if two trials with live hosts, two trials with an *H. downesi*-killed host and four trials with a freeze-killed host were included one set of trials, each in an arena with a wire scratching the bark, the same number of trials for each host type was set up in arenas receiving no scratching (controls). The arenas along the scratching platform were labelled 1 through 10 and the position of each pair of a 'scratching' arena and its corresponding control arena along the scratching platform was randomly determined with each new set of trials to control for differences in the vibration created by the wires. Not all ten arenas on the scratching platform were used in each experiment.

The number of eggs on each host was determined after the trial had ended and the presence of scratching marks on the underside of bark patches from scratching arenas was confirmed to ensure that bark in scratching arenas had indeed been scratched during the 24 h trial period.

In trials with artificial poking wasps were placed into arenas which had a host in the chamber receiving a poking stimulus (either a freeze-killed *H. abietis* larva or a *H. abietis* larva killed by *H. downesi*). All other methods used were identical to the ones used in standard arena oviposition experiments.

6.2.12 Instantaneous behavioural observation of wasp and host during oviposition trials

In a subset of the oviposition trials described in section 6.2.9, wasps and hosts were observed for 2 h. Trials in which naïve and experienced wasps were offered the following hosts were observed: control (live *H. abietis*), *H. abietis* infected with EPN (*S. carpocapsae* or *H. downesi*) but alive at beginning of trial, *H. abietis* killed by EPN (*S. carpocapsae* or *H. downesi*), freeze-killed *H. abietis*, prepupal *H. abietis*, paralysed *H. abietis*, empty host chamber and *G. mellonella* and *R. bifasciatum* hosts.

Observations spanned a 2 h period beginning immediately after wasps had been introduced to arenas and all observations commenced between 14:00 and 20:00. No more than 24 trials were observed at any given time. Each wasp was observed for five seconds every five minutes, resulting in 24 separate instances of recorded behaviour for each wasp ('instantaneous behavioural observation'). The following component behaviours were distinguished and recorded as binomial data (i.e. it was noted whether they did or did not occur at least once within the five seconds a wasp was observed for each record):

1.) **Movement**: Any locomotion by wasp, including flight
2.) **Antennation:** Wasp was seen antennating the substrate
3.) **Turning:** Wasp was seen performing any turn (either on the spot or while walking on the substrate) that resulted in a change of orientation of at least 90°
4.) **Waiting:** Wasp was seen assuming a characteristic, still position that was apparently associated with host searching behaviour. This behaviour could be distinguished from the normal sitting behaviour the wasps usually assumed when still (Fig 6.3)
5.) **Probing:** Wasp was seen probing the substrate with its ovipositor (Fig 6.3).

It was also recorded whether or not the wasp was seen on the bark patch at any time during the five second observation period or not.

Immediately after the behaviour of the wasp was recorded, the arena was gently lifted and the host was observed in its chamber through the base of the arena for five seconds. For hosts, it was only recorded whether or not they showed any movement during each five second observation period, including any movement of head or body contractions). Moving the arena in this way had no apparent effect on wasp behaviour. Since paralysis induced by host venom affected host movement, the proportion of records with movement for each host was calculated by excluding observations made after oviposition had commenced. Since the component behaviours '**probing**' and '**waiting**' were almost exclusively observed when the wasps were on the bark patch covering the host, these component behaviours were considered to be closely associated with wasp searching behaviour and host location within the experimental host microhabitat as represented by the bark patch and host chamber. Since 'antennation' of the substrate and 'turning' were observed frequently when wasps were both on and off the bark patch, these were assumed to be associated with general wasp foraging behaviour, but not necessarily indicative of localised searching behaviour in the host microhabitat. For data analysis, wasp '**searching**' was therefore defined according to the criteria listed in Table 6.1: a given record was only thought to represent '**searching**' by a wasp if the wasp was on the bark patch and performed the component behaviours 'moving', 'antennation' and 'turning' at least one time each within the five second observation period, or if the wasp was

'waiting' on the bark patch. Probing was treated as a separate response variable in data analysis as it was clearly distinguishable from all other behaviours and was directly indicative of wasps attempting to locate a host. Probing records when a wasp was not on the bark patch were very rare (< 0.1 % of probing records) and were thus not included in data analysis (Table 6.1).

A '**bout**' of behaviour was any stretch of uninterrupted behavioural records for a wasp. For example, if a wasp was probing on the bark for three successive records, this was classified as a probing '**bout**' of 15 minutes duration. When calculating the duration of '**bouts**' or the time to the first record of a certain behaviour, each record represented a unit of 5 minutes (i.e. a 'bout' that covered 5 records was considered to have lasted 25 minutes and if probing was first observed on the tenth record, time to first probing was considered to be 50 minutes). If wasps were recorded off the bark patch after they had been recorded searching or probing on the record immediately previous, this was considered to constitute an aborted searching or probing '**bout**'.

Table 6.1: Criteria for wasp and host records taken every five minutes during 2 h instantaneous observation of no-choice trials with *B. hylobii* females.

Subject	Record type	Alternative	Criteria
Wasp	*Location*	**'On bark'**	Wasp was on the bark patch
		'Off bark'	Wasp was not on the bark patch
	Behaviour: ***'Searching'***	**'Searching'**	Wasp was either (**a**) on the bark patch and was seen *moving*, *turning* and *antennating* bark within the five seconds it was observed or (**b**) it was in *waiting* posture at any time during five second observation period (see Fig 6.3)
		'Not searching'	All other combinations of wasp behaviour
	Behaviour: ***'Probing'***	**'Probing'**	Wasp was seen 'probing' on the bark patch at least once during the five second observation period
		'Not probing'	Any other wasp behaviour
Host	*Behaviour:* ***'Movement'***	'Moving'	Any movement by the host during the five second observation period, including head movements and body contractions
		'Not moving'	No visible host movement

Whenever probing behaviour was recorded, it was also noted whether the wasp was probing directly over the host or not (most commonly, if the wasp was not probing over the host chamber, it was doing so at the edge of the bark patch) and, if the wasp had been probing before, whether the wasp was probing in a new location (thus recording whether wasps were interrupting individual probing bouts for repositioning). If a wasp probed in one location over the host for a prolonged period of

time (> 20 minutes) and it assumed a posture characteristic of oviposition (ovipositor usually thrust deep into bark, contracting abdomen that curved toward the bark [see Fig 6.4], in some cases eggs visible in host chamber), this was recorded as oviposition.

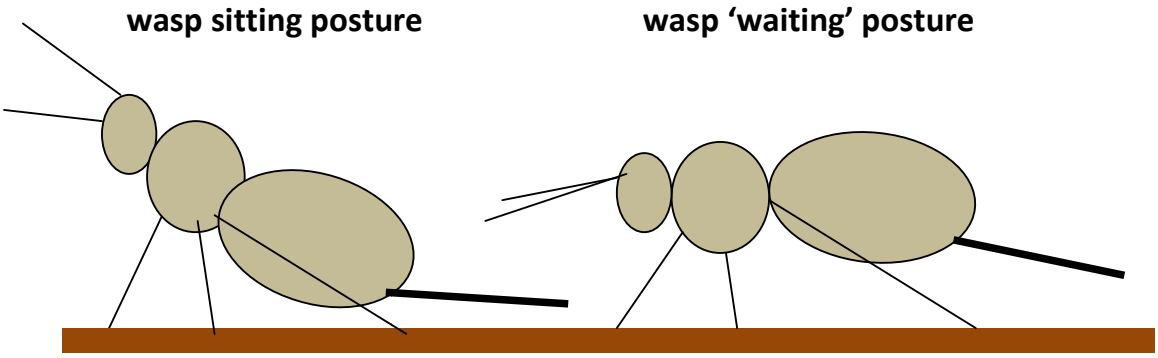


Fig 6.3: Distinction between wasps that were sitting on the bark with no apparent response to host or bark (left) and wasps that were 'waiting', apparently in response to host and/or bark substrate (right).

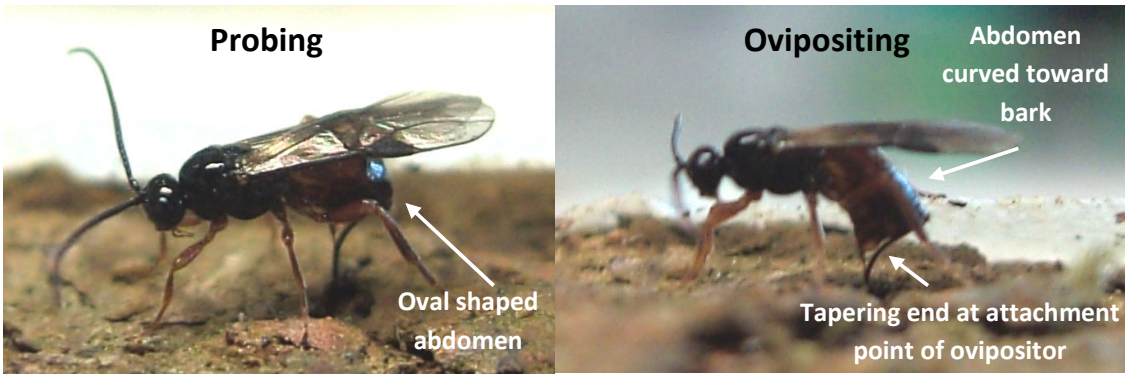


Fig 6.4: Distinction between probing behaviour (left) and signs of oviposition in *B. hylobii* wasps (right). Note the curving of the abdomen and the tapering at the base of the ovipositor.

6.2.13 Continuous observation trials

For these trials, continuous observation arenas were used as described in section 6.2.3. The bark patch in arenas for continuous observation was larger than in the standard arena (20.25 cm^2 versus 2.25 cm^2, respectively). Trials were conducted one at a time and from 12:00 to 20:00 (April 2010 to June 2010). Trials with four combinations of wasp/host were continuously observed (all hosts were *H. abietis*): naïve waps were offered live hosts and experienced wasps were offered a live host, a freeze-killed host or a dead host killed by *H. downesi* (dead for 24 – 48 h prior to the trial). If trials with more than one wasp/host type combination were conducted on a day, they were conducted in random order and in equal numbers. In total, 39 trials with experienced wasps and live hosts, 10 trials with naïve wasps and live hosts, 10 trials with experienced wasps and freeze-killed hosts

with manual scratching and 9 trials with *H.downesi*-killed hosts with manual scratching were observed. Each arena was placed on a stand 10 cm high and fastened to it with strips of masking tape. The stand itself was fastened to the bench with masking tape to prevent shifting of the arena or stand, especially when scratching the bark (Fig 6.5).

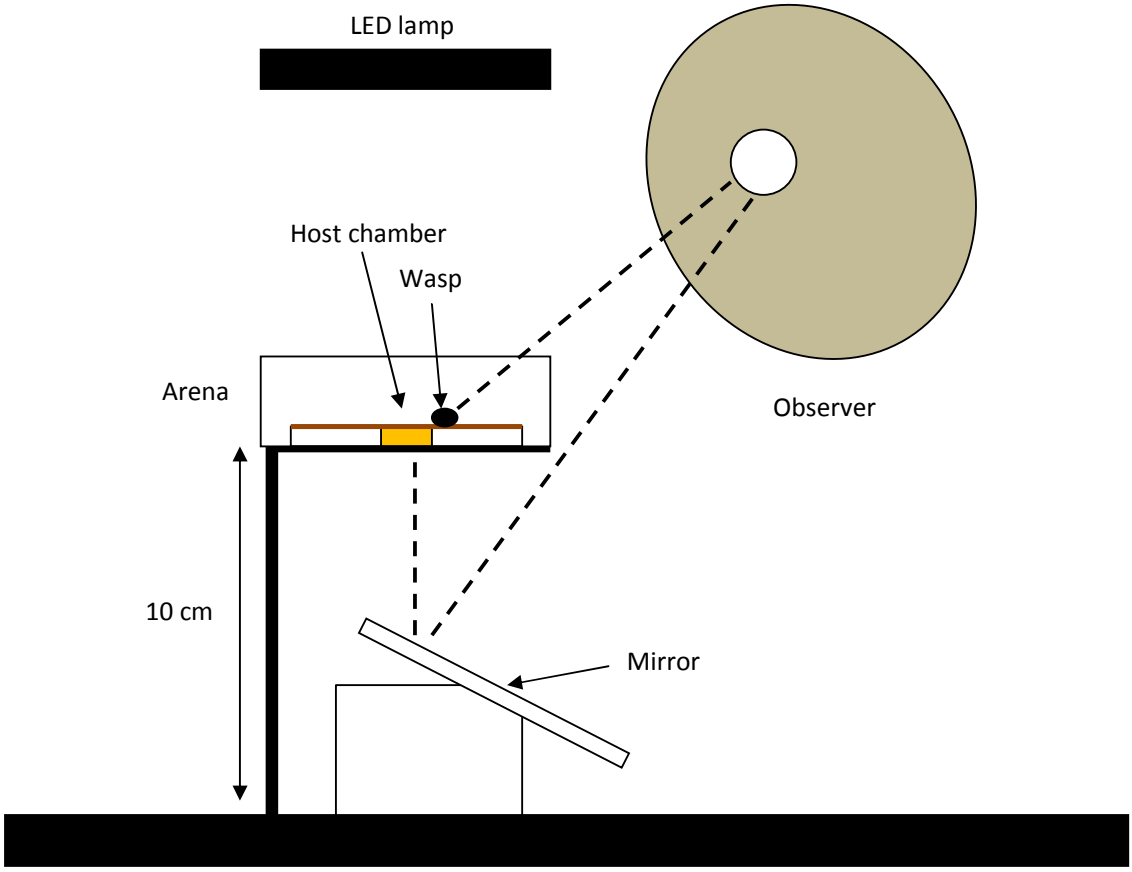


Fig 6.5: Schematic representation of the setup used for continuous 30 minute observations of *B. hylobii* females with *H. abietis* host larvae.

An angled mirror was located midway between the bench and the top of the stand where the arena was placed that allowed the observer to simultaneously keep the wasp and the host in its chamber within the field of view at all times (the stand had a gap in its platform to afford a view of the host). An LED desktop lamp was used to illuminate the arena during experiments (Rochester TM2034, B&Q, Eastliegh, England) (Fig 6.5). The setup allowed the observer to view the wasp and the host larvae simultaneously and record behaviour for both.

Behaviour was recorded for 30 minutes using The Observer™ software (Noldus Information Technology; Wageningen, Netherlands). The observation module of the programme was set to 30 minutes after which time the observation was automatically stopped. Due to the constraints of the observational setup (to keep host in view, wasp could only be observed from above, not from the

side) and the operation of the software (each change in subject behaviour is recorded by a stroke on the computer keyboard), the definition for certain wasp behaviours previously outlined for instantaneous observations (6.2.12; see Table 6.1) had to be changed for continuous observations.

Three different basic wasp behaviours were distinguished (for definition of component behaviours given in italics, see 6.2.12):

'Searching:' Wasp was '*on the bark*' patch and was '*moving*'. In most instances, wasps were also '*antennating*' the bark and/or '*turning*' when this behaviour was recorded, but the latter two component behaviours did not have to be present to classify behaviour as '*searching*' in this experimental protocol.

'Pausing:' The wasp was on the bark patch, but not moving. Whether wasps were '*sitting*' or '*waiting*' at this time was not recorded (see Fig 6.3).

'Probing:' The wasp was '*probing*' the substrate with its ovipositor.

Each of these three behaviours was further subdivided by location: A wasp was considered to be '**close**' to the host if it was located in an area described by a circle around the host chamber with a radius one and a half times that of the host chamber. Wasps were considered to be '**away**' from the host when they were in any other position on the bark. To avoid possible effects on host behaviour by marking the area '**close**' to the host on the bark directly, location judgements were estimated visually as best as possible based on the location of the wasp on the bark and the position of the host chamber as seen in the mirror reflection (Fig 6.6).

This meant that six different behaviour/location combinations could occur during observation: **searching close** or **away**, **pausing close** or **away** and **probing close** or **away**. Each of these combinations was set as a separate behaviour in The Observer™ to allow the separate grouping of data for analysis. The final and seventh behaviour that was recorded for wasps was termed 'other' and included any and all behaviour the wasp performed off the bark patch.

Only two host behaviours were distinguished and recorded separately (wasps and host were set as separate subjects in the software): '**Movement**' or '**no movement**'. The relation of wasp behaviour to host movement was reflected in the recorded data by adding a modifier for host behaviour (moving or not moving) to each of the six behaviour/location combinations possible for wasp behaviour recorded on the bark patch. The modifier was recorded only at the time the wasp behaviour changed. For instance, if a wasp was pausing close to the host as the latter was moving and then changed its behaviour to '**pausing close**' while the host was still moving, this was recorded as such (i.e. the behaviour was changed to 'Wasp: probing close, host moving; Host: moving').

However, if the host then ceased to move after another ten seconds of observation while the wasp was still probing in the same location, this was not recorded as 'new' wasp behaviour so as not to misrepresent the number of records and mean time spent by wasps performing each of the behaviours.

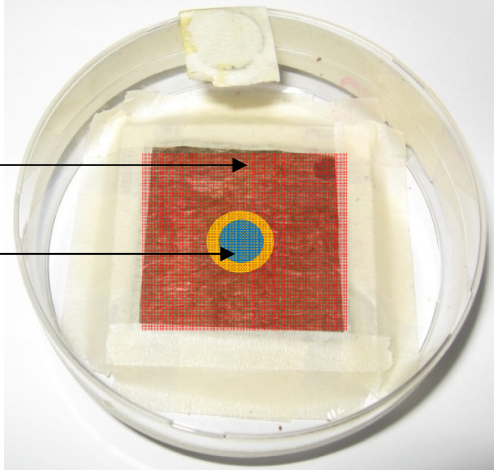


Fig 6.6: Areas of bark patch in continuous observation arenas which were considered to be located 'close' to and 'away' from the host.

6.2.13 Continuous observation with scratching stimulus

In the two sets of trials in which wasps were offered dead host larvae (freeze-killed or killed by *H. downesi* infection), the bark was scratched manually from below. Scratching commenced 15 minutes into each of these trials to allow comparison between equal time periods in which scratching was and was not being applied. The bark was scratched with a stainless steel needle that was bent at a 90° angle. Its tip was inserted into the host chamber from the underside of the arena through a small hole melted into its base (2 mm diameter).

The bark was gently agitated in an effort to simulate the feeding activity and movement of a live *H. abietis* host. To this end, the needle was used to gently scrape the bark in half-circular back and forth motions (approximately 1 mm in each direction). The bark was scratched continuously at a rate of approximately one scratch every 2 seconds. It was possible to monitor the intensity and efficacy of the manual scratching by observing the movement of the bark patch.

In five of the ten trials with freeze-killed hosts in which the scratching stimulus was added, scratching was continued after the end of the observation period in an effort to induce oviposition by the respective wasp on the dead host in the chamber. The same was done in two of the nine trials with *H. downesi*-killed hosts.

6.3 Results

6.3.1 Oviposition trials: General observations

Most *H. abietis* larvae that died of EPN infection during the course of the experiment showed clear signs of infection (i.e. colouration associated with nematode infection and, in the case of infection with *H. downesi*, luminescence). Based on mortality and cadaver appearance observed when *H. abietis* larvae were infected for the trials (some larvae died during exposure to EPN), it was concluded that colouration usually takes at least three to four hours to clearly manifest after the death of a larva. This suggested that some EPN-infected larvae died early on during the 24 h trial period. In some cases, colouration and/or luminescence was so pronounced that it was reasonable to conclude that the host larvae had died at least 10 h prior to the end of the trial period.

Paralysis of host larvae was restricted to *H. abietis* larvae. Longhorn beetle larvae (*R. bifasciatum* and *A. striatum*) as well as *G. mellonella* showed no sign of paralysis during or after being parasitized by wasps. While egg clutches on *H. abietis* hosts were generally tightly packed and found closely together in one bunch, on *G. mellonella* and longhorn beetle larvae, eggs were almost always randomly distributed around the surface of the host and inside the chamber, indicating that the egg clutch laid by the wasp had been severely disturbed by host movement after oviposition.

By contrast, *H. abietis* larvae remained paralysed for several days after the end of the trial. Paralysis was not complete. Venomised *H. abietis* larvae responded to prodding with a metal probe, but showed no signs of unprovoked locomotion or movement.

Only a single instance of a wasp probing directly over a dead host and then rejecting it was observed (experienced wasp on a freeze-killed *H. abietis*). Any other probing recorded during trials with dead hosts occurred at the edge of the bark patch, not over the host chamber.

6.3.2 Host weight

A General Linear Model showed that there was no significant difference in host weight among trials, regardless of host type (DF = 8, F = 1.40. P = 0.192) or wasp experience (DF = 1, F = 1.30, P = 0.254) and there was no significant interaction between these two variables (DF = 8, F = 0.85, P = 0.563). Data for prepupae (only used in trials with naïve wasps) and hosts with a weight of less than 100 mg (*A. striatum* [N = 2] and *R. bifasciatum* [N = 4] used in trials with experienced and naïve wasps, respectively) were excluded from this analysis, for mean weights and N see Appendix A.12.

6.3.3 Parasitism of hosts in oviposition trials by naïve wasps

a) Control trials

In trials with control *H. abietis* hosts that had been incubated in the same way as EPN-infected hosts (48 h at 20°C, see 6.2.6a) parasitism was approximately 76.7 % for naïve wasps (33 out of 43 hosts parasitized). This rate of parasitism was compared with trials with *H. abietis* control hosts that had only been incubated at room temperature for 2 h before trials (102 out of 141 parasitized; 72.3%). The difference in parasitism was not significant ($\chi^2 = 0.327$, DF = 1, P = 0.567). Since exploratory data analysis using only this control data set to compare against trials with EPN-infected *H. abietis* yielded no difference in the outcome of statistical tests (i.e. significant or not significant) compared with using data from all control trials together (N = 184), data for control trials was combined for subsequent data analysis including observational data.

b) Trials with *H. abietis* hosts infected with EPN

These hosts had been exposed to a high dose of EPN before the beginning of a trial (see 6.2.5). All hosts included in this data set were alive when introduced into the trial, but died either during the trial (24 h) or within five days after it had ended. Note that control hosts also included *H. abietis* larvae that had been exposed to EPN but that did not die within a five day period after the experiment (these were considered to be free of EPN at the time of the trial).

Host type had a highly significant effect on the proportion of trials in which eggs were laid ($\chi^2 = 71.277$, DF = 4, P < 0.001) (Fig 6.7). Approximately 73% of 184 naïve *B. hylobii* females laid a clutch of eggs on control hosts, a proportion similar to those females that had been exposed to an EPN – infected host that survived the trial (88 % of 24 wasps and 70% of 23 wasps, respectively). There was no significant difference in the parasitism rate among these three groups of wasps (Table 6.2). The rate of oviposition was dramatically reduced to 15 % (3 out of 20) and 10 % (3 out of 30) for hosts infected with *S. carpocapsae* or *H. downesi*, respectively, in those trials where the host larvae was found dead at the end of the 24 h trial period. The difference was highly significant in all cases (P < 0.001). It was also highly significant when parasitism rates were compared between surviving and dead hosts exposed to each of the two EPN species (P < 0.001).

c) Dead *H. abietis* hosts (freeze-killed and EPN-killed)

Naïve wasps were offered 23 host larvae (*H. abietis*) that had been killed by EPN infection 24 h to 48 h prior to the beginning of the trial. Of these, 11 had been killed by *S. carpocapsae* and 12 by *H. downesi*. No eggs were found on any of these hosts at the end of trials. No parasitism of the 27 freeze-killed hosts that were offered to naïve wasps was recorded.

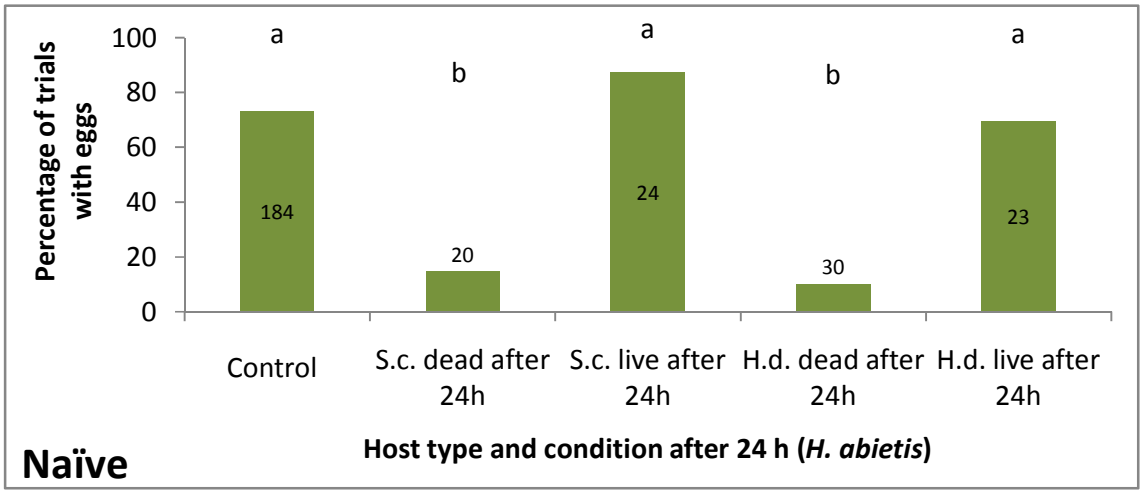


Fig 6.7: Percentage of hosts infected with EPN but alive at beginning of trial parasitized by **naïve wasps**. Numbers inside bars give N. Bars that are marked with the same letter above them are not significantly different from each other (χ^2 – test, 10 cross-comparisons, Bonferroni-adjusted significance level of $\alpha = 0.005$).

Table 6.2: Statistical cross-comparison of parasitism by **naïve wasps** depending on host condition at the end of the trial (live or dead due to infection) (χ^2 – test, 10 cross-comparisons, Bonferroni-adjusted significance level of $\alpha = 0.005$). DF = 1 for all comparisons, for N see Fig 6.7.

	S.c. dead after 24h	*S.c.* live after 24h	*H.d.* dead after 24h	*H.d.* live after 24h
Control	$\chi^2 = 28.082$, **P < 0.001**	$\chi^2 = 2.261$, **P = 0.133**	$\chi^2 = 45.229$, **P < 0.001**	$\chi^2 = 0.150$, **P = 0.699**
S.c. dead after 24h		$\chi^2 = 23.127$, **P < 0.001**	$\chi^2 = 0.284$, **P = 0.594**	$\chi^2 = 12.915$, **P < 0.001**
S.c. live after 24h			$\chi^2 = 32.434$, **P < 0.001**	$\chi^2 = 2.255$, **P = 0.133**
H.d. dead after 24h				$\chi^2 = 20.085$, **P < 0.001**

d) Prepupal hosts

In the course of 30 trials with prepupal hosts, eggs were laid on five of them (16.7 %). This was a significantly lower rate of oviposition in comparison to the control ($\chi^2 = 66.661$, DF = 1, $P < 0.001$).

e) Paralysed hosts

Out of 25 previously parasitized hosts (these hosts were alive but paralysed by wasp venom), only one was oviposited upon by naïve *B. hylobii* (approximately 4 % oviposition rate, 26 eggs in clutch). This was a rate lower rate than and significantly different from that in the control (Fisher's exact test, $P < 0.001$).

f) Hosts other than *H. abietis*

When offered longhorn beetle larvae of the species *R. bifasciatum*, 12 out of a total of 34 naïve wasps laid eggs, whereas only 3 of the 38 wasps offered *G. mellonella* did so. The difference was significant (χ^2 = 8.168, DF = 1, P = 0.004, N = 34 for *R. bifasciatum* and N = 38 for *G. mellonella*) (Fig 6.8). Parasitism rate was significantly different from the control (*R. bifasciatum*: χ^2 = 18.944, DF = 1, P < 0.001; *G. mellonella*: χ^2 = 57.404, DF = 1, P < 0.001). While wasps did lay egg clutches on these host larvae and these eggs did hatch, wasp larvae did not develop beyond the first larval instar on these hosts.

a) Empty chamber

None of the 15 naïve wasps that were presented with an empty chamber for 24 h laid any eggs.

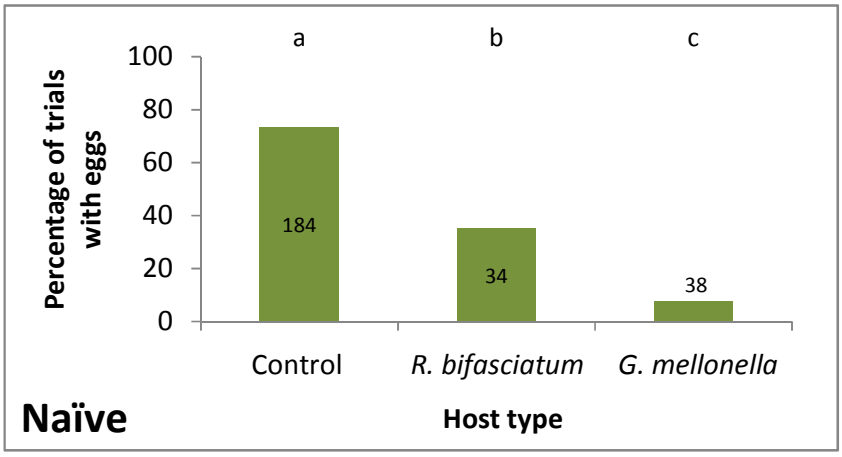


Fig 6.8: Percentage of *H. abietis* control hosts and hosts other than *H. abietis* parasitized by **naïve wasps**. Numbers inside bars give N. Bars that are marked with the same letter above them are not significantly different from each other (χ^2 – test, 3 cross-comparisons, Bonferroni-adjusted significance level of α = 0.017).

6.3.4 Parasitism of hosts in oviposition trials by experienced wasps

Of the 86 experienced wasps used in trials with infected *H. abietis* hosts, 31 (36 %) had laid their first clutch of eggs on an infected host. Only seven of the 75 hosts used in the control had first laid eggs on an infected host (9.3%). Exploratory analysis of the parasitism rate and behavioural observations made on these groups of experienced wasps indicated that they laid eggs no more or less frequently or behaved in any way different from wasps experienced on an uninfected host (data not shown). Data for these experienced wasps was therefore included in the overall analysis. The parasitism rate in the control was not significantly higher than that for naïve wasps (χ^2 = 3.344, DF = 4, P = 0.067).

a) Control trials

In trials with control *H. abietis* hosts that had been incubated in the same way as EPN-infected hosts (48 h at 20°C, see section 6.2.6a) parasitism was approximately 82.5 % for experienced wasps (32 out of 41 hosts parasitized). This rate of parasitism was compared with trials with *H. abietis* control hosts that had only been incubated at room temperature for 2 h before trials (32 out of 35 parasitized; 91.4 %). The difference in parasitism was not significant (Fisher's exact test, P = 0.321). Since exploratory data analysis using only this control data set to compare against trials with EPN-infected *H. abietis* yielded no difference in the outcome of statistical tests (i.e. significant or not significant) compared with using data from all control trials together (N = 82), data for control trials was combined for subsequent data analysis, including observational data.

b) Hosts infected with EPN

Out of 75 trials with control hosts, experienced wasps laid eggs in 63 out of 75 trials (approximately 84 %). By comparison, the rate of oviposition was slightly lower in the group of *H. abietis* hosts that were found dead at the end of the 24 h trial (73 % for *S. carpocapsae* and 78 % for *H. downesi*). A χ^2 – test was not possible due to low expected cell counts, but a comparison of the host types with the highest and lowest parasitism (*H. downesi* live and *S. carpocapsae* dead after 24 h) using Fisher's exact test found no significant difference (P = 0.418; Fig 6.9).

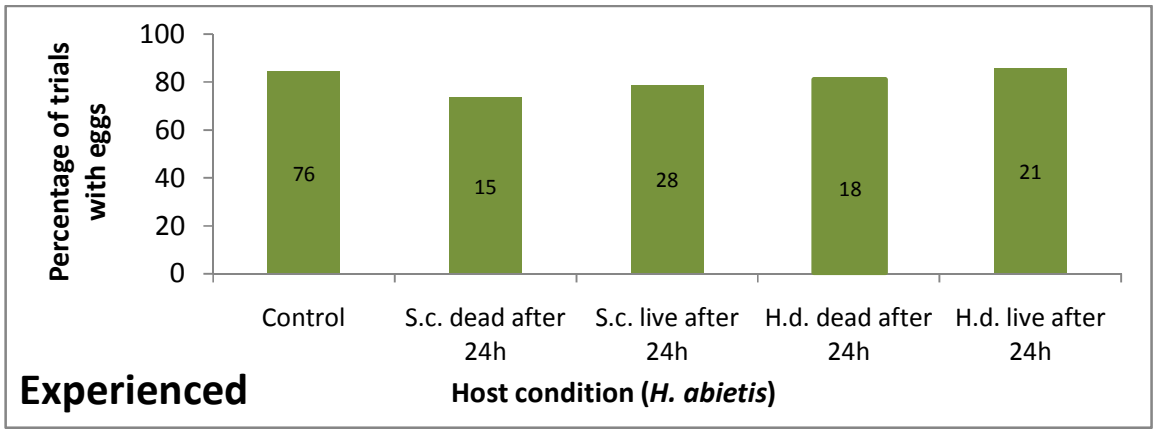


Fig 6.9: Percentage of hosts infected with EPN but alive at beginning of trial parasitized by **experienced wasps**. The data is grouped by which EPN species hosts were exposed to (*S. carpocapsae* or *H. downesi*) and the condition of the host at the end of the trial (live or dead due to infection). Numbers inside bars give N.

c) Dead *H. abietis* hosts (freeze-killed and EPN-killed)

Experienced wasps were also offered *H. abietis* hosts that had been killed by EPN infection 24 h to 48 h before they were introduced into trials, but none of these hosts were parasitized (12 with

S. carpocapsae infection, 21 with *H. downesi* infection). No experienced wasps parasitized hosts that had been freeze-killed (18 trials).

d) Paralysed hosts

Of the 19 previously parasitized and thus paralysed hosts they were offered, experienced wasps laid eggs on two (approximately 10 %). This represented a highly significant difference in comparison to the control (Fisher's exact test, $P < 0.001$).

e) Hosts other than *H. abietis*

Experienced wasps laid eggs on only one out of twelve longhorn beetle larvae (*R. bifasciatum*) and on five out of ten *G. mellonella* (Fig 6.10). Cross-comparisons of the data sets revealed a significant difference in parasitism rate on longhorn beetle larvae compared with *H. abietis* control hosts (Fisher's exact test, $P < 0.001$). After Bonferroni-adjustment of the significance level for three cross-comparisons ($\alpha = 0.017$), there was no significant difference between *G. mellonella* hosts and the control in terms of parasitism frequency, though significance was approached (Fisher's exact test, $P = 0.024$). No significant difference in parasitism was found between longhorn beetle larvae and *G. mellonella* (Fisher's exact test, $P = 0.056$). Experienced wasps were also offered *A. striatum* larvae (two trials only), with one being parasitized.

f) Empty chamber

No eggs were laid In 20 trials in which experienced wasps were offered an empty chamber.

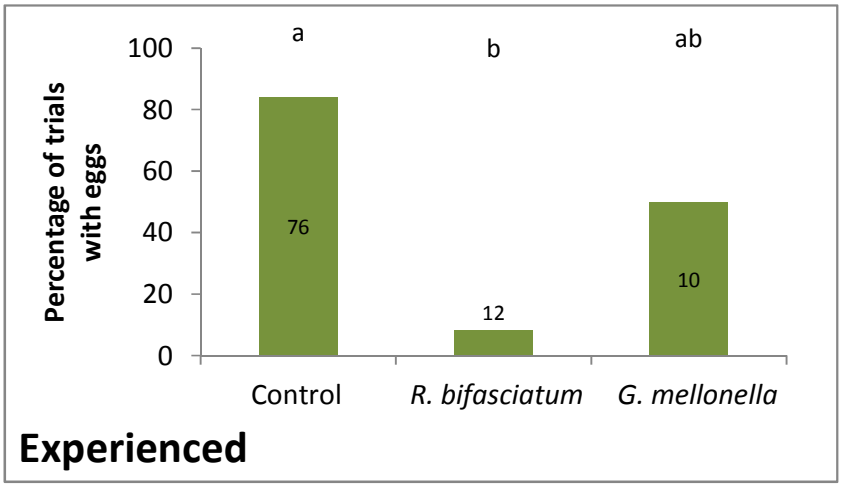


Fig 6.10: Percentage of *H. abietis* control hosts and hosts other than *H. abietis* parasitized by **experienced wasps**. Numbers inside bars give N. Bars that are marked with the same letter above them are not significantly different from each other (Fisher's exact test, 3 cross-comparisons, Bonferroni-adjusted significance level of $\alpha = 0.017$).

6.3.5 Clutch size in trials with naïve and experienced wasps

In control trials, egg clutches laid by naïve wasps (ranging from 1 egg to 39 eggs in size for a mean of 20.6 eggs per clutch) tended to include more eggs than those laid by experienced wasps (1 to 34 eggs in a clutch for a mean of 14.0 eggs per clutch) (Fig 6.11). A One-way ANOVA that compared the mean number of eggs per clutch among all host types for naïve and experienced wasps with an N > 10 detected a highly significant effect ($F = 8.25$, $DF = 8$, $P < 0.001$). In the control, the difference observed in the mean egg clutch size between naïve and experienced wasps was found to be significant (Tukey's test, $\alpha = 0.05$).

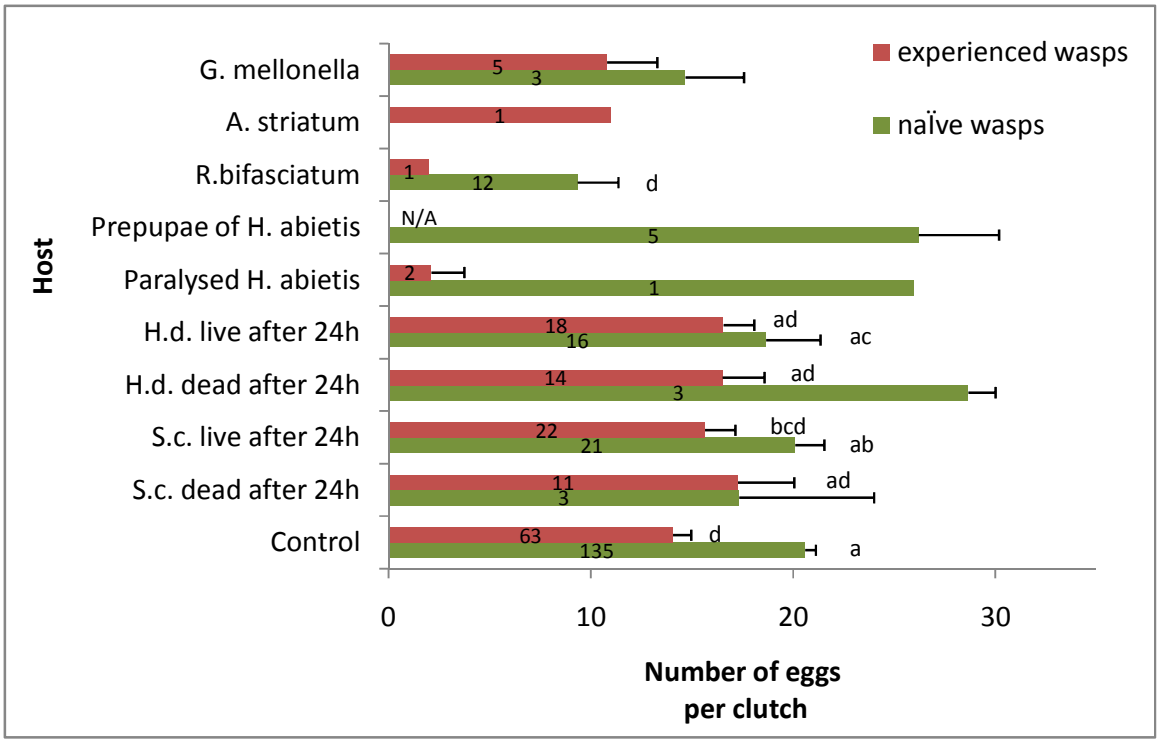


Fig 6.11: Mean number of eggs per clutch laid by **naïve and experienced wasps** (all host types with oviposition included). The data are grouped by host type (*H. abietis* hosts further segregated by *S. carpocapsae* or *H. downesi* infection and condition at end of trial and whether larvae was in prepupal stage or had been previously parasitized an paralysed). Numbers inside bars give N. Error bars represent standard errors. Bars that are marked with the same letter above them are not significantly different from each other (One-way ANOVA with Tukey's post-hoc test [$\alpha = 0.05$]). Bars without letters were not included in statistical test due to low N.

Overall, the mean number of eggs laid per clutch by naïve wasps exceeded that laid by experienced wasps per clutch for all host types, though this difference was not always significant (Fig 6.11). With regards to infected *H. abietis* hosts, whether or not a larvae died during the 24 h trial did not have a significant effect on the size of the egg clutch laid by either naïve or experienced wasps (Tukey's test, $\alpha = 0.05$). On average, naïve wasps laid 9.3 eggs per clutch on longhorn beetle larvae (*R. bifasciatum*).

This was less than half the number found on control hosts exposed to this wasp group, which represented a significant difference (Tukey's test, $P < 0.05$). While the number of egg clutches laid on *G. mellonella* or *A. striatum* was very low, their size fell within the ranges observed in the control. The two egg clutches laid by naïve wasps on a paralysed and a pupating host included more eggs than the control mean (26 eggs each).

6.3.6 Chewing on bark by hosts in oviposition trials and its effect on parasitism

The amount of bark chewed within the 24 h trials was recorded for a portion of each of the host types by measuring the area in pixels that had been chewed on the underside of the bark patch with which each host was covered. Fig 6.12 shows scanned images of bark patches that had been fed on to varying degrees by a variety of hosts.

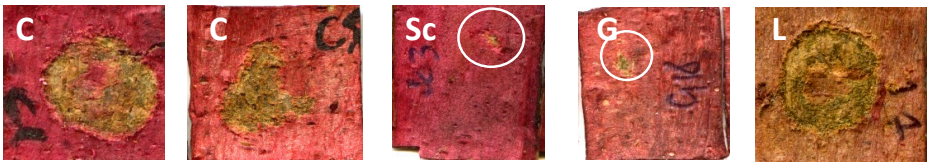


Fig 6.12: Chewed areas on the underside of bark patches used to cover hosts in oviposition trials. From left: C = control host (live *H. abietis*), Sc = *S. carpocapsae* infected host that died during trial, G = *G. mellonella* (*G. mellonella*), L = longhorn beetle larva (*R. bifasciatum*). Small areas are highlighted with white circles.

Most of the *H. abietis* larvae used in control trials for both naïve and experienced wasps chewed on the bark at some stage during the 24 h trial period (Fig 6.13). The occurrence of chewed bark was slightly higher in trials with naïve wasps (73 %) than in those with experienced wasps (63 %), but this difference was not significant ($\chi^2 = 3.100$, DF = 1, $P = 0.078$; for N see Fig 6.13). Pupating *H. abietis* and paralysed *H. abietis* larvae are not included in Fig 6.12, since they only chewed the bark in two instances (one pupating host with 30, 67895 pixels of chewed area; one paralysed host with 43, 1341 pixels of chewed area). There was a marked difference in the pattern of the numbers of infected *H. abietis* hosts chewing on bark between the trials with naïve and experienced wasps, mainly due to the difference in trials with hosts infected with *S. carpocapsae* that survived the trial period (For statistical test results see Appendix A.13). The probability of chewed bark occurring in a trial was similar to that of eggs being laid in trials with naïve wasps (Fig 6.7 and Fig 6.13). A significant difference in the occurrence of chewed areas on the underside of the bark was recorded for those hosts that died within the trial period, regardless of EPN species and in comparison to both control hosts and those that were infected but died at a later stage ($P < 0.001$ in all cases). No significant difference was found when comparing infected hosts that did not die within the 24 h trial period and control hosts ($P = 0.840$ for *S. carpocapsae* and $P = 0.432$ for *H. downesi*).

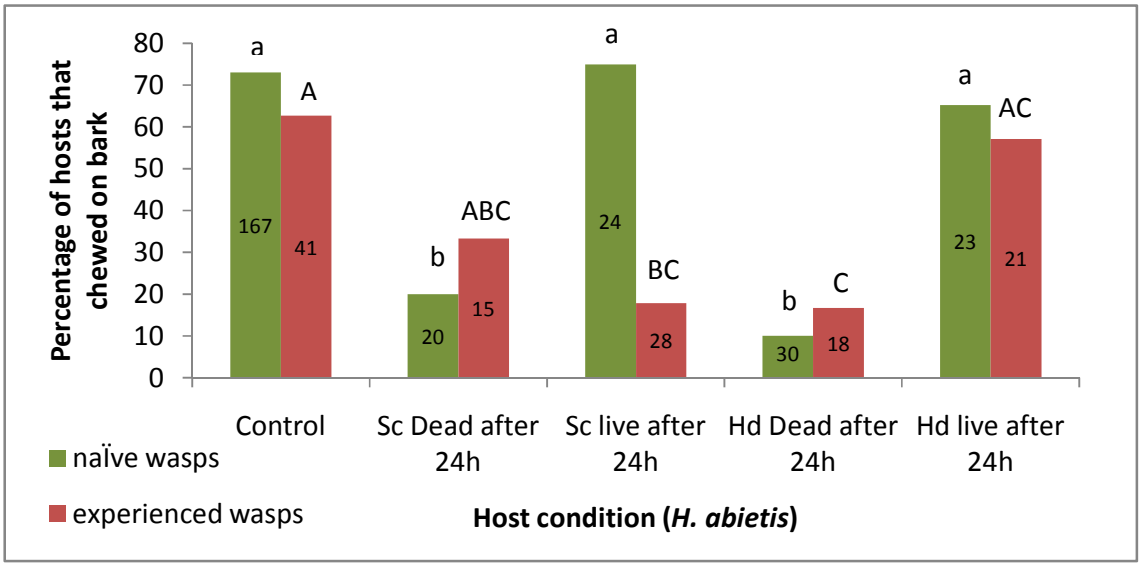


Fig 6.13: Percentage of trials with **naïve and experienced wasps** in which the bark was chewed by hosts. The data are grouped by which EPN species hosts were exposed to (*S. carpocapsae* or *H. downesi*) and the condition of the host at the end of the trial (live or dead due to infection). Numbers inside bars give N. Bars for each group of wasps (naïve or experienced) that are not marked with the same letter above them are significantly different from each other (χ^2 – test and Fisher's exact test, 10 cross-comparisons, Bonferroni-adjusted significance level of $\alpha = 0.005$). For statistical data on cross – comparisons see Appendix A.13.

No clear pattern could be identified in the same host groups in experienced wasp trials (Fig 6.13). A significant reduction in the occurrence of chewed bark in relation to the control was only recorded for hosts that were infected with *S. carpocapsae* but survived the trial ($P < 0.001$) and those infected with *H. downesi* that did not ($P = 0.001$). No significant differences were detected when comparing the occurrence of chewed bark for hosts infected with *S. carpocapsae* and *H. downesi* amongst each other ($P = 0.281$ and $P = 0.010$, respectively [adjusted $\alpha = 0.0005$]).

6.3.7 Area of chewed bark in oviposition trials with EPN infected hosts

The area of bark that was chewed by hosts in the course of the trials varied considerably both among and within the groups of *H. abietis* hosts that had been exposed to EPN before the trials (Fig 6.14). Hosts that did not chew the bark were excluded from analysis. Due to the low number of hosts chewing on the bark, few replicates were obtained for most host types (e.g. N = 2 and N = 3 for *H. downesi* infected hosts that died within the trial period for naïve and experienced wasps, respectively). A one-way ANOVA including only those data sets for naïve and experienced wasps with an N > 10 (controls, *S. carpocapsae* infected hosts that survived the trials [naïve only] and *H. downesi* infected hosts that survived the trials) did not reveal a significant difference among them ($F = 1.74$, $DF = 4$, $P = 0.144$; for N see Fig 6.14), indicating that if a larva did chew on the bark during the trial,

the extent of the area it chewed on was not affected by infection with EPN. To investigate whether a host chewing on the underside of the bark was more likely to be parasitized by a wasp, the number of control hosts for which chewed bark and parasitism coincided after the trial was tabulated against all other possible combinations for both naïve and experienced wasps. The results can be found in Table 6.3. This tabulation was not carried out for other host types due to the restrictively low number of replicates in most cases.

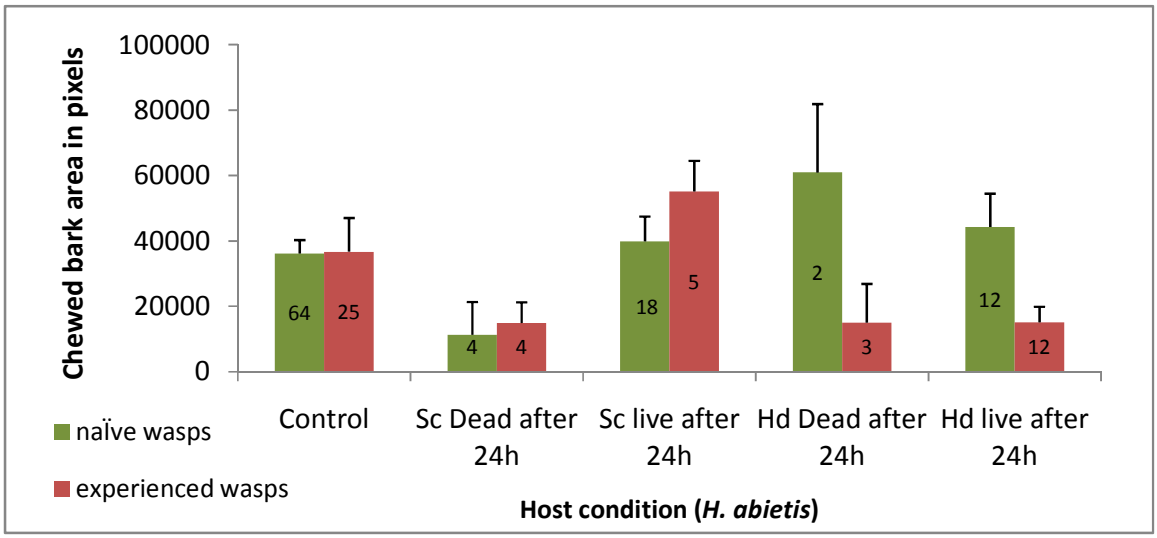


Fig 6.14: Chewed area on underside of bark in trials with **naïve and experienced** wasps (hosts that did not chew the bark at all were excluded). The data is grouped by which EPN species hosts were exposed to (*S. carpocapsae* or *H. downesi*) and the condition of the host at the end of the trial (live or dead due to infection). Error bars represent standard errors. Numbers inside bars give N.

Table 6.3: Incidence of chewed bark tabulated against the incidence of parasitism for trials with **naïve and experienced wasps** and control hosts (*H. abietis*). P – values resulting from statistical tests on the data (χ^2 – test or Fisher's exact test) are given for each group of wasps.

Wasps		*Parasitized*	*Not parasitized*	Test results
Naïve	Chewed bark	101	21	$\chi^2 = 18.640$
	No chewed bark	26	25	DF = 1
				P < 0.001
Experienced	Chewed bark	43	4	**P = 0.049**
	No chewed bark	21	8	

Both naïve and experienced wasps were significantly more likely to lay eggs on a host that chewed the bark at some stage during the 24 h trial period than on a host that did not do so. As was shown above (see section 6.3.6), once a host was paralysed, it was very unlikely (< 2.5%) to chew on any more bark in addition to what it had already chewed up to that point in the trial. It was therefore concluded that in those trials in which oviposition had occurred, the data only represented chewing that had occurred up to the time of oviposition.

6.3.8 Egg clutch size and parasitism in relation to host weight and frass

To assess the relationship between a wasp laying eggs on a given host and the amount of frass that host produced, multiple linear regression analysis was conducted (control hosts only, data for naïve and experienced wasps examined separately). Host weight was also included to test for any possible effects it may have had on the egg clutch size or the frequency of parasitism in a set of trials. Table 6.4 shows the results of this analysis. Multiple binary logistic regression on data for trials with experienced wasps did not yield reliable results based on a comparison of the frequency distribution of Pearson residuals against a normal distribution and was thus not included. Neither the area of bark that was chewed by a host nor its weight had a significant impact on the size of egg clutches or the frequency with which they were laid by naïve or experienced wasps.

Table 6.4: Multiple regression analysis results for oviposition trials in which **naïve and experienced** (Exp.) **wasps** were offered control hosts (live pine weevil larvae). Link function with Pearson Goodness-of-Fit P-value is given for binary logistic regression, equations are given for linear regression and test variables are listed (T for linear regression, Z for binary logistic regression). P value for regression analysis is given in the last column. N = 31 for naïve/linear, N = 36 for naïve/binary and N = 20 for experienced/linear.

Wasp	*Type*	*Regression*	*Link function*	*Equation/ Coefficient*	R^2	*T or Z*	*P*
Naïve	Linear	Egg clutch size (***y***) vs. chewed bark area (***xa***) and host weight (***xb***)	-	y = 60.0 – 14.1xb – 2.08xa	0.11	xa: -1.81 xb: -0.46	**xa: 0.080 xb: 0.647**
Naïve	Binary logistic	Parasitism (***y***) vs. chewed bark area (***xa***) and host weight (***xb***)	Normit (0.736)	xa: -4.900 xb: -0.667	-	xa: -1.50 xb: -0.84	**xa: 0.135 xb: 0.402**
Exp.	Linear	Egg clutch size (***y***) vs chewed bark area (***xa***) and host weight (***xb***)	-	y = 53.2 – 19.7xb + 1.32xa	0.03	xa: 0.30 xb: -0.61	**xa: 0.764 b: 0.552**

6.3.9 The effect of preventing hosts from chewing bark on parasitism rate

a) Parasitism rate

In a separate set of trials, *H. abietis* larvae with glued mandibles were offered to naïve and experienced wasps. These hosts were incapable of chewing the bark, though they were able to move freely in their chamber. Live hosts without glue as well as hosts with a drop of glue on the back of their head capsule were presented as controls and some of the hosts with glued mandibles were supplemented by adding wood shavings into their well in addition to covering them with bark patches that hosts had been allowed to chew on before the experiment. Statistical analysis of the mean weight of hosts from each of the eight trial groups (naïve and experienced wasps offered four host types each) detected no significant difference among treatments in this variable (One-way ANOVA, F = 0.96 DF = 7, P = 0.459).

In this set of trials, for both wasp groups parasitism rates were highest in the control without glue (86 % for naïve wasps and 97 % for experienced wasps) and lowest for hosts that had had their mouthparts glued shut (52 % and 81 % for naïve and experienced wasps, respectively) (Fig 6.15). There was a significant difference across the four data sets for naïve wasps (χ^2 = 17.269, DF = 3, P = 0.001, for N see Fig 6.15).

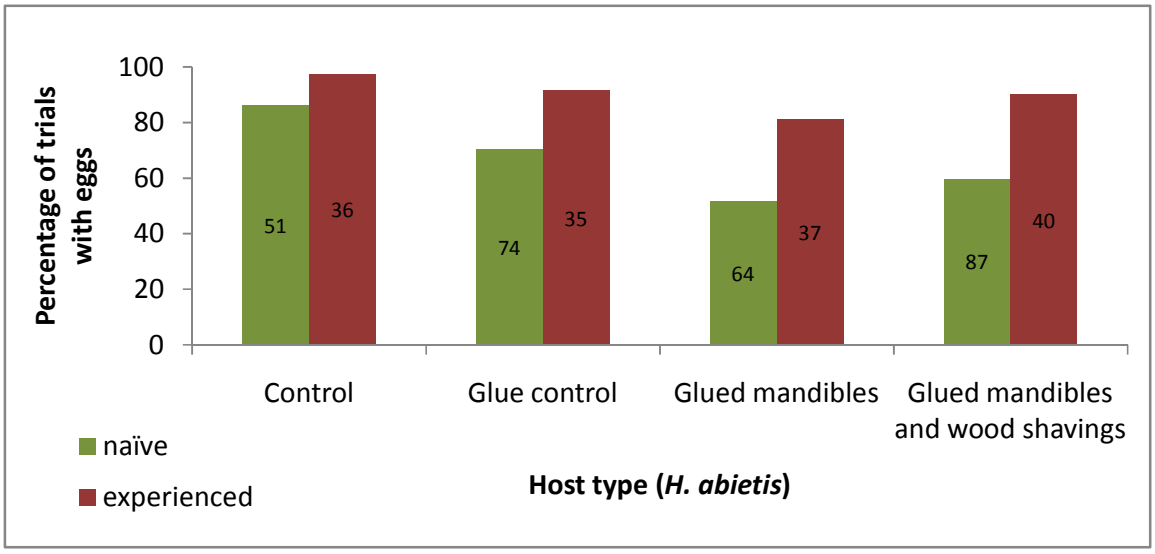


Fig 6.15: Percentage of control hosts and hosts with mandibles that had been glued shut parasitized by **naïve and experienced wasps**. Numbers inside bars give N.

Table 6.4: Statistical cross-comparison of number of trials with **naïve wasps** laying eggs on controls or hosts with glued mandibles (χ^2 – test, 3 cross-comparisons for glue control, Bonferroni-adjusted significance level of $\alpha = 0.017$). DF = 1 for all comparisons, for N see Fig 6.14.

	Glue control	Glued mandibles	Glued mandibles with wood shavings
Control	$\chi^2 = 4.340$, **P = 0.037**	$\chi^2 = 15.457$, **P < 0.001**	$\chi^2 = 10.668$, **P = 0.001**
Glue control		$\chi^2 = 5.077$, **P = 0.024**	$\chi^2 = 1.928$, **P = 0.165**
Glued mandibles			$\chi^2 = 1.010$, **P = 0.315**

For experienced wasps, the χ^2 – test result for a comparison including all treatments was not reliable due to expected counts < 5 for three out of eight cells in the test. Instead, Fisher's exact test was used to compare the host type with the highest rate of egg-laying (control without glue) for this group of wasps, with that for the host type with the lowest rate (glued mandibles). The difference was not significant (P = 0.205) and it was concluded the chewing of bark had no significant effect on the rate of parasitism by experienced wasps. Cross-comparisons of the parasitism frequency in trials with naïve wasps did not yield any significant differences between the glue control and any of the other host types after the significance level had been adjusted using Bonferroni's method. There was, however, a significant difference between the control and hosts with glued mandibles, regardless of whether the latter were offered with or without chewed bark and wood shavings (P < 0.001) (Table 6.4).

b) Occurrence of chewed bark

None of the host larvae that had had their mouthparts glued together prior to the trials chewed on the bark during the trial, indicating that the gluing procedure was effective. In trials with naïve wasps there was a significant difference in the number of trials in which chewing was recorded after 24 h between control (90 %) and glue control (58 %) ($\chi^2 = 15.160$, DF = 1, P < 0.001, for N see Fig 6.15). While there was also a change in the occurrence of chewing between these two host groups in trials with experienced wasps, this difference did not reach significance (experienced: $\chi^2 = 3.773$, DF = 1, P = 0.052, for N see Fig 6.16).

c) Area of chewed bark

The mean area of chewed bark that was recorded on the underside of bark patches at the end of 24 h trials is presented in Fig 6.17 (glued mandible hosts not included due to no chewing in this group). The mean for each type of host was higher in trials with naïve wasps in all three cases, with

the greatest mean area recorded for hosts with glued mandibles which had been supplemented with a patch of bark that had previously been chewed. In a General Linear Model in which the host type and wasp experience were used to predict the chewed area, both of these variables were found to have a significant effect on the extent of bark that was chewed (host type: DF = 2, F = 8.09, P < 0.001; wasp experience: DF = 1, F = 9.49, P = 0.002). There was no significant interaction between these two factors, however (DF = 2, F = 0.37, P = 0.691). Individual comparisons of data sets (Tukey's test, $\alpha = 0.05$) can be found in Fig 6.17.

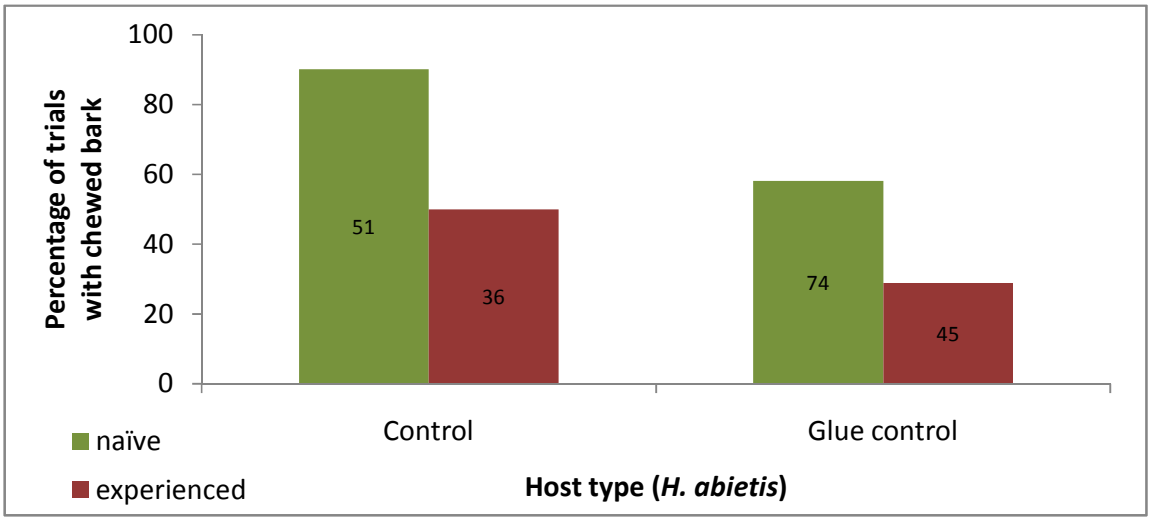


Fig 6.16: Percentage of trials in which bark was chewed when offering **naïve and experienced** wasps control hosts and hosts (*H. abietis*) with a drop of glue on the back of the head. N given inside bars.

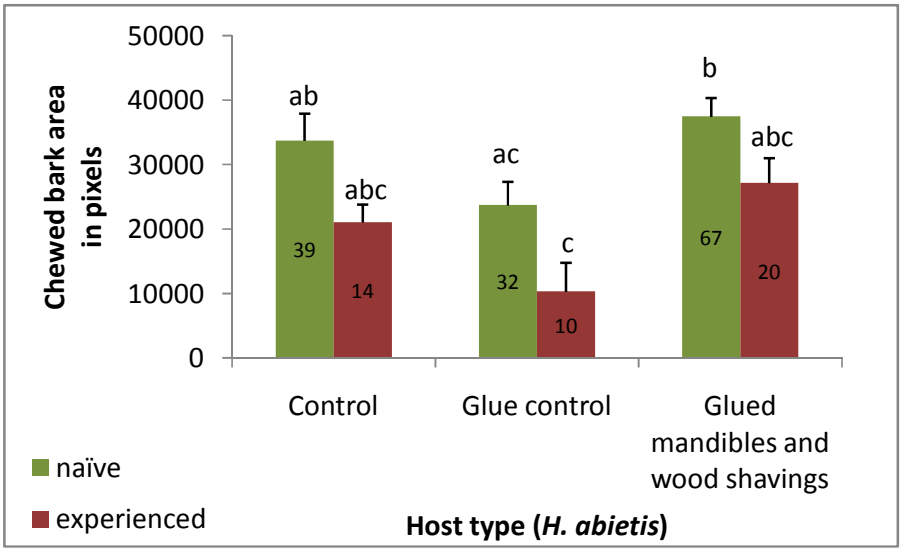


Fig 6.17: Chewed area on underside of bark in trials with **naïve and experienced wasps** offered *H. abietis* with glued mandibles (hosts that did not chew the bark at all were excluded). The data is grouped by host type (control, control with glue on head capsule and glued mandibles with wood shavings). Error bars represent standard errors. N given inside abrs. Bars that do not share the same letter above them have significantly differing means (One-way ANOVA with Tukey's test [$\alpha = 0.05$]; F = 5.46, DF = 5, P < 0.001).

d) Parasitism of hosts with glued mandibles corrected for presence of chewed bark in controls

Not all of the glue control hosts had chewed the bark during the course of the trials. To correct for this and allow a more representative comparison between the control trials and those trials in which hosts with glued mandibles were augmented with wood shavings and chewed bark, those glue control trials in which chewed bark was found at the end of the trial were selected for further statistical analysis. Doing so would control for chemical cues originating from the wood shavings and chewed bark in both treatments and allow detection of effects due primarily to vibrations created by the chewing behaviour of the host (Fig 6.18 A). Conversely, data for those glue control hosts that had not chewed on the bark during the course of the experiment were compared with data for the hosts with glued mandibles and without the wood shavings (Fig 6.18 B). For test results see Table 6.5.

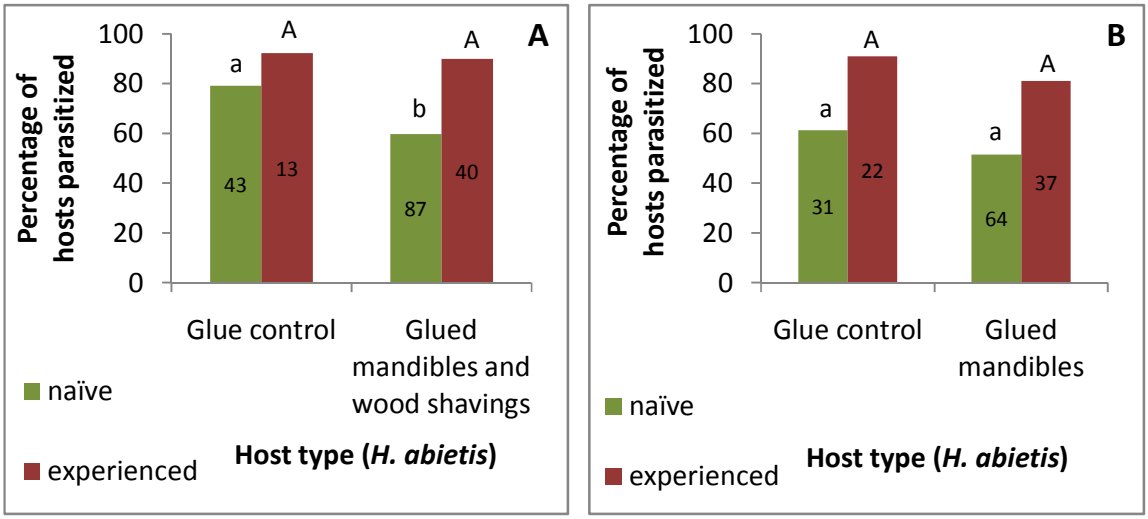


Fig 6.18: A Parasitism rate in glue control trials in which the bark had been chewed during the trial compared with the parasitism rate in trials with hosts that had their mandibles glued shut but were supplemented with wood shavings and a bark patch that had previously had its underside chewed. **B** Parasitism rate in trials with glue control hosts that did not chew the bark during the trial compared with the parasitism rate in trials with hosts that had had their mouthparts glued shut and were not supplemented. Numbers inside bars give N. Bars that share the same letter above them are not significantly different within that group of wasps (naïve or experienced) (χ^2 – test for naïve wasps and Fisher's exact test for experienced wasps; $\alpha = 0.05$).

When only those trials in which glue control hosts chewed the bark during the 24 h of trials were included in analysis, parasitism frequency was higher than when all glue control trials with and without chewed bark were taken together (79 % of trials, up from 70 %) (Fig 6.15 and Fig 6.18). Comparing these corrected data for glue controls with the data for trials in which hosts had glued mandibles but were supplemented with wood shavings and chewed bark patches revealed a significant difference (P = 0.029) (Fig 6.18 A). This was not the case when making the same correction

for experienced wasps (P = 1) (Fig 6.18 B). When the data for the parasitism rate by naïve wasps in glue control trials only included hosts that had not chewed the bark, a significant difference to the parasitism rate in trials using hosts with glued mandibles was no longer detected (P = 0.372). No difference was found for this data set when looking at experienced wasps only (Fisher's exact test, P = 0.461).

Table 6.5: Statistical cross-comparison of parasitism frequency in glue control trials with **naïve and experienced wasps** in which bark was chewed on by hosts. (χ^2 – test for naïve wasps and Fisher's exact test for experienced wasps. DF = 1 for all comparisons, for N see Fig 6.17.

	Naïve wasps		**Experienced wasps**	
	Glued mandibles with wood shavings	Glued mandibles	Glued mandibles with wood shavings	Glued mandibles
Glue control + chewed bark	$\chi^2 = 4.787$, **P = 0.029**	-	**P = 1**	-
Glue control - chewed bark	-	$\chi^2 = 0.798$, **P = 0.372**	-	**P = 0.461**

e) Egg clutch size on hosts with glued mandibles

The mean number of eggs per clutch ranged from an average of 20 to 23 on hosts offered to naïve wasps in this experiment and from 10 to 14 eggs per clutch in trials with experienced wasps (Fig 6.19). As in the standard oviposition trial controls (6.3.5), naïve wasps laid significantly more eggs per clutch than experienced wasps.

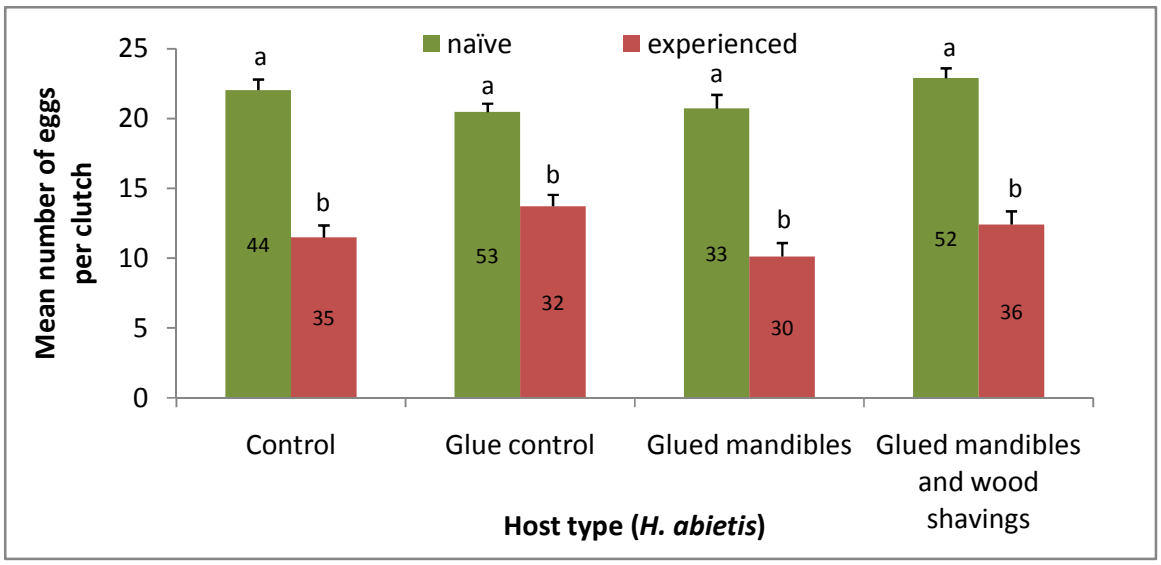


Fig 6.19: Mean number of eggs per clutch laid by naïve and experienced wasps in glued mandible trials. The data is grouped by host type. Numbers inside bars give N. Error bars represent standard errors. Bars that are not marked with the same letter above them are significantly different from each other (One-way ANOVA with Tukey's post-hoc test [α = 0.05]).

A statistical test revealed no significant difference in the mean number of eggs per clutch across host types within either group of wasps (naïve or experienced), but egg number in clutches laid by experienced females were significantly lower than those found in egg clutches laid by naïve wasps for all host types (One-way ANOVA with Tukey's post-hoc test [$\alpha = 0.05$], $F = 39.67$, $DF = 7$, $P < 0.001$, for N see Fig 6.19).

6.3.10 Parasitism of dead hosts with added scratching stimulus

In control trials without added scratching stimulus to the bark, naïve wasps did not parasitize dead hosts, regardless of whether they were killed by infection with *H. downesi* or by freezing and thawing. Only five out of nine control hosts were parasitized (Table 6.6). In trials in which scratching was added, parasitism by naïve wasps was lower in both the control, but two out of 23 wasps laid eggs on a freeze-killed host, a proportion not significantly different from the control (P = 0,120). Experienced wasps laid eggs more frequently in no scratching controls (75 %), but also did not parasitize dead hosts. Eggs were laid on dead hosts with added scratching stimulus in a total of four cases out of a possible 57, a rate significantly different from the scratching control ($P < 0.001$) (Table 6.6). There was no significant difference between the no-scratching control trials and the trials with scratching for any of the host types used, regardless of wasp experience (Table 6.6).

Table 6.6: Number of trials in which **naïve and experienced wasps** parasitized live and dead hosts with and without added scratching. Number after slash is total number of trials conducted. Superscript numbers indicate pairwise comparisons to which the accordingly labelled P – values apply (Fisher's exact test).

Wasps	*Trials*	Control	Dead (*H. downesi*)	Freeze-killed	*Test result (Fisher's exact test)*
Naïve	No scratching	5/9	0/7	0/23	N/A
	Scratching	3/9[1]	0/7	2/23[1]	**[1]P = 0.120**
	Test result (Fisher's exact test)	**P = 0.637**	N/A	**P = 0.489**	N/A
Experienced	No scratching	16/22	0/21	0/38	N/A
	Scratching	12/22[1 2]	1/21[1 3]	3/38[2 3]	**[1]P < 0.001 [2]P < 0.001 [3] P = 1**
	Test result (Fisher's exact test or χ^2 - test)	χ^2 = 1.909, DF = 1 **P = 0.167**	**P = 1**	**P = 0.240**	

6.3.11 Parasitism of dead hosts with added poking stimulus

When the bark was poked from below, naïve wasps laid eggs in only one out of 27 trials with dead hosts (a freeze-killed host). Experienced wasps were more likely to lay eggs on dead hosts when poking was added. Out of thirteen trials with dead hosts, five were parasitized (38 %) (Table 6.7). No controls were included in trials with added poking stimulus, so no statistical comparisons were made.

Table 6.7: Number of times **naïve and experienced** wasps parasitized hosts in trials with added poking out of total number of trials conducted.

Wasps	Dead (*H. downesi*)	Freeze-killed
Naïve	0/12	1/15
Experienced	2/6	3/7

6.3.12 Wasp behaviour in oviposition trials (instantaneous observation over 2 h): General observations

Wasp behaviour in control trials was consistent among trials across the entire one year period trials were conducted. Wasps did not respond to hosts in all trials and on occasion would show no movement or response to the host during the entire 2 h observation period. Wasp activity as reflected by the proportion of records in which a wasp was moving during the observation period was fairly similar across all host types used, ranging from 20 % to 60 % of records (with the exception of the paralysed *H. abietis* host trials, where wasp movement was recorded on an average 78 % of records).

Wasps were frequently seen antennating and turning on the plastic of the Petri dish as well as on bark patches but probing behaviour was generally restricted to the bark patch covering the host chamber (records of searching or probing on plastic were not included in data analysis). Occasionally, a wasp would be seen probing on a piece of masking tape used to fasten the bark patch to the perspex host chamber and sometimes a wasp inserted its ovipositor in between the glass slide and perspex from the side of the host chamber. These instances were rare (< 1 % of wasps, < 0.1 % of records) and were thus not separately recorded as a behavioural category. Wasps displayed searching and waiting behaviour in trials with dead hosts as well as live hosts.

Duration of oviposition varied considerably from wasp to wasp but lasted longer than 30 min in every case oviposition was completed within the 2 h observation period. Judging by observations made after the 2 h recording period, some wasps seemed to oviposit for more than two hours. It is

possible, however, that they had aborted oviposition and shifted position at some point in those instances, since wasps were only intermittently monitored during and after the 2 h recording period.

Paralysis of *H. abietis* hosts due to wasp venom accompanied oviposition in almost all cases and generally set in 10 – 30 minutes after wasps had first probed over the host. This was reflected in a decrease and ultimately a cessation of host movement after wasps had commenced oviposition. However, if the host seemed particularly agitated and especially when it was actively and extensively chewing on the underside of the bark, wasps seemed to abort individual oviposition attempts and reposition.

6.3.13 Wasp probing and oviposition behaviour in oviposition trials and its association with host behaviour (instantaneous observation over 2 h)

In this section, data for the individual behaviours that were considered to be most closely associated with host location and host acceptance - probing and oviposition by wasps (see 6.2.12) - are presented. Individual results for each behaviour (e.g. what percentage of the observational records wasps were seen probing and how long it took them to begin doing so) are presented first (subsections a to d). Observational data on host movement and production of wood shavings are presented next (subsections e and f) and the section concludes with data relating wasp probing to host movement (subsection g).

a) Probing behaviour

On average, naïve wasps probed on the bark patch less than half as frequently as experienced wasps did (19 % versus 44 %) when the host was a live *H. abietis* larva (control) (Table 6.8). This difference was highly significant (M.-W. U-test, $W = 7020.0$, $P < 0.001$). A comparison of the probing records across all host types for naïve wasps (including hosts other than *H. abietis*) showed a significant effect of the host type on probing behaviour (K.-W. test, $H = 47.58$, $DF = 12$, $P < 0.001$). The same was true for experienced wasps (K.-W. test, $H = 58.76$, $DF = 11$, $P < 0.001$). When this analysis was restricted to only *H. abietis* hosts which had been exposed to EPN but were alive at the beginning of the trail and the control (five data sets), a significant effect due to the host type was only found for naïve wasps (K.-W. test, $H = 14.53$, $DF = 4$, $P = 0.006$), but not experienced wasps (K.-W. test, $H = 2.81$, $DF = 4$, $P = 0.591$). In trials with both naïve and experienced wasps, probing frequency on average was lower if a *H. abietis* host died of EPN infection during the trial than when an infected host did not die of infection. This difference in probing was greater for naïve wasps (e.g. 38 % versus 10 % records spent probing when host was infected with *S. carpocapsae* and survived or died of infection, respectively).

Cross-comparisons of probing record frequency among naïve wasps in trials with these host types revealed a significant difference only between those trials in which hosts had been infected with *S. carpocapsae* and had survived the trial and those that had died during the trial (M.-W. U-test, W = 328.0, P = 0.007). For both groups of wasps, naïve and experienced, probing incidence among the remaining host types was highest for *R. bifasciatum* or *G. mellonella*. A mean of 30 % of records had experienced wasps probing on bark patches covering *G. mellonella* hosts, a rate not significantly different from that seen in the control (M.-W. U-test, W = 2698.0, P = 0.196). Likewise, naïve wasps probed in trials with longhorn beetle larvae no less frequently than they did in trials with control hosts (M.-W. U-test, W = 6311.0, P = 0.435).

Table 6.8: Mean percentage of records naïve and experienced wasps were observed proving on bark patch over 2 h of observation (24 records per trial, each 5 minutes apart).

	Probing (% of records)		*St. error*		*N (trials)*	
Host type (* = *H.abietis*)	**Naïve**	**Exp.**	Naïve	Exp.	Naïve	Exp.
**Control*	**18.8**	**43.97**	*2.96*	*4.97*	99	60
**S.c. dead after 24h*	**10.0**	**36.94**	*4.71*	*9.52*	20	15
**S.c. live after 24h*	**38.26**	**53.72**	*7.00*	*6.39*	22	30
**H.d. dead after 24h*	**7.78**	**40.51**	*4.13*	*8.36*	30	18
**H.d. live after 24h*	**27.08**	**42.06**	*7.02*	*9.45*	23	21
**S.c. dead from beginning of trial*	**3.41**	**1.79**	*3.41*	*1.79*	11	11
**H.d. dead from beginning of trial*	**2.08**	**5.00**	*0.81*	*2.55*	12	10
**Freeze-killed*	**0.00**	**1.67**	*0.00*	*0.68*	9	15
**Prepupae*	**1.53**	**N/A**	*0.71*	N/A	30	N/A
**Paralysed*	**1.54**	**0.28**	*1.32*	*0.28*	19	15
R. bifasciatum	**22.22**	**7.64**	*5.83*	*4.49*	30	6
G. mellonella	**7.57**	**29.17**	*2.85*	*10.99*	38	10
Empty	**0.56**	**9.03**	*0.56*	*5.87*	15	20

b) Time to first probing behaviour

The mean amount of time that passed before wasps began probing the bark was greater for naïve wasps than it was for experienced wasps for all the host types examined (trials in which wasps which did not probe at all excluded) (Table 6.9). For instance, in the control, naïve wasps on average took ten minutes longer to begin probing than experienced wasps did (39 minutes versus 29 minutes, respectively). This difference was significant (M.-W. U-test, W = 1705.0, P = 0.047). Though the mean time that elapsed before first probing varied considerably among hosts other than *H. abietis*, paralysed and prepupal *H. abietis* as well as dead *H. abietis* (freeze-killed and killed by EPN), no significant differences were detected when the data was compared (both naïve and experienced wasps included, K.-W.-test, H = 14.50, DF = 8, P = 0.070; only data sets with N > 7 included).

Statistically, experienced wasps started to probe on infected hosts (both those that died during and those that survived the trial) as early as they did on the control.

Table 6.9: Mean time that elapsed until probing behaviour was first observed in trials with naïve and experienced wasps. In trials with host types not listed, no probing occurred during the 2 h observation period.

	Mean time to first probing (min)		*St. error*		*N (trials)*	
Host type (* = *H.abietis*)	**Naïve**	**Exp.**	Naïve	Exp.	Naïve	Exp.
**Control*	**39.47**	**28.91**	*5.18*	*4.96*	36	45
**S.c. dead after 24h*	**25.00**	**15.00**	*10.41*	*5.40*	3	9
**S.c. live after 24h*	**25.33**	**22.61**	*7.15*	*5.69*	15	23
**H.d. dead after 24h*	**44.29**	**20.91**	*13.11*	*7.50*	7	11
**H.d. live after 24h*	**26.83**	**13.18**	*6.34*	*4.87*	10	11
**S.c. dead from beginning of trial*	**20.00**	**14.29**	*N/A*	*N/A*	1	1
**H.d. dead from beginning of trial*	**23.00**	**27.00**	*7.18*	*19.33*	4	4
**Freeze-killed*	**N/A**	**N/A**	N/A	N/A	N/A	N/A
**Prepupae*	**75.00**	**40.00**	*35.00*	N/A	4	N/A
**Paralysed*	**75.00**	**N/A**	*35.00*	*0.00*	2	1
R. bifasciatum	**43.85**	**36.00**	*10.26*	N/A	13	N/A
G. mellonella	**49.17**	**N/A**	*10.66*	*20.40*	12	5
Empty	**20.00**	**11.67**	*N/A*	*1.67*	1	3

c) Proportion of females commencing oviposition during observation period

The rate at which naïve wasps oviposited or commenced oviposition within the 2 h period of observation followed the general trend seen in terms of parasitism results after 24 h (see 6.3.3 and 6.3.4) (Table 6.10). In the control trials, 26 % of naïve wasps successfully oviposited or commenced oviposition. This proportion was almost twice as high for experienced wasps (51 %), which represented a significantly higher rate of oviposition (χ^2 = 10.484, DF = 1, P = 0.001; Bonferroni – adjusted α = 0.0033). Within the set of trials with naïve wasps, oviposition rate fluctuated between 4 % (*H. abietis* infected with *S. carpocapsae* that died within 24 h) and 45 % (*H. abietis* infected with *S. carpocapsae* that survived the 24 h trial). Oviposition during the observation period occurred less frequently in those trials in which host died of infection than it did in the control and in trials with their surviving counterparts. A χ^2 – test on these data sets revealed a significant effect of host survival on the occurrence of oviposition (χ^2 = 14.091, DF = 4, P = 0.007). Results for the cross-comparison of these data sets are presented in Table 6.11.

The difference in the occurrence of oviposition between infected hosts that died of infection and those that did not die approached significance in most instances (e.g. *H. downesi* infected hosts that survived the trial and those that died, P = 0.007), but due to the adjustment of P – levels according to Bonferroni for the cross-comparisons carried out, only one of these differences fulfilled the required

conditions for significance (*H. downesi* infected hosts that died during the trial and *S. carpocapsae* infected hosts that did not) (Table 6.10). An overall comparison of these same host types for trials with experienced wasps yielded no significant difference among them in the rate of oviposition (χ^2 = 2.982, DF = 4, P = 0.561).

Occurrence of oviposition during observation in this group ranged from 68 % to 47 %. In trials with each of the four EPN-infected host types, oviposition rates were higher for experienced wasps than they were for naïve wasps, though significantly so only in the case of *H. downesi* infected larvae that died within the trial period (*S. caprocapsae* dead after trial period: Fisher's exact test, P = 0.0619; *S. carpocapsae* live after trial period: χ^2 = 2.538, DF = 1, P = 0.111; *H. downesi* dead after trial period: Fisher's exact test, P < 0.001; *H. downesi* live after trial period: χ^2 = 0.748, DF = 1, P = 0.387).

Table 6.10: Percentage of trials in which wasps commenced oviposition within the 2 h observation period.

	Percentage of trials in which wasps commenced oviposition within observation period		*N (trials)*	
Host type (* = *H.abietis*)	**Naïve**	**Exp.**	Naïve	Exp.
**Control*	**26.3**	**50.8**	99	60
**S.c. dead after 24h*	**15.0**	**46.7**	20	15
**S.c. live after 24h*	**45.5**	**67.9**	22	30
**H.d. dead after 24h*	**3.6**	**55.6**	28	18
**H.d. live after 24h*	**34.8**	**47.6**	21	21
**S.c. dead from beginning of trial*	**0.00**	**0.0**	11	11
**H.d. dead from beginning of trial*	**0.00**	**0.0**	12	10
**Freeze-killed*	**0.0**	**0.0**	9	15
**Prepupae*	**0.0**	**N/A**	30	N/A
**Paralysed*	**0.0**	**0.0**	19	15
*Longhorn (*R. bifasciatum*)*	**3.3**	**0.0**	30	6
*Waxmoth (*G. mellonella*)*	**2.6**	**0.0**	38	10
Empty	**0.0**	**0.0**	15	20

Table 6.11: Statistical cross-comparison of trials in which naïve wasps initiated oviposition within 2 h observation period. Only trials with *H. abietis* control (live larvae) and infected *H. abietis* (infection with *S. carpocapsae* or *H. downesi* but alive at beginning of trial) included. (χ^2 – test or Fisher's exact test, Bonferroni-adjusted significance level of α = 0.0042). DF = 1 for all tests. For N see Table 6.15.

	S.c. dead after 24h	*S.c.* live after 24h	*H.d.* dead after 24h	*H.d.* live after 24h
Control	**P = 0.396**	χ^2 = 3.172 **P = 0.075**	χ^2 = 6.713 **P =0.010**	χ^2 = 0.674 **P= 0.674**
S.c. dead after 24h		χ^2 = 4.456 **P = 0.033**	**P = 0.294**	**P = 0.007**
S.c. live after 24h			**P < 0.001**	χ^2 = 0.534, **P = 0.465**
H.d. dead after 24h				**P = 0.007**

d) Time to oviposition

Experienced wasps took less time to commence oviposition than naïve wasps did in control trials and trials EPN-infected *H. abietis* that died during trials (Table 6.12). Time to oviposition was longest for naïve wasps offered *H. downesi* infected hosts that died within the trial period (80 minutes). In the control trials, naïve wasps took approximately 16 minutes longer on average to commence oviposition than experienced wasps did (55 minutes versus 35 minutes). A K.-W.-test that included data sets for time to oviposition for both groups of wasps (naïve and experienced) and all host types with an N ≥ 7 for this variable found no significant difference among them (H = 15.71, DF = 9, P = 0.073). Oviposition within the 2 h observation period on prepupal, paralysed or dead *H. abietis* (freeze-killed or EPN-killed) was of such low frequency that statistical testing was precluded.

Table 6.12: Mean time that elapsed until oviposition was first observed in trials with naïve and experienced wasps. In trials with host types not listed, no oviposition occurred during the 2 h observation period.

	Mean time to oviposition (min)		*St. error*		*N (trials)*	
Host type (* = *H.abietis*)	**Naïve**	**Exp.**	Naïve	Exp.	Naïve	Exp.
**Control*	**54.62**	**38.59**	*5.92*	*5.94*	26	31
**S.c. dead after 24h*	**26.67**	**25.71**	*10.14*	*7.51*	3	7
**S.c. live after 24h*	**49.00**	**53.42**	*10.80*	*9.26*	11	19
**H.d. dead after 24h*	**80.00**	**20.00**	*10.14*	*6.67*	1	10
**H.d. live after 24h*	**31.78**	**35.00**	*10.93*	*11.81*	7	10
*Longhorn (*R. bifasciatum*)*	**85.00**	N/A	N/A	N/A	1	N/A
*Waxmoth (*G. mellonella*)*	**50.00**	N/A	N/A	N/A	1	N/A

e) Percentage of records with host movement

Only observational records for hosts made before oviposition commenced were included in this set of data as hosts were usually paralysed at oviposition. For naïve wasp trials, records of host movement were most frequent for *S. carpocapsae* infected host larvae that lived through the trial (68 %) and lowest for those *H. abietis* hosts that died during the experiment (15 %). In the control, the proportion was 41% (Table 6.13). Host larvae that were infected and that died within the trial tended to move less than their counterparts that lived through the experiment, regardless of the EPN species used for infection. Host movement in trials with experienced wasps was very similar among these host types, with all but the hosts infected with *S. carpocapsae* that died within the trial period (60 % host movement records) falling within a narrow range of 37 % records (*H. downesi*, dead after 24 h) to 45 % (*S. carpocapsae*, live after 24 h) with host movement. A comparison of these data sets (control and infected hosts alive at beginning of trial) revealed a highly significant difference among

groups of hosts used in naïve wasp trials (K.-W.-test, H = 29.84, DF = 4, P < 0.001), but not experienced wasps (K.-W.-test, H = 3.22, DF = 4, P = 0.522). There was no significant difference in host movement between naïve and experienced wasp controls (M.-W. U-test, W = 8071.0, P = 0.591). Results for statistical cross-comparison of the data sets for naïve wasps can be found in Table 6.14. Cross-comparisons indicated that there was no significant reduction in the percentage of movement records between control host larvae and those that died of *S. carpocapsae* infection within the 24 h trial (P = 0.606). The difference in movement between the control and *S. carpocapsae* infected hosts that survived the trial did approach significance (P = 0.010 [not significant due to Bonferroni-adjustment of α]). Reduction in host movement of hosts killed by *H. downesi* during the trail was significant in relation to the control (P < 0.001), but not compared with the hosts infected by this EPN species that survived the trial period (P = 0.006 [not significant due to Bonferroni-adjustment of α]).

None of the 34 host larvae that had been previously parasitized and thus paralysed by wasps moved at any time they were observed in the trials and only one record of movement out of a total of 720 was taken for pupating *H. abietis* larvae (30 hosts in total, 24 records taken each). In trials with naïve wasps, longhorn beetle larvae (*R. bifasciatum*) moved on 49 % of records and this rate was not significantly different from the control (M.-W. U-test, W = 6197.0, P = 0.184). No data were available for experienced wasps for this host type. *Galleria mellonella* moved less frequently than *H. abietis* control hosts (17 % in naïve wasp trials, 23 % in experienced wasp trials), but only in the case of naïve wasps was this difference to the control significant (M.-W. U-test; naïve: W = 7529.5, P = 0.001; experienced: W = 2175.0, P = 0.449). Both paralysed and pupating hosts did not move of their own volition when being prepared for the trials. Paralysed wasps responded to agitation and prodding with head capsule movements, body contractions and opening and closing of mandibles while pupating hosts responded to prodding with uncoordinated rolling body movements typical of pupating insects.

Table 6.13: Percentage of observational records with host movement in trials with naïve and experienced wasps. Trials in which hosts were dead and trials with empty chamber excluded.

	Percentage of records with host movement		*St. error*		*N (trials)*	
Host type (* = *H.abietis*)	**Naïve**	**Exp.**	Naïve	Exp.	Naïve	Exp.
**Control*	**41.13**	**41.08**	*3.21*	*3.35*	99	60
**S.c. dead after 24h*	**36.87**	**60.35**	*5.95*	*6.88*	20	15
**S.c. live after 24h*	**68.37**	**45.26**	*8.30*	*6.42*	15	30
**H.d. dead after 24h*	**15.39**	**37.31**	*4.43*	*3.58*	28	18
**H.d. live after 24h*	**43.14**	**38.95**	*9.10*	*3.74*	12	21
**Prepupae*	**0.14**	N/A	*0.34*	N/A	30	N/A
**Paralysed*	**0.00**	**0.00**	*0.00*	*0.00*	19	15
*Longhorn (*R. bifasciatum*)*	**48.75**	N/A	*5.87*	N/A	30	N/A
*Waxmoth (*G. mellonella*)*	**17.11**	**23.33**	*2.09*	*6.52*	38	10

Table 6.14: Statistical cross-comparison of percentage of records with host movement during 2 h observation of **naïve wasps**. Only trials with *H. abietis* control (live larvae) and infected *H. abietis* (infection with *S. carpocapsae* or *H. downesi* but alive at beginning of trial) included. (M.-W. U- test, Bonferroni-adjusted significance level of $\alpha = 0.00556$). For N see Table 6.12.

	S.c. dead after 24h	*S.c.* live after 24h	*H.d.* dead after 24h	*H.d.* live after 24h
Control	W = 6013.0, **P = 0.606**	W = 5578.5, **P = 0.002**	W = 7202.5, **P < 0.001**	W = 6058.5, **P = 0.847**
S.c. dead after 24h		W = 329.0, **P = 0.010**		
H.d. dead after 24h				W = 660.0, **P = 0.006**

While records of host movement reflect whether or not a host was moving at any given observation time during the trial, they do not reflect the quality or intensity of the movement. In general, host movement intensity (i.e. the amount of movement when observed and the speed of movement) was greatest for (*R. bifasciatum*), followed by *H. abietis* larvae. When these two host types moved, it was generally readily visible that they were doing so (i.e. clear head capsule movements [> 2 mm radius of movement], pushing the head capsule up against the bark while chewing on its underside, muscle contractions running along the length of the body). *G. mellonella* moved with much less intensity, often making detection of movement difficult. If movement was seen in this host type it was usually restricted to limited movements of the head capsule (< 2 mm radius of movement) and slight contractions of the body, usually not running the length of the larvae.

f) Occurence of wood shavings

Wood shavings appearing in the host chamber indicated that the host was chewing the bark. The absence of wood shavings did not preclude chewing by hosts, however. In trials with naïve wasps, wood shavings were observed more frequently in trials with hosts that lived through the 24 h trial than in those in which they died within that time frame (e.g. wood shavings appeared in 60 % of trials with *S. carpocapsae* infected *H. abietis* hosts that survived trials compared with none at all in trials in which such hosts died of infection within 24 h) (Table 6.15). A χ^2 – test indicated that there was a significant difference in the proportion of naïve wasp trials with wood shavings among these host types ($\chi^2 = 32.825$, DF = 4, $P < 0.001$) (Table 6.16). Statistical cross-comparison identified a significant difference in the frequency of wood shavings appearing in those trials in which *H. downesi* infected larvae went on to die during the trial, but only when compared with the control ($P < 0.001$), not trials in which these host did not die of infection by the end of the trial ($P = 0.031$, Bonferroni-adjusted $\alpha = 0.0042$). Trials with hosts infected with *S. carpocapsae* that died within 24 h showed a significantly reduced occurrence of wood shavings compared with the control ($P < 0.001$) and survivors infected with this EPN species ($P < 0.001$).

In trials in which these host types were offered to experienced wasps, on average, wood shavings were seen more frequently than in corresponding naïve wasp trials (e.g. 33 % for *S. carpocapsae* infected hosts that died during the trial in comparison to none all for this host in naïve wasp trials). However, there was no significant difference in the proportion of trials with wood shavings between the controls for the two wasp groups (χ^2 = 0.634, DF = 1, P = 0.426). Comparison among the host groups infected with EPN and the control for experienced wasp trials revealed no significant difference among them (χ^2 = 4.538, DF = 4, P = 0.338). No wood shavings were seen at any time during observation of pupating *H.abietis*, paralysed *H. abietis* or *G. mellonella*. Wood shavings were recorded in roughly 18 % of trials with naïve wasps and longhorn beetle larvae (*R. bifasciatum*). This did not represent a significant difference from the control (χ^2 = 8.827, DF = 1, P = 0.003; Bonferroni – adjusted α = 0.0042). Test results are shown in Table 6.16.

Table 6.15: Percentage of observational trials with naÏve and experienced wasps in which wood shavings were recorded in the host chamber. Trials with dead hosts (freeze-killed or EPN killed) and hosts for which no wood shavings were recorded excluded.

	Percentage of trials in which wood shavings were recorded during observation		*N*	
Host type (* = *H.abietis*)	**Naïve**	**Exp.**	Naïve	Exp.
**Control*	**46.5**	**40.0**	99	60
**S.c. dead after 24h*	**0.0**	**33.3**	20	15
**S.c. live after 24h*	**60.0**	**28.6**	20	30
**H.d. dead after 24h*	**6.7**	**16.7**	28	18
**H.d. live after 24h*	**30.4**	**23.8**	23	21
*Longhorn (*R. bifasciatum*)*	**17.7**	**0.0**	30	6

Table 6.16: Statistical cross-comparison of percentage of trials with wood shavings seen in host chamber during 2 h observation of oviposition trials with naïve wasps. Only trials with *H. abietis* control (live larvae) and infected *H. abietis* (infection with *S. carpocapsae* or *H. downesi* but alive at beginning of trial) included. (χ^2 – test or Fisher's exact test, 12 cross-comparisons, Bonferroni-adjusted significance level of α = 0.0042). DF = 1 for all tests. For N see Table 6.13.

	S.c. dead after 24h	*S.c.* live after 24h	*H.d.* dead after 24h	*H.d.* live after 24h
Control	χ^2 = 15.149 **P < 0.001**	χ^2 = 1.220 **P = 0.269**	χ^2 = 15.608 **P < 0.001**	χ^2 = 1.952 **P = 0.162**
S.c. dead after 24h		χ^2 = 17.143 **P < 0.001**	**P = 0.510**	**P = 0.010**
S.c. live after 24h			χ^2 = 16.931, **P < 0.001**	χ^2 = 3.792, **P = 0.052**
H.d. dead after 24h				**P = 0.031**

g) First probing record coinciding with host movement

In the majority of instances when a wasp first started probing, host movement was observed for the same record (Table 6.17). The rate of coincidence was above 80 % of trials for all host types infected with EPN but alive when entering the trial when naïve wasps were observed and as high as 100 % (*S. carpocapsae* infected hosts). The proportion of trials with first probing and host movement coinciding was only 62 % in the control for experienced wasps. Coincidence in trials with experienced wasps was more frequent than in trails with naïve wasps when the host was infected with *H. downesi* and less frequent when *S.carpocapsae* was used to infect hosts (Table6.17). In trials with hosts other than *H. abietis*, coincidence of host movement and wasp probing was highest for *R. bifasciatum* when offered to naïve wasps (50 %), whereas *G. mellonella* larvae were only moving in 17 % of cases where naïve wasps were first seen probing.

Extensive analysis for all host types with the aid of a χ^2 – test was not possible due to the low number of replicates available for those trials where wasps did not probe very frequently (e.g. naïve wasps on infected *H. abietis* that died during the trial period). However, a χ^2 – test comparing only the controls for naïve wasps and experienced wasps yielded no significant difference between the two for the coincidence of host movement and wasp probing, though significance was approached (χ^2 = 3.224, DF = 1, P = 0.073). To examine whether the rate of coincidence between host and wasp behaviour was explained by mere chance, a χ^2 – Goodness-of-Fit test was carried out for the control trials with naïve and experienced wasps. In each case, the observed coincidence was compared to that expected by chance based on the data for host movement (see Table 6.10). This was 41 % in control trials with both naïve wasps and experienced wasps, thus indicating that there was a 41 % chance of hosts moving at the time of first probing due to chance alone (6.13). The Goodness-of-fit test found a significantly higher coincidence of wasp probing and host movement than would have been expected due to chance alone, both for naïve wasps (χ^2 = 23.285, DF = 1, P = 0.01; N = 36) and experienced wasps (χ^2 = 8.378, DF = 1, P = 0.004, N =45).

Table 6.17: Mean percentage of first probing records that coincided with host movement.

	First probing record coinciding with host movement (%)		*N*	
Host type (* = *H.abietis*)	**Naïve**	**Exp.**	Naïve	Exp.
**Control*	**80.56**	**62.22**	36	45
**S.c. dead after 24h*	**100**	**88.89**	3	9
**S.c. live after 24h*	**100**	**60.87**	15	23
**H.d. dead after 24h*	**42.86**	**63.64**	7	11
**H.d. live after 24h*	**80.00**	**90.91**	10	11
**Prepupae*	**0.00**	N/A	2	N/A
**Paralysed*	**0.00**	**0.00**	2	1
*Longhorn (*R. bifasciatum*)*	**50.00**	N/A	13	N/A
*Waxmoth (*G. mellonella*)*	**16.67**	**20.00**	12	5

6.3.14 Regression analysis of host movement and wasp oviposition

To see whether wasps made decisions about oviposition based on host movement, regression analysis on this matter was conducted for naïve and experienced wasps. Host movement was chosen as the predictor variable since it was the most basic variable associated with host behaviour. Oviposition by wasps was chosen as the response variable since it indicated that the wasp had located the host and was committed to laying a clutch of eggs on that host. Residuals for binary logistic regression models using oviposition data for individual wasps (oviposition commencing or not within two hours) did not conform to a normal distribution sufficiently well for the models to be deemed reliable, irrespective of whether all host types were included, or only the control. It was therefore decided to use the aggregate variable of the proportion of trials in which oviposition commenced within the 2 h observation period for each host type and regress it against the mean proportion of records for host movement (Fig 6.20). Only data from *H. abietis* hosts that were alive when introduced into a trial (control, EPN infected [four data sets], prepupae and paralysed) and thus capable of movement were included in this linear regression model to exclude a possible effect of host species. Regression was run separately for naïve wasps (N = 7) and experienced wasps (no data for prepupae, N = 6). For N for each host movement mean see Table 6.15. All data were arcsine transformed.

There was a positive relationship between *H. abietis* host movement and the occurrence of oviposition initiation within the 2 h observation period for naïve wasps. This relationship was found to be highly significant (T = 7.49, Coef. = 0.799, R^2 = 0.92, F = 56.04, DF = 1, P = 0.001) (Fig 6.20). For experienced wasps, the rate of oviposition also increased with the rate of host movement. However, the slope of the linear trendline was less steep, the fit was less significant and the R^2 obtained was lower than that for naïve wasps (T = 2.93, Coef. = 0.582, R^2 = 0.68, F = 8.59, DF = 1, P = 0.043). Due to the low number of data points each trendline was based on, it was not possible to compare the slopes and intersects of the two for significant differences. Data points representing hosts that were infected with EPN and that died within the 24 h trial period fell within or very close to the confidence interval boundaries, both for naïve wasps and experienced wasps (Fig 6.19).

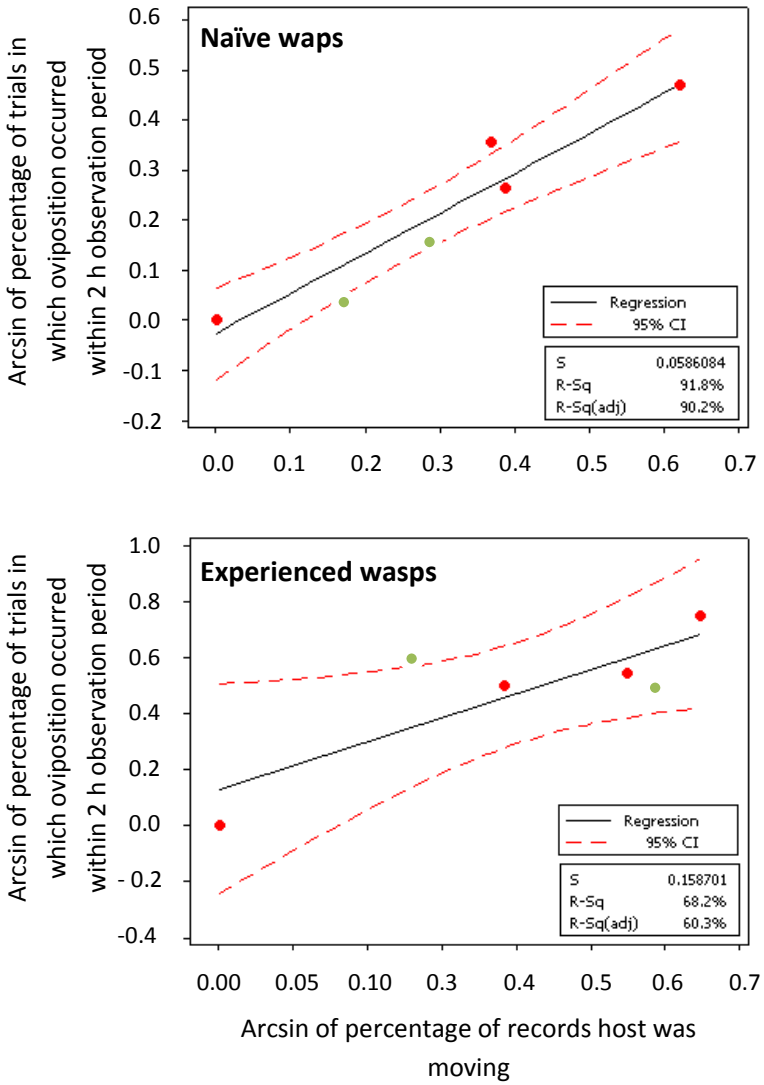


Fig 6.20: Linear regression fitted lines for the arcsin of host movement (x-axis) against the arcsin of the proportion of trials in which wasps initiated oviposition within 2 h observation period (y-axis). Red dashed lines represent upper and lower 95 % confidence interval. Green data points represent *H. abietis* larvae infected with EPN that died during the experiment.

6.3.15 Regression analysis for host movement and wasp probing location

To elucidate the connection between host movement rates and the likelihood with which wasps would probe over the host chamber, a similar approach to the one used for wasp oviposition regression was taken. Aggregate variables for the mean proportion of probing records in which wasps were probing over the host chamber were regressed against the mean proportion of records of host movement for the respective trails (linear regression). N was 7 for naïve wasps and N was 6 for experienced wasps (only data for *H. abietis* that were capable of movement was included, as above) (Fig 6.21).

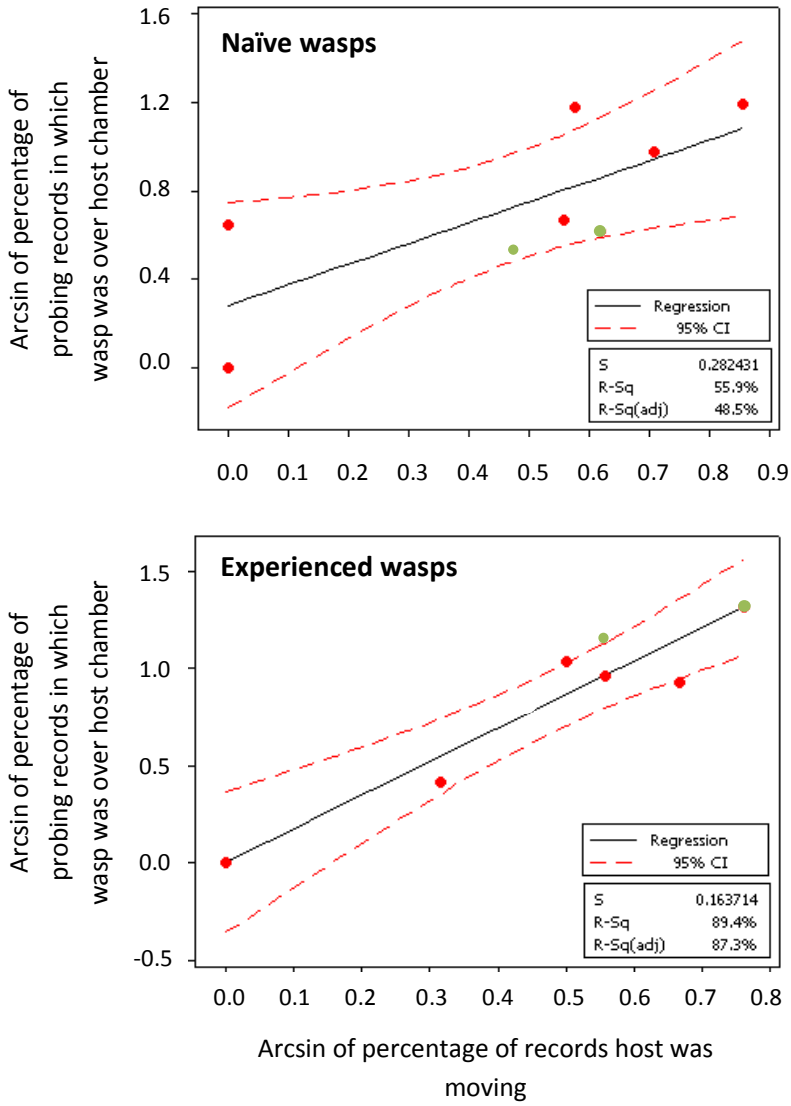


Fig 6.21: Linear regression fitted lines for arcsin of host movement (x-axis) against the arcsin of the proportion of probing records in which wasps were located over the host chamber. Red dashed lines represent upper and lower 95 % confidence interval. A = naïve wasps, B = experienced wasps. Green data points represent *H. abietis* larvae infected with EPN that died during the experiment.

While the linear fits for naïve wasps and experienced wasps both had a positive slope, indicating a positive relationship between wasps probing in a location over the host chamber and host movement, the fit was significant for naïve wasps (T = 2.76, Coef. = 0.937, R^2 = 0.56, F = 7.59, DF = 1, P = 0.033) and experienced wasps (T = 6.50, Coef. = 1.456, R^2 = 0.92, F = 56.94, DF = 1, P = 0.001).

6.3.15 Connections between host movement and wasp behaviour based on individual records of probing and searching

Observation records for control hosts as well as EPN infected *H. abietis* hosts that either survived the trials or perished during them (naïve and inexperienced wasps) were analyzed for whether or not this first probing bout directly led on to oviposition by the wasp. It was recorded how often wasps aborted probing and searching bouts in relation to host movement (abortion was constituted by the wasp moving off the bark patch). Due to the limited number of instances in which wasps probed in trials with some wasp/host combinations, statistical comparison was limited to control data for naïve and experienced wasps as well as the overall totals for the five different host types, again separately for naïve and experienced wasps (Tables 6.18 and 6.19).

Table 6.18: Wasp behaviour in response to host movement recorded in trials with **naïve wasps** (data for controls and *H. abietis* infected with EPN but alive at beginning of trial included). Each set of data is grouped in accordance with whether host moved (**+**) or did not move (**-**) when oviposition followed on from the first probing bout or a searching or probing bout was aborted. Numbers in brackets represent N or, if in italics, the number in trials in which oviposition occurred against the total. Asterisks indicate a significant difference between the respective data sets within paired columns (+ and -), or for totals of aborted searching and probing bouts, an asterisk represents a significant difference from chance occurrence. For test results, see Appendix A.16.

Wasp	Host type (*H. abietis*)	Frequency of oviposition after first probing bout		Aborted searching bouts		Aborted probing bouts	
		+	-	+	-	+	-
Naïve	***Control***	**45.83** *(11/24)*	**0.00** *(0/6)*	2 *	14	2	5
	S.c. dead	**50.00** *(1/2)*	**0.00** *(0/2)*	1	7	N/A	3
	S.c. live	**28.57** *(4/18)*	N/A	1	2	N/A	2
	H.d. dead	**33.33** *(1/3)*	**0.00** *(0/1)*	1	4	N/A	1
	H.d. live	**75.00** *(6/8)*	**0.00** *(0/1)*	N/A	3	N/A	N/A
Totals for infected hosts		**23/55** *	**0/10**	**5** *	**30**	**2**	**11**

Table 6.19: Wasp behaviour in response to host movement recorded in trials with **experienced wasps.** Each set of data is grouped in accordance with whether host moved (**+**) or did not move (**-**) when oviposition followed on from the first probing bout or a searching or probing bout was aborted. Numbers in brackets represent N or, if in italics, the number in trials in which oviposition occurred against the total. Asterisks after numbers indicate a significant difference between the respective data sets within paired columns (Frequency of oviposition) or from chance (Abortion of searching and probing bouts). For test results, see Appendix A.16.

		Frequency of oviposition after first probing bout (%)		**Aborted searching bouts**		**Aborted probing bouts**	
Wasp	Host type (*H. abietis*)	**+**	**-**	**+**	**-**	**+**	**-**
	Control	**62.5** * *(15/24)*	**9.09** *(1/11)*	2	10	2	8
	S.c. dead	**37.50** (3/11)	**0.00** (0/1)	1	2	1	3
Exp.	***S.c. live***	**57.14** (8/14)	**14.29** (1/7)	1	5	2	6
	H.d. dead	**80.00** (4/5)	**50.00** (1/2)	N/A	5	N/A	4
	H.d. live	**66.67** (6/9)	**50.00** (1/2)	N/A	4	1	N/A
Totals for infected hosts		**36/53** *	**4/23**	**4** *	**26**	**6** *	**21**

In trials with all five host types, wasps were more likely to oviposit following on from their first probing bout when the host was seen moving at the time the probing bout began, regardless of wasp experience. These differences were either significant (e.g. for experienced control, P = 0.004) or approached significance (Appendix A.16). Wasps were consistently more likely to abort bouts of searching or probing by moving off the bark patch when hosts were not moving at the same time. For instance, of the 16 searching bouts that were aborted by naïve wasps in control trials, 14 were aborted at a time the host was not moving. For experienced wasp controls, this number was 10 out of 12. When these frequencies were statistically compared with the mean proportion of records of host movement, to test whether they were due to chance alone, a significant difference to chance occurrence was detected in all cases or approached significance (see Appendix A.16).

6.3.17 Continuous observation trials (30 minute observation period)

There was a clear restriction in the area wasps were most likely to search and probe the bark in continuous observation trials, especially when the host larva was not moving (or when no scratching stimulus was supplied together with a dead host). Just as was seen during the 2 h instantaneous observations with the smaller bark patches (2.25 cm^2), if wasps were probing in a position other than over the host chamber, they tended to stay at the very edge of the bark patch, in the area in which bark and tape met (Fig 6.22). In some instances, wasps would spend protracted periods of time

(> 5 minutes) probing in a single location at the edge of the bark patch without retracting their ovipositor. Wasps were rarely seen (approximately < 10 % of time they were on the bark patch) in the area between the edge of the bark patch and the centre where the host was located if the host was not moving.

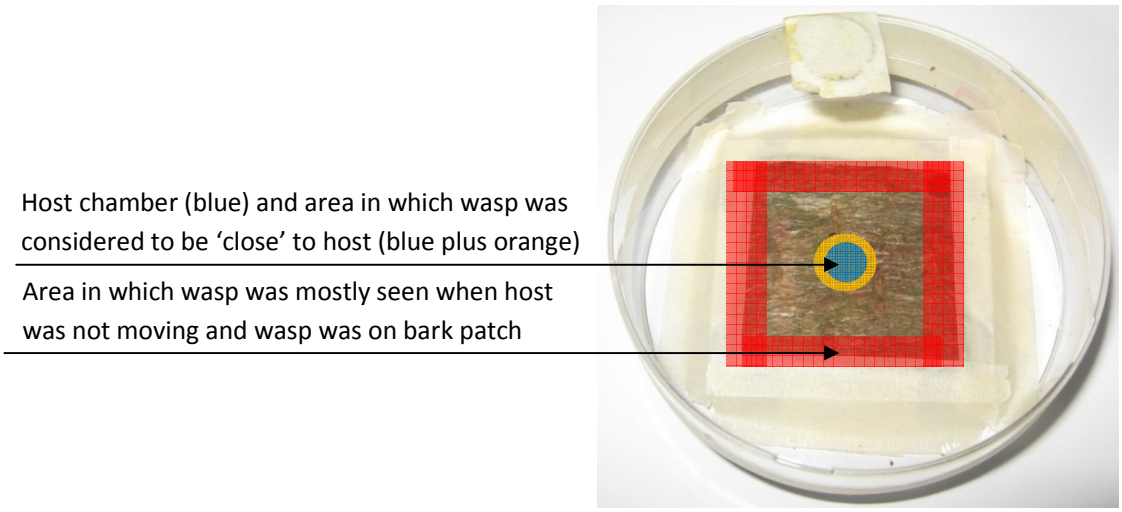


Fig 6.22: Area in which wasps (naïve and experienced) were most commonly seen searching, pausing and probing on the bark patch in trials that were continuously observed).

Once the host began moving or when a scratching stimulus was introduced, wasps would frequently begin a gradual approach toward the location of the host chamber, i.e. the centre of the bark patch. This was particularly apparent in trials with experienced wasps. The approach would consist of a slow, zig-zagging pattern of intermittent movement, often associated with wasps antennating the bark and turning ('searching'), interrupted by periods of stillness in which wasps most often spread out their legs and raised their abdomen from the bark ('waiting') (Fig 6.23; see also 6.2.12 and Fig 6.3). However, all instances of wasps being on the bark patch but not moving were categorised as 'pausing' behaviour during data recording so this component behaviour was not recorded separately.

In some cases, wasps would probe intermittently on their approach to the centre of the bark patch. By contrast, when the host was not moving or no scratching was applied, wasps that did cross the centre of the bark patch often did so rapidly, rarely pausing or showing any signs of intensified localised searching as evidenced by a rapid succession of bouts of searching, pausing and probing.

In trials in which the wasp approached the location of the host and made its way to the centre of the bark patch, it would occasionally spend a portion of time repositioning itself over the host. In some cases, wasps probed the bark in several places only a few millimetres apart when directly over the host, while in others they probed only once and quickly began ovipositing. In several instances (four

trials) in which wasps were located directly above a host that was moving and chewing the bark below them, wasps repeatedly turned and then paused for several seconds at a time in alternation. In these instances, turns the wasp made and the way in which it repositioned its body over the host seemed to track the position of the head capsule of the host under the bark as the host was chewing on it (i.e., if the host was chewing on the bark on the right side of its chamber, but then shifted its position and began chewing on the bark on the left side of its chamber, the wasp would turn and move so as to track the epicentre of the hosts chewing activity). Similar observations were made when wasps were responding to artificial scratching of the bark with a needle. In two of the cases in which wasps were successfully attracted to the centre of the bark patch to where the scratching stimulus was being applied, wasps were observed to change their position and probing location in response to the scratching epicentre (i.e. where the needle physically touched the bark as it was scratching it) being moved from one side of the host chamber to the other side.

a) Host movement

Live host larvae (*H. abietis*), moved during 34.70 % of the observation time in trials with naïve wasps (standard deviation 12.50) and 27.94 % of the time in trials with experienced wasps (standard deviation 33.50). Medians were not significantly different from each other for these two data sets (M.-W. U-test, W = 262.5, P = 0.7619, N = 10 for naïve wasps and N = 39 for experienced wasps). The rate of movement was similar to the proportion for records for host movement in 2 h instantaneous observation trials which was approximately 41 % for trials with naïve and experienced wasps.

Mean host weight was similar across all four trial groups (live hosts for experienced wasp trials: 216.22 mg, standard deviation ± 46.53; live hosts for naïve wasp trials: 224.00 mg, standard deviation ± 23.36; freeze-killed hosts: 230.55 mg, standard deviation ± 21.52, *H. downesi*-killed hosts: 204.78 mg, standard deviation ± 24.14). A significant difference among means was found (One-way ANOVA, F = 2.89, DF = 3, P = 0.042) and Tukey's test showed that this difference was due to a significant difference between freeze-killed hosts and *H. downesi* hosts [$\alpha = 0.05$]. As in the oviposition trials, hosts chewed the bark when inside their chamber in some continuous observation trials. When the host was chewing the bark, it was often possible to see the bark move up and down above it as it did so (approximately 1 mm to 2 mm amplitude).

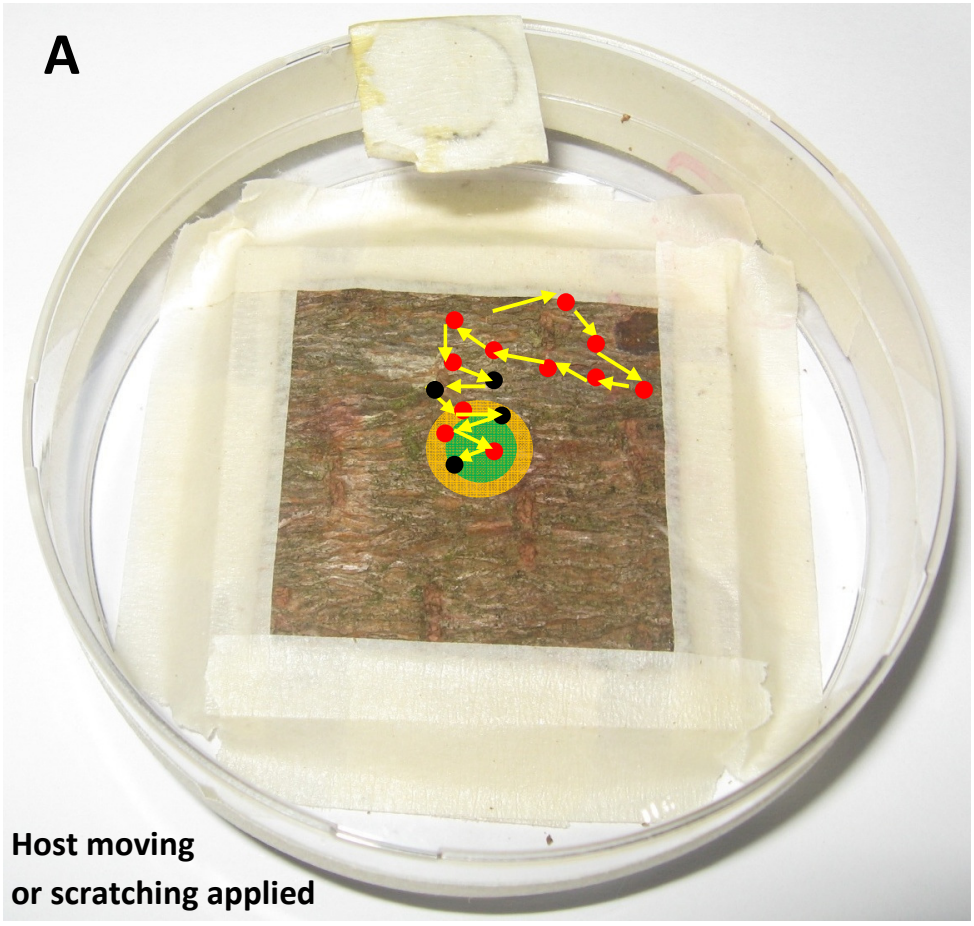
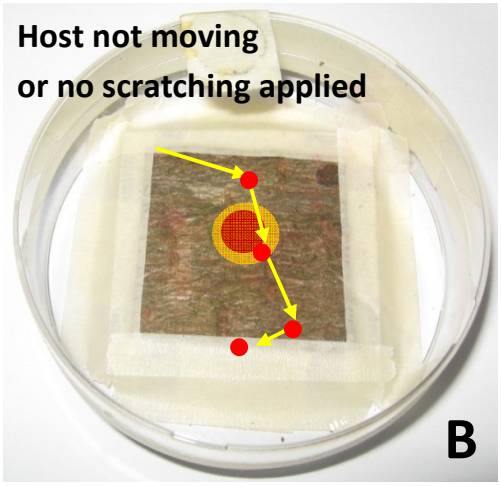


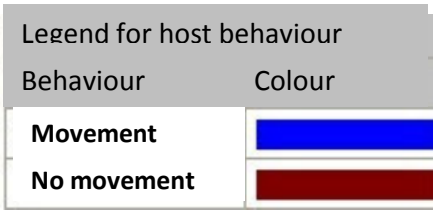
Fig 6.23: Schematic illustration of frequently observed wasp movement patterns across the bark patch in continuously observed experiments. (**A**): Host larvae moving (or scratching being applied). (**B**): Host not moving (or no scratching applied). Yellow arrows indicate movement of the wasp ('**searching**'), red dots indicate locations in which wasps '**paused**' and a black dot indicates a '**probing**' site.

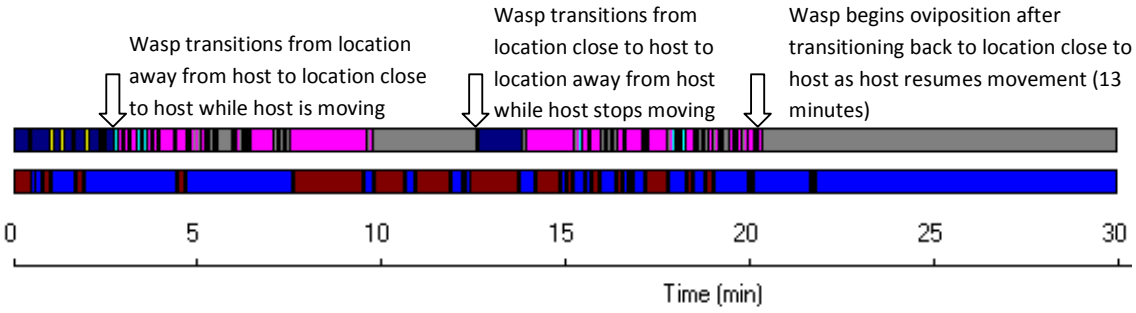


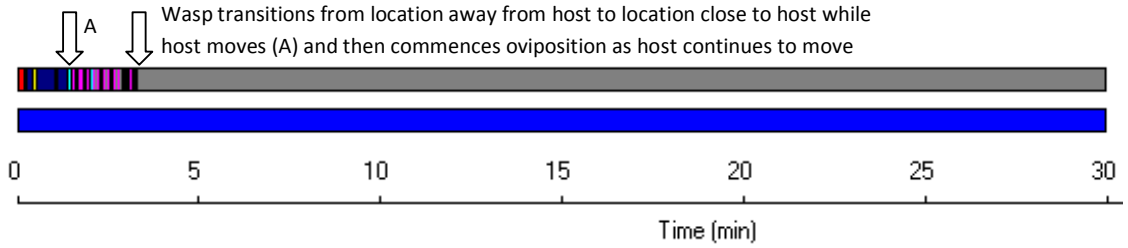
b) Time – event plots relating wasp behaviour to host behaviour

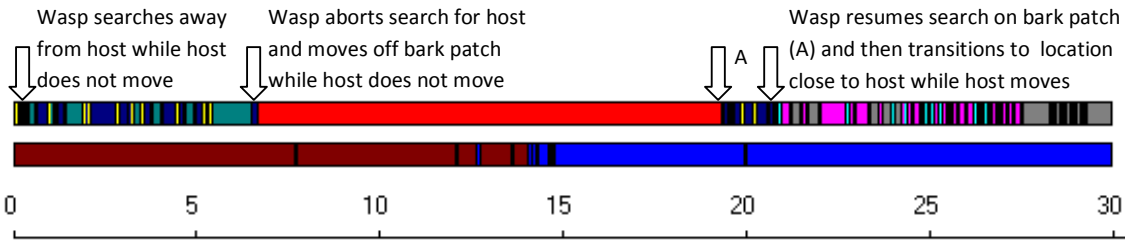
Using The Observer™ software package, time-event plots were created for the observations recorded. These plots show the wasp and host behaviour during the course of an individual 30 minute observation. Some representative time-event plots for each of the four wasp/host combinations for which observations were recorded are presented in Fig 6.24 through Fig 6.26.

Fig 6.24: Time – event plots for continuous observation trials with experienced wasps and live *H. abietis* as hosts [created with The Observer™]. In each plot, the wasp behaviour is given on top and host behaviour is given on bottom.









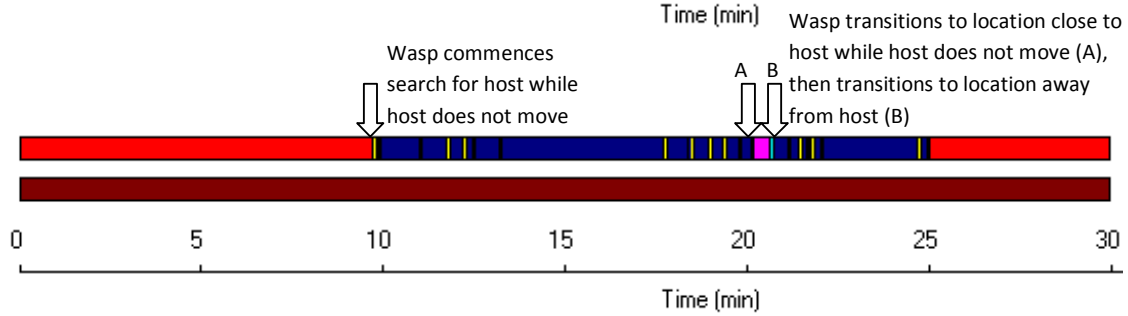
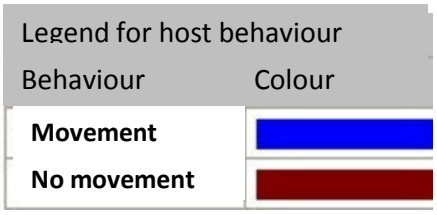
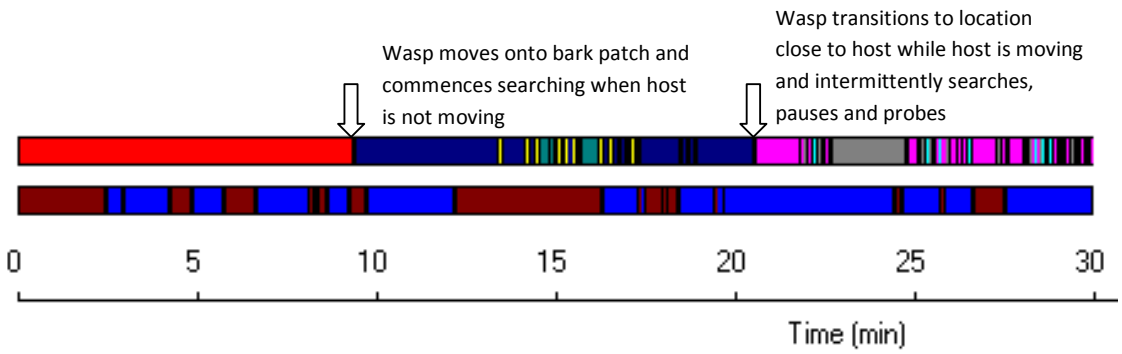
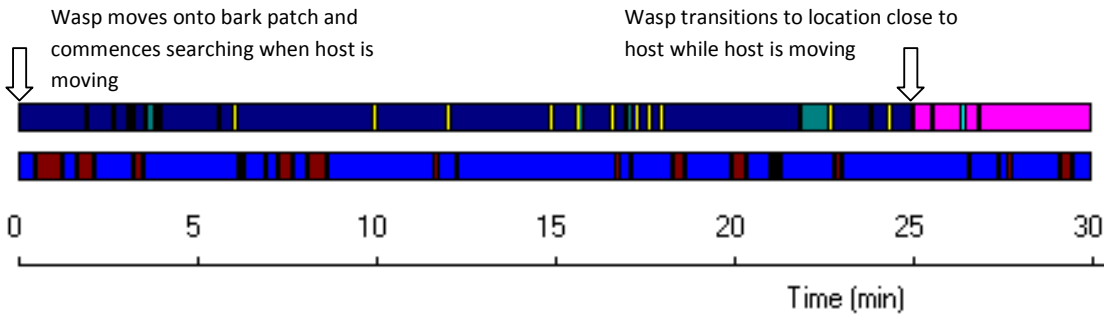


Fig 6.25: Time – event plots for continuous observation trials with naïve wasps and live *H. abietis* as hosts [created with The Observer™]. In each plot, the wasp behaviour is given on top and host behaviour is given on bottom.







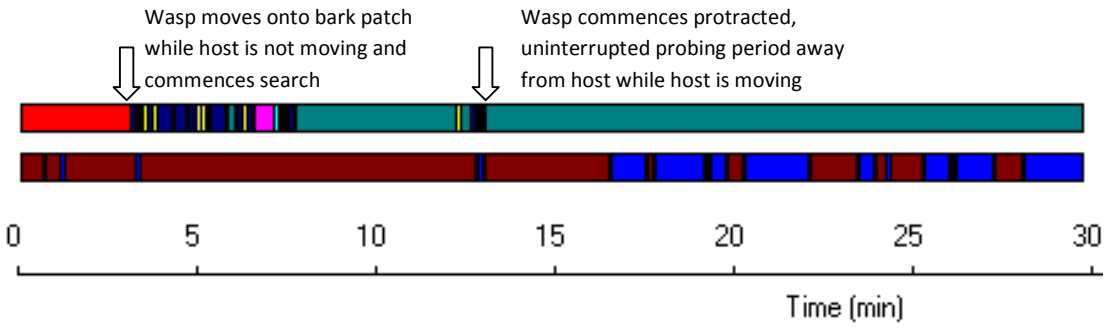


Fig **6.26:** Time – event plots for continuous observation trials with experienced wasps and freeze-killed *H. abietis* (plots A and B) and *H. downesi*-killed *H. abietis* (graph C) with added manual scratching [created with The Observer™]. In each plot, the wasp behaviour is given on top and scratching is given on bottom.

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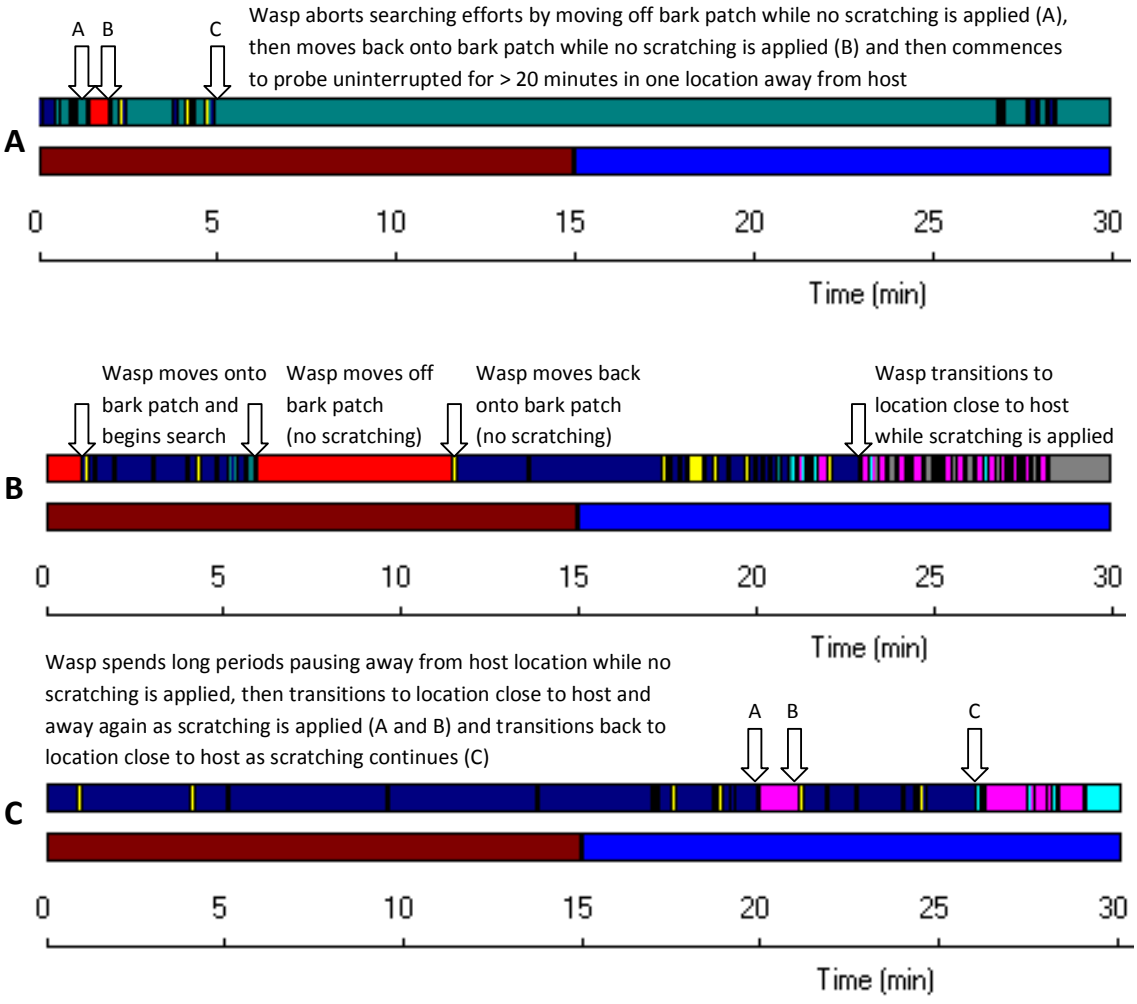

c) Wasp behaviour in response to host movement

Naïve wasps were less likely to forage on the bark patch than experienced wasps. In three out of the ten trials conducted with naïve wasps, wasps did not move onto the bark patch at all during the 30 minutes of observation (30.0 %). This was the case for three of 39 trials with experienced wasps and a live *H. abietis* host (7.7 %). On average, naïve wasps spent 56.2 % of the observation time off the bark patch – this figure was 44.4 % for experienced wasps that were offered a live host (37.1 % for freeze-killed hosts with scratching commencing 15 minutes into the observation, 51.1 % if the host had been killed by *H. downesi*). There was no significant difference between the time wasps spent off the bark patch among these four sets of trials, however (K.-W.-test, H = 1.90, DF = 3, P = 0.592, N = 39 [exp. live host], N = 10 [naïve live host], N = 10 [freeze – killed + scratching], N = 9 [*H. downesi*-killed + scratching]).

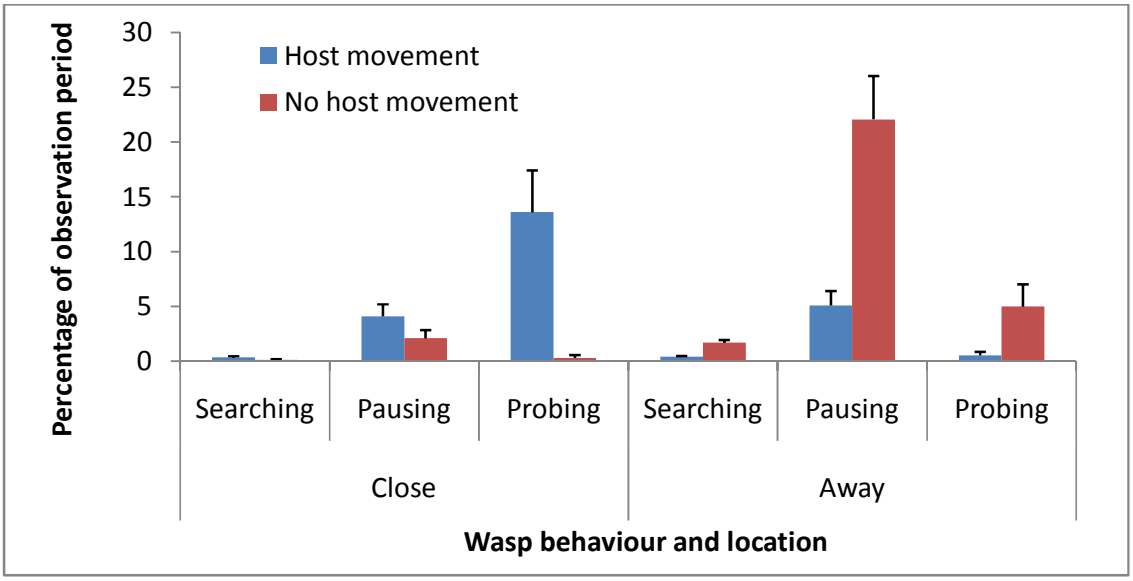

Fig 6.27: Mean proportion of 30 minute observation period spent by **experienced wasps** displaying each of the three behaviours that were distinguished in an area of the bark patch close to or away from the host. The data is taken from trials with live hosts and grouped by whether or not the host was moving at the time. N =39 for each bar.

Experienced wasps spent more time close to the host at the centre of the bark patch when the host was moving than when it was not doing so (Fig 6.27). The difference in wasp location was particularly striking for probing behaviour close to the host, which was much more prevalent when hosts were moving (approximately 14 % of the observation time compared with < 1 % when the host was not moving Fig 6.27). Overall, only three of the 39 experienced wasps observed probed close to the host when it was not moving, whereas 14 wasps did so when it was moving, a significant difference (χ^2 = 9.101, DF = 1, P = 0.003). The time spent close to the host also was greater for naïve wasps

when the host was moving and for experienced wasps when host movement was simulated by manually scratching the bark in trials with freeze-killed and EPN-killed host larvae (Appendix A.17). Wasps spent more time pausing than they did searching in all four trial types (Fig 6.27, Appendix A.17). Experienced wasps spent more time pausing away from the host location than they did searching away from the host location when live host were offered. The data was not statistically analysed as the predicting variable in each case (host movement or no movement) was interdependent (i.e. the more a time a host moved during the observation period, the less time it did not move).

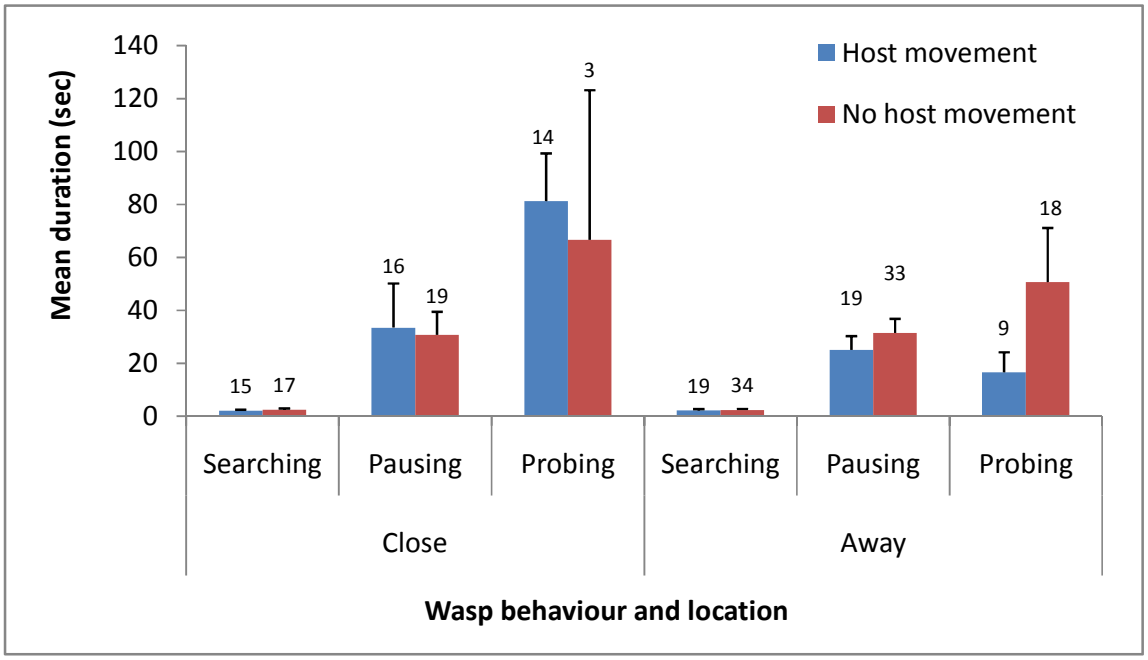

Fig 6.28: Mean duration of each bout of behaviour performed by **experienced wasps** in an area of the bark patch close to or away from the host. The data were taken from trials with live hosts and grouped by whether or not the host was moving at the time the respective behaviour was observed.

The mean duration for each bout of behaviour during the 30 minute observation period is presented in Fig 6.28. In this case, only data for experienced wasps with live hosts is shown as N was low (< 8) for most behaviour/location data from other trials. The mean amount of time experienced wasps spent performing each of the three behaviours that were distinguished during observations (searching, pausing and probing) was almost identical when the host was moving or not moving, regardless of where the wasp was located (close or away from the centre of the bark patch). Pairwise comparison of data sets for each behaviour/location combination with respect to host movement revealed no significant differences (for test results see Appendix A.14). Individual bouts of pausing

were also significantly longer than bouts of searching, regardless of the location of the wasp and whether or not the host was moving ($P < 0.001$; see Appendix A.15 for test results).

6.3.18 Behavioural transitions of wasps in response to host mosvement

With The Observer™ software package, a table was created for each group of trials (naïve wasps with live hosts, experienced wasps with live hosts and experienced wasps with dead hosts killed by freezing or *H. downesi* infection with added manual scratching) in which the number of instances in which wasps transitioned from one type of behaviour in one location (e.g. 'searching close') to another behaviour and/or location (e.g. 'pausing away'). Whether or not the host was moving at the time the transition occurred was also taken into account. The resulting transition matrices are presented in Appendix A.18. Subsets of this data that represent the most relevant transitions for relating wasp behaviour to host vibrational cues are presented here.

a) Location transitions of wasps in response to host movement

In all four groups of trials, wasps more often made the transition from a location away from the host to close to the host when the host was moving or scratching was being applied. In trials with experienced wasps and live hosts, wasps transitioned from an 'away' location to a 'close' location almost five times as frequently as they did the reverse (19 versus 4, respectively). When the host was not moving, wasps were equally likely to make the transition in either direction. The difference between the frequency with which these location changes occurred in relation to host movement was found to be significant only in trials with experienced wasps and live hosts, however ($P = 0.004$) (Table 6.20).

Table 20: Location transitions of foraging *B. hylobii* females while host is moving/scratching is applied (**+**) or not moving/scratching is not applied (**-**) with statistical test results.

Trial group	N	Location transition	+	-	χ^2 or Fisher's exact test
Experienced wasp with live host	39	*Away → Close*	19	26	$\chi^2 = 8.296$, DF = 1, **P = 0.004**
		Away ← Close	4	26	
Naïve wasp with live host	10	*Away → Close*	2	7	**P = 0.486**
		Away ← Close	0	6	
Experienced wasp with freeze-killed host	10	*Away → Close*	4	4	**P = 0.565**
		Away ← Close	1	4	
Experienced wasp with H. downesi-killed host	9	*Away → Close*	3	4	**P = 1**
		Away ← Close	2	4	

b) Behavioural transitions of wasps in response to host movement

To examine which behaviour of wasps most commonly preceded probing behaviour and what effect host movement had on this, wasp behaviour transitions from searching behaviour (close to or away from host) to probing behaviour (close to or away from host) when the host larva was moving and not moving were compared (Table 6.21). The same was done for behaviour transitions between pausing and probing. This was done for each of the four trial groups and the results are presented in Table 6.21. It was found that in all trials with experienced wasps, they were more likely to start probing after pausing rather than after searching. In addition, the likelihood of doing so was significantly affected by host movement (live hosts, $P < 0.001$) or the bark was being scratched to supplement a freeze-killed host ($P < 0.001$). With a *H. downesi*-killed host, the result was significant ($P = 0.051$). In trials with naïve wasps, there was no significant difference in the ratio of these behavioural transitions with regard to host movement ($P = 0.605$).

c) Abortion of searching, pausing and probing behaviour in response to host movement

Abortion of a searching, probing or pausing was considered to have occurred when the wasp moved off the bark (i.e. when it transitioned from any of the behaviours mentioned to the 'other' category which was used during observations when the wasp was off the bark). The number of times a wasp moved off the bark (transition to 'other' behaviour) was compiled for when the host was moving and not moving, and the ratio found was compared with the ratio that would be expected if the wasps aborted their foraging efforts randomly. This was accomplished by comparing the observed ratio (i.e. 6/65 for experienced wasps and live hosts) to the ratio expected by random chance (this was the proportion of the observation period that hosts moved on average in each of the trial groups [experienced wasps with live hosts: 27.94 %; naïve wasps with live hosts: 34.70 %; for trials with scratching 50.00%]) using a χ^2 – Goodness-of-Fit test.

Table 6.21: Behavioural transitions of wasps in continuously observed trials recorded while host is moving (**+**) or not moving (**-**) with statistical test results.

Trial group	N	Behavioural transition	+	-	χ^2 or Fisher's exact test
Experienced wasp *with live host*	39	*Searching → Probing*	12	41	$\chi^2 = 48.021$, DF = 1, **P < 0.001**
		Pausing → Probing	164	59	
Naïve wasp *with live host*	10	*Searching → Probing*	8	10	$\chi^2 = 0.267$, DF = 1, **P = 0.605**
		Pausing → Probing	7	6	
Experienced wasp *with freeze-killed host*	10	*Searching → Probing*	0	4	**P < 0.001**
		Pausing → Probing	74	12	
Experienced wasp *with H. downesi-killed host*	9	*Searching → Probing*	1	2	**P = 0.051**
		Pausing → Probing	34	4	

Wasps were at least 7 times (*H. downesi*-killed host plus scratching) and up to eleven times (naïve wasps with live hosts) more likely to move off the bark and abort foraging efforts when the host was not moving than when it was moving (Table 6.22). In the two instances where a statistical comparison against the random chance of this occurring was possible, the difference was found to be significant ($P < 0.001$ for experienced wasps and live hosts and $P = 0.008$ for freeze-killed hosts with scratching).

Table 6.22: Location transitions of wasps (from any behaviour on bark to 'other', i.e. off the bark) in continuously observed trials recorded while host is moving (**+**) or not moving (**-**) with statistical test results (χ^2 Goodness-of-Fit test against mean time host spent moving in respective trial group).

		Transitions from on the bark to off the bark		
Trial group	N	**+**	**-**	χ^2 Goodness-of-Fit test
Experienced wasp *with live host*	39	6	65	$\chi^2 = 13.384$, DF = 1, **P < 0.001**
Naïve wasp *with live host*	10	1	11	N/A
Experienced wasp *with freeze-killed host*	10	3	14	$\chi^2 = 7.118$, DF = 1, **P = 0.008**
Experienced wasp *with H. downesi-killed host*	9	1	7	N/A

6.3.19 Oviposition during and after continuously observed trials

In continuously observed trials with experienced wasps and live hosts, wasps oviposited or commenced oviposition during the 30 minute observation period in eleven instances out of 39 trials in total (28.2 %). Naïve wasps oviposited within the 30 minutes in none of the ten trials. In trials in which the bark was scratched and a freeze-killed host was offered, wasps moved close to the host during the observation period in three out of ten trials (30 %) and in one case initiated oviposition. Using *H. downesi*-killed hosts, only one wasp was attracted to the centre of the bark patch and no oviposition occurred.

After the observation period ended, it was attempted to goad wasps into ovipositing on the dead host (freeze-killed). This was done in five instances: the three trials in which wasps were already probing, searching or pausing close to the host at the end of the trial plus two additional trials in which wasps were probing, searching or pausing away from the host at the end of the trial. It was also done in the case of two trials in which hosts were dead and infected with *H. downesi*. In all of

these cases where wasps were not already close to the host chamber, it was possible to lure the wasp to the centre of the bark patch close to the host chamber within 30 minutes of the end of the experiment by continuously scratching the bark (if they were not already close to the host chamber when the exposure period ended). By continuing to scratch and then gently moving the host with the needle as the wasp was probing over it, it was possible to cause wasps to lay eggs in three of the five trials it was attempted with freeze-killed hosts (60 % oviposition rate). Oviposition occurred in one of the two trials in which this was attempted with an *H. downesi* – killed host.

Once the wasps in these attempts appeared to be ovipositing (long period of probing over host, contraction of abdomen), they were allowed to complete oviposition. The egg clutches that were laid in these instances were of a size typical for experienced wasps (12, 4 and 4 eggs on freeze-killed hosts and 11 eggs on *H. downesi*-killed host).

To compare oviposition rates in the continuous observation arena with the large bark patch and the standard arena with the small bark patch, ten arenas each with live hosts and an experienced or naïve wasp were incubated the same way arenas in no-choice trials were for 23.5 h after continuous observation had ended. Eggs were found on 9 out of these ten trials for each of the two wasp types (naïve and experienced), a rate slightly higher than in standard arena trials, but not significantly so (Fisher's exact test; naïve: $P = 0.175$, experienced: $P = 1$).

6.4 Discussion

Both naïve and experienced *B. hylobii* females laid eggs on hosts infected with the two nematode species investigated (*H. downesi* and *S. carpocapsae*). Naïve wasps were, however, much less likely to parasitize a moribund host larva that died within the trial period. Hosts that died after a trial had ended were parasitized in frequencies no different from the control and nematode species had no effect on parasitism rates. Everard et al. (2009) report similar results: the more time that passed before *H. abietis* larvae previously infected with *H. downesi* were offered to naïve *B. hylobii* females, the lower the likelihood of wasps parasitizing such a host when given a choice. Cocoons of codling moth larvae exposed to the LC_{95} of *S. carpocapsae* IJs for 12 h prior to being offered to parasitoid wasps for 24 h were parasitized significantly less frequently than uninfected control hosts (Lacey et al. 2003). However, in neither of the two mentioned studies is parasitism directly compared between hosts that survived the 24 h trials period and those that did not. Other examples of parasitoids that discriminated between EPN infected hosts and healthy hosts are the leafminer parasitoids *Diglyphus begini* and *D. isea* (Sher et al. 2000; Head, 2003). In the present study, host larvae were exposed to EPN IJs for 48 h prior to experiments. This means that hosts that died of EPN infection during the 24 h trials had been infected up to 72 h earlier. Parasitism of infected hosts was reduced as early as twelve hours after host infection in the study conducted by Everard et al. (2009). All of the *H. abietis* hosts that were offered to wasps 72 h after EPN infection (4 h exposure to 8,000 IJs) in that study were dead by the time oviposition trials began and were thus not parasitized by wasps. Since Everard et al. do not give parasitism rates for infected hosts grouped by their condition or progress of infection (i.e. dead or alive at the end of the trial) it is not possible to make a direct comparison to the results of the present study. Also, in both of the cited studies wasps were offered a healthy host as an alternative to an infected host, which may account for effects on parasitism rates being observed so early on after infection. In the present study, wasps were only provided one host at a time.

In most cases in which parasitoid response to pathogen infected host larvae has been investigated, wasps were not able to distinguish between healthy hosts and those in advanced stages of infection by pathogens including *Beauveria bassiana* and baculovirus (Sait et al. 1996; Chilcutt & Tabashnik 1999; Lord 2001; Down et al. 2005). In those instances that a parasitoid wasp did refuse to oviposit on infected hosts, the hosts that were rejected had already died of an infection or were in advanced stages of infection (Brobyn et al. 1988; Mesquita & Lacey 2001). In some cases, dead hosts were handled and even probed by these parasitoids, however. Such behaviour would indicate that wasps are basing their oviposition decisions on the quality of a host they have located. Parasitoid wasps have been shown to be able to evaluate the quality of a host by integrating visual and chemical cues,

assessing host texture and size as well as the host response to handling and probing. This process is termed 'host acceptance' (Turlings & Wäckers 1993; Godfray 1991; Strand & Obrycki 1996; Pexton & Mayhew 2005; Xiaoyi & Zhongqi 2008). The fact that *B. hylobii* females, both naïve and experienced, did not reject *H. abietis* larvae in advanced stages of nematode infection (and the finding that wasps would oviposit on dead infected hosts if provided with a vibrational stimulus as discussed below) indicates that they were not capable of detecting or evaluating the condition of host larvae to any significant extent. If they did so, it had no apparent detrimental effect on the acceptance of EPN infected hosts.

Though dead hosts (both freeze-killed and nematode infected) were not parasitized by *B. hylobii* females, wasps did probe on bark patches sheltering them, in only a single instance was a wasp observed probing over a dead host and then declining to oviposit (freeze-killed host). Everard et al. (2009) report that host larvae that were dead from *H. downesi* infection from the beginning of a trial were not parasitized at all, an observation also made in the present study. Similar findings have been published for other wasps that parasitize cryptic hosts. For example, the braconid *Biosteres longicaudatus* showed no searching or probing behaviour when offered dead or etherised hosts (Lawrence 1981). The wasps did, however, occasionally probe dead or etherised hosts when they were offered in combination with a live host. Some of the dead hosts that were probed had oviposition scars but were not parasitized, indicating that host movement was a prerequisite for host acceptance or that wasps were able to detect that hosts were dead based on contact cues (volatiles or texture).

The failure of *B. hylobii* to parasitize dead and incapacitated hosts and the low rates of parasitism found for prepupal and paralysed *H. abietis* larvae begs the question as to how *B. hylobii* locates hosts. Volatiles alone, whether released by the bark substrate or the host, appeared to be insufficient to account for successful host location and oviposition by wasps on their own. As a cryptic host that is sheltered under the bark of tree stumps, *H. abietis* is inaccessible to direct handling or visual location by the wasp (Henry 1995). Plant and host volatiles as well as substrate vibrations caused by the host have been identified as two of the major cues used by most parasitoids of cryptic hosts to locate host insects at short-range (Meyhöfer et al. 1997; Xiaoyi & Zhongqi 2008). Volatiles may be emitted by the host itself, either directly or via its excretions (e.g. larval frass), or the plant it is feeding on. In addition, the substrate texture itself may be used by wasps to infer whether or not it is in an area likely to harbour hosts (Xiaoyi & Zhongqi 2008). For example, host volatiles released by the cryptic bark beetle *Ips typographus* not only attract parasitoids to infested trees trees, but also initiate and sustain probing by parasitoid wasps even after beetles have been removed from bark (Mills et al. 1991; Pettersson & Boland 2003).

Both naïve and experienced *B. hylobii* did occasionally probe in trials in which the host chamber was empty, indicating that bark substrate may play a role in stimulating foraging in this species. The incidence of probing was not significantly increased when a dead host was present in the chamber, suggesting either that host-associated cues did little to increase wasp searching activity, or that dead hosts emit quantitatively or qualitatively less attractive cues than live hosts. However, the fact that wasp response to live prepupal hosts and paralysed hosts was no different from that to dead hosts makes this explanation less likely (though it cannot be excluded that quantity or quality of cues emitted by all of these immobile hosts [dead or alive] was different from those emitted by healthy, live larval hosts). The majority of probing that occured in trials with dead hosts was not conducted over the host chamber. It appears that while host volatiles or physical or chemical cues of the bark substrate can elicit foraging and even probing in female wasps, they do not seem to be sufficient for wasps to precisely locate the host even at close range. Evidence that suggests that bark volatiles are not essential components required by *B. hylobii* for successful host location and oviposition is provided by the fact that wasps can be cultured using a thin layer of nylon mesh to cover hosts instead of bark (author's observation; Cliff Henry, unpublished data).

Frass or faeces produced by the host can be important attractants to parasitoids (Lewis & Tumlinson 1988; Agelopoulos et al. 1995; Röse et al. 1997; Xiaoyi & Zhongqi 2008). In this study, trials did not last long enough to allow hosts to excrete any significant amount of faeces into the host chamber. Wood shavings were, however, significantly more likely to appear in the chambers of *H. abietis* larvae that were either healthy or that did not die from EPN infection during the trial than they were in trials with moribund infected hosts. There was also a significant correlation between bark being chewed in a trial and the host being parasitized, both for naïve wasps and experienced wasps. Chewing by hosts may have released volatiles from the bark patch that allowed wasps to detect and locate these hosts more readily, but it may also have been the vibrations associated with chewing activity that did so (Meyhöfer & Casas 1999; Faccoli & Henry 2003; Pettersson & Boland 2003). Evidence suggesting that volatile emission was a contributing factor is contained in the observation that hosts with glued mandibles that were prevented from chewing bark were less likely to be parasitized by naïve and experienced wasps than the same type of host supplemented with chewed bark and wood shavings (though this difference was not significant). In olfactometer assays, Faccoli and Henry (2003) found that *H. abietis* larvae feeding on pine bark produced volatiles that were more attractive to *B. hylobii* than those emitted by a host larva or bark alone, but this attraction was stronger for experienced wasps than it was for naïve wasps. In summary, it appears unlikely that an alteration in the volatile bouquet emitted by moribund EPN-infected *H. abietis* can account for the reduction in parasitism by naïve wasps. It may have been a contributing factor, however.

Some parasitoids are known to actively search for cryptic hosts by echolocation: they will drum the substrate with their antennae and then use the reflected vibrational signals to locate host larvae (Broad & Quicke 2000; Fischer et al. 2001; Kroder et al. 2007). Broad & Quicke (2000) have reported that have During *B. hylobii* trials, females were often observed antennating the bark patch, a behaviour that was apparently part of their general foraging behaviour. If indeed *B. hylobii* females were using echolocation to locate hosts, wasps would be expected to pinpoint quiescent hosts (both dead and alive) with some level of efficiency. This was not the case in the present study as the frequency of wasps probing over the host chamber decreased along with host movement. Moreover, published data on echolocating parasitoid wasps that target leaf-mining larvae suggests that the smaller the wasp (within a range of 0.8 to 1.3 cm) and the denser the substrate (45 to 590 g per m^2) being searched, the less effective echolocation becomes as a foraging strategy (Otten et al. 2001; Fischer et al. 2003). Since females of *B. hylobii* are comparatively small (no longer than 8 mm including ovipositor) and the substrate they forage on in the field (mainly pine and spruce bark on tree stumps one to three years after felling) is usually much thicker and denser than the leaves on which most echolocating parasitoids forage, this is not likely to represent an efficient strategy in the natural foraging habitat of *B. hylobii.*

Vibrations caused by moving or feeding host have been shown to be an important cue to aid in location and host acceptance (Lawrence 1981; Meyhöfer et al. 1997; Xiaoyi & Zhongqi 2008). Meyhöfer and Casas (1999) and, more recently, Xiaoyi and Zhongqi (2008) have summarised the literature on this subject. The instantaneous observation of wasps and hosts in oviposition trials gave some insight into the relationship between host movement and wasp foraging and oviposition. Wasps, both naïve and experienced, were significantly more likely to begin probing on bark patches when the host was moving than could be expected by chance alone. Furthermore, for naïve and experienced *B. hylobii* alike, the first probing bout was significantly more likely to result in oviposition if the host was moving when probing began. Regression analysis demonstrated that overall, wasps were significantly more likely to parasitize *H. abietis* hosts (control, prepupal, paralysed or infected with EPN) the more these tended to move during observation. Moreover, both naïve and experienced wasps were significantly more likely to move off the bark patch and thus abort searching and/or probing bouts when the host was not moving (this was the case for both instantaneous and continuous observations of wasp behaviour).

Comparable data for other parasitoids that target bark- or wood-dwelling hosts is scarce as most parasitoids in which vibrokinesis has been reported target leaf mining lepidopteran hosts (Cheah & Coaker 1992; Meyhöfer et al. 1994). The general observations that have been published on this group of parasitoid wasps are similar to those reported here. For instance, the parasitoid wasp

Diglyphus isaea was not able to detect dead hosts of the chrysanthemum leaf miner but increased the frequency of probing in the vicinity of live hosts (vibrokinesis) (Cheah & Coaker 1992). Probing behaviour of another leaf-miner parasitoid, *Phyllonorycter malella,* is affected by host movement as well (Meyhöfer et al. 1997). The rate of movement of *H. abietis* larvae that were infected with either *H. downesi* or *S. carpocapsae* and that died of infection during oviposition trials was lower than that of their surviving counterparts. If host movement is a key component of host location by *B. hylobii*, this observation could explain the reduced parasitism of these hosts as recorded for naïve wasps.

The incidence of bark being chewed during trials was also significantly affected by nematode infection – but only in hosts that died during the trial period. In trials with naïve wasps, almost none of the bark patches were chewed on when the host died of EPN infection during the trial. The area of bark that was chewed was not affected according to any clear trend, however, thus suggesting that on average those hosts that did chew the bark did so in equal measures, regardless of EPN infection. When *H. abietis* hosts were prevented from chewing the bark patches covering them by gluing their mandibles shut, naïve wasps were significantly less likely to oviposit on such hosts when compared with the control. Parasitism by experienced wasps was not affected, however. In continuous observation trials, wasps were apparently tracking host movement, in particular the movement of the head capsule when the host was chewing the underside of the bark patch. Feeding activity of *H. abietis* may represent an important source of vibrational cues. Feeding by host larvae has been investigated as a potential source of vibrational cues triggering vibrotaxis or vibrokinesis in leaf miner parasitoid *Phyllonorycter malella* (Meyhöfer et al. 1997). While host movement did seem to affect wasp foraging behaviour in that instance, host feeding, however, did not appear to do so. Henry & Day (2001) speculated that host feeding was a contributing cue aiding *B. hylobii* in host location based on behavioural observations they made of foraging naïve and experienced wasps.

While instantaneous observations suggested that host movement may trigger probing behaviour in wasps and affect the outcome of searching and/or probing bouts, continuous observations revealed no clear indication of general changes to wasp foraging behaviour in response to host movement. For example, the duration of searching, pausing or probing bouts was not affected by host movement, Bouts of pausing were, however, found to last significantly longer than bouts of searching irrespective of host movement or scratching, indicating that the former plays an important role in *B. hylobii* foraging. This connection is underlined by the fact that probing in *B. hylobii* was significantly more likely to be directly preceded by a bout of pausing when the host was moving than when it was not. When the host was not moving, wasps were about equally likely to probe after a bout of either searching or pausing. This may suggest that in the absence of host vibrations, probing decisions by *B. hylobii* are either random or based on other cues such as volatiles or bark texture that

are more likely to be detected during searching bouts (e.g. while antennating the bark). Similar behaviour has been observed for other parasitoids as well: in an extensive study on 57 fruit fly parasitoids, Vet and van Alphen (1985) report that especially those wasp species that responded to host vibrations frequently stopped and paused intermittently while searching for hosts. They also state that a pattern of alternating brief searching bouts, often comprising only a few steps, and extended pausing bouts lasting several seconds was prevalent when wasps that showed strong vibrotaxis were foraging for hosts. This is very much representative of the data collected for *B. hylobii*, especially when wasps were approaching a moving host at the centre of the bark patch. Based on their observations of the leaf-miner parasitoid *Phyllonorycter malella*, Meyhöfer et al. (1997) suggest that pausing (sitting 'still' as termed by the authors) in this species also was a behaviour associated with sensing host vibration. The hypothesis is that wasps can sense vibrations created by host activity more accurately if they are not disrupting them by their own movements.

In the present study, *B. hylobii* wasps displayed a characteristic behaviour that was apparently associated with localized searching behaviour in response to host movement in the host microhabitat (i.e. on the bark patch). While pausing, wasps would splay their legs and lift their abdomen off the bark, a posture termed 'waiting', which was usually distinguishable from the typical sitting posture wasps assumed when not moving. Adopting such a 'waiting' posture may allow wasps to detect vibrations travelling through the substrate more easily, possibly even permitting extraction of directional information from vibrations. Several parasitoid wasps that use echolocation to detect hosts feature mechanoreceptors on their tarsi that they require to detect and interpret vibrational stimuli (Meyhöfer 1997; Xiaoyi & Zhongqi 2008). Since 'waiting' behaviour also occurred in trials with dead hosts, it must be assumed that wasps can assume a 'waiting' position in anticipation of vibratory stimuli, perhaps in response to volatile cues or the substrate texture.

The area covered by the bark patch in oviposition trials was relatively small (2.25 cm^2) and the portion of the bark directly over the host chamber amounted to approximately 25 % of this area, making the detection of vibrotaxis in these trials difficult. Despite the limited foraging area the bark patch represented, wasps seemed to be incapable of locating the host if it was not moving. In oviposition trials, the more frequently a *H. abietis* host moved during the 2 h instantaneous observation period, the more likely the wasp was to probe over the host chamber. Failure of wasps to locate a host was especially evident in trials with dead, paralysed and prepupal hosts in which almost all of the probing occurred at the edge of the bark patch. This was true for naïve and experienced wasps alike. It is not clear whether wasps preferred the very edge of the bark patch because of its texture (i.e. the fissure between tape and bark patch) or because this was where host volatiles or volatiles emitted by the bark were escaping. Experienced *B. hylobii* are attracted to

volatiles emitted by feeding hosts, but show little to no response to bark or host volatiles alone (Faccoli & Henry 2003). Parasitoid wasps are capable of learning novel cues – in laboratory trials conducted by Vinson et al. (1977), *Bracon mellitor*, a parasitoid of the boll weevil, learned to associate an antimicrobial compound in the host diet with the presence of a host and subsequently would probe in response to being offered this compound alone. Since naïve *B. hylobii* probed at the very edge of the bark patch just as experienced wasps did and probing on the masking tape was a very rare occurrence in trials, it can safely be assumed that wasps were not probing between bark and tape as a consequence of responding to cues associated with the tape that had been learned.

Clear instances of vibrotaxis were observed in continuously observed trials with a larger bark patch (20.25 cm^2), especially for experienced wasps that were offered a live *H. abietis* host. Even though the host chamber only occupied about 4 % of the bark patch area, wasps were seen making their way toward it in a series of short, alternating bouts of searching and pausing with occasional periods of probing along the way. This kind of behaviour was only observed when the host was moving or when a dead host was being supplemented with artificial scratching - when the host was not moving, wasps rarely strayed into the centre of the bark patch. One response of wasps to vibrational cues may be to adjust the time they spend searching in an area where such cues are prevalent. When offered an artificially generated signal designed to imitate host vibrations, the leaf-miner parasitoid *Sympiesis sericeicornis* spent significantly longer periods of time searching for a host than when the vibration was not offered (Djemai et al. 2004).

In continuous observation trials, both naïve and experienced wasps were more likely to search, pause or probe close to the host chamber when the host was moving – irrespective of whether the vibrations were being created by a live host or by manually scratching the bark. Wasps were also significantly more likely to transition from a position away from the host chamber to a position close to it when host movement or scratching was occurring. These data represents the strongest evidence that *B. hylobii* is not only capable of progressing toward the exact location of a host from a distance (approximately 2 cm) via successive bouts of searching and pausing, but that it also will be attracted toward a host it would not usually be able to locate (freeze-killed or killed by EPN infection) by artificial vibrations.

Vibrotaxis has been reported for other parasitoid wasps (Vet & Alphen 1985; Meyhöfer & Casas 1999; Xiaoyi & Zhongqi 2008). In the majority of these studies, vibrotaxis was, however, not observed directly, but inferred indirectly from other parameters that were recorded. For example, van Dijken and van Alphen (1998) found that the host encounter rate of a fruit fly parasitoid was significantly reduced when fruit fly larvae were immobilised by a genetic defect and deduced that host movement was attracting wasps. Similarly, Sokolowski and Turling (1987) concluded that vibrotaxis determined

the differing encounter rates of a fruit fly parasitoid with moving and incapacitated hosts. To the author's knowledge, the present study is the first to provide direct evidence that vibrations caused by host movement as well as artificially created vibrational stimuli will guide a parasitoid wasp directly and precisely to a cryptic host.

Artificial vibrational stimuli have been tested for their ability to elicit a parasitoid response in a number of studies. By scratching of the plexiglass wall of a wasp arena from the outside with a needle immediately below a wasp, Lawrence was able to elicit probing efforts from females of *Biosteres longicaudatus,* a braconid parasitoid of dipteran larvae (Lawrence 1981). Djemai et al. (2004) recorded host vibrations of a leaf-miner host of eulophid parasitoid wasp *Sympiesis sericeicornis* and when these were reproduced inside vacated mines in a leaf, the wasps responded with searching and probing of the mine. Preliminary experiments in which *B. hylobii* was introduced into arenas with an empty host chamber in which the bark patch was being scratched from below showed that wasps would probe on these chambers. Since, however, probing on the bark was also observed in oviposition trials with empty chambers, it appears that vibrations are not an obligatory cue required for wasps to initiate probing. Cheah and Coaker (1992) found that *Diglyphus iseae*, a parasitoid of the chrysanthemum leaf miner, on occasion searches and probes even on uninfested leaves of the host plant.

As discussed above, providing an experienced *B. hylobii* female with a dead host that was augmented by a scratching stimulus resulted in positive vibrotaxis by the wasp (i.e. movement toward the source of vibration). These artificial vibrations did not appear to be as attractive as a those created by a live, healthy host, however. This may indicate that artificial vibrations did not fully match those produced by a live host. Meyhöfer et al. (1999) have argued for the importance of recording and accurately reproducing the vibrational stimulus of a normal host. This was unfortunately not possible within the scope of the present study, but the results that were obtained conclusively demonstrate that artificial vibration induced vibrotaxis and if the host was moved manually, together these cues were sufficient to induce a considerable proportion of wasps to oviposit on dead hosts - including those killed by EPN infection – that they did not parasitize in oviposition trials without scratching (e.g. 60 % induced oviposition on freeze-killed host where attraction of wasps by scratching was successful). This is clear evidence that vibrations created by a moving host are the main cue affecting both host location and host acceptance by *B. hylobii*.

Artificial vibrations (scratching and poking of the bark) that were produced using an automated system to supplement dead hosts also induced oviposition by naïve and experienced *B. hylobii*, in one case (naïve wasps with freeze-killed hosts) to such a degree that there was no difference to the live host control without added scratching. The poking stimulus was more successful in eliciting wasp

oviposition than the scratching stimulus, suggesting that it represented a more accurate approximation of vibrational cues produced by movement or feeding activity of live hosts. Since dead host larvae were offered together in tandem with artificial vibrations, it is possible that host cues played some role in host acceptance and/or host localization in these cases as well, though vibrotaxis or oviposition was never observed when such hosts were offered without scratching or poking the bark. To the author's knowledge this study is the first to report oviposition by a parasitoid in response to artificially created vibrations.

The data collected during oviposition trials and observations revealed some striking differences in foraging behaviour and oviposition rates between naïve and experienced *B. hylobii* females, some of which have been discussed above. Research on learning in parasitoid wasps has shown that female wasps emerging from cocoons are genetically programmed with a set of innate responses to host cues that will elicit foraging, determine host acceptance and ultimately the oviposition decision of the naïve wasp (Geervliet et al. 1996; Hedlund et al. 1996). These innate cues are thought to be relatively specific and host associated (Vet et al. 1995). During the adult life of the wasp and even when still developing inside the cocoon, wasps may, however, learn to associate cues they encounter with their local habitat or a particular host in addition to the innate, programmed responses ('associative learning') (Turlings et al. 1993; Meiners et al. 2003). Learning is thought to increase the fitness of wasps by enhancing their abilities to locate and thus parasitize hosts, especially when the host habitat is variable and therefore can encompass a range of different secondary cues (i.e. cues produced by the host plant or other organisms associated with the host) (Vet et al. 1995; Meiners et al. 2003).

Despite the larger foraging area (20.25 cm^2 bark patch) and shorter time span (30 minutes) that was available to wasps in continuous observation trials in comparison to standard oviposition trials (2.25 cm^2 bark patch, 2 h of observation), almost 30 % of experienced wasps initiated oviposition within the 30 minutes of observation when offered a live host. This was a rate similar to that observed in oviposition trials with a small bark patch and a live host within the 2 h (roughly 50 %). None of the naïve wasps initiated oviposition within the half hour of continuous observation in arenas with a large bark patch. When incubated for 24 h in such arenas with a live host, naïve and experienced wasps parasitized hosts at the same rate as they had done in oviposition trials with a small bark patch. This suggests that experienced wasps were quicker at parasitising the host when presented with a large foraging area, either because they initiated foraging on the bark sooner than naïve wasps did or because they required less time to locate the host once they were on the bark patch (20.25 cm^2).

In a standard arena with a small bark patch that represented a restricted microhabitat, naïve wasps initiated oviposition significantly less frequently than experienced wasps did in the first 2 h of trials. If they did initiate oviposition, however, it took them no longer to do so than it did experienced wasps. Since experienced and naïve wasps were equally successful at parasitizing live hosts over 24 h, this would indicate that in an arena with a restricted host microhabitat area (i.e. 2.25 cm^2 bark patch with host chamber) the difference in frequency of oviposition between experienced and naïve wasps was down to experienced wasps being able to locate the host microhabitat quicker (i.e. the bark patch) than naïve wasps could (thus resulting in a higher oviposition rate within the 2 h of observation).

Differences between the wasp groups become more apparent when comparing the time it took each to initiate probing during 2 h instantaneous observations. Experienced wasps needed significantly less time to do so than naïve wasps did when they were presented with a live host. Naïve wasps took significantly longer to begin probing in oviposition trials with those infected *H. abietis* hosts that died during the trials than in trials with hosts that did not die. Time to probing was, however, not significantly different between trials with surviving and dead infected hosts in trials with experienced wasps. In addition to the observations made for wasp oviposition as discussed above, this is a strong indication that the difference in parasitism rates for infected hosts between naïve and experienced wasps was due to experienced wasps either (a) being quicker to locate the bark patch and begin foraging or (b) having a lower host cue threshold for initiating foraging behaviour. The outcome of either scenario would be that experienced wasps were more likely to parasitize a host dying of EPN infection during a 24 h trial before its movement ceased. Moribund *H. abietis* larvae that died of EPN infection during trials tended to move slightly more and chewed the bark more frequently during the 2 h observation period in trials with experienced wasps than they did in those with naïve wasps. This may explain some of the difference in the parasitism of such hosts between naïve and experienced wasps. Observations covered only about 8 % of the trial period and it can only be speculated how much hosts moved during the remaining 92 %. The occurrence and area of chewed bark was not significantly different between trials for each wasp type, so it appears that chewing of bark over the entire trial period does not account for the difference between naïve and experienced wasps.

Host and substrate volatiles are the main cues for which associative learning has been demonstrated in parasitoid wasps. Substrate volatiles learned when first ovipositing can influence which substrate experienced wasps will gravitate toward when foraging subsequently (Dukas & Duan 2000). Several studies have shown that experienced wasps are both more likely and also quicker to locate a microhabitat with hosts than naïve wasps are. For instance, Steidle (1998) observed that experienced females of *Lariophagus distinguendus,* a Pteromalid parasitoid of the grain weevil *Sitophilus granarius*, found and parasitized more hosts than naïve ones at low host densities. Experienced

wasps also were quicker to locate hosts than naïve wasps were. It was later demonstrated that the wasps learned to associate host cues with successful oviposition and were able to retain the learned information for at least 144 h (Müller et al. 2006). Experienced females of the fruit fly parasitoid *Leptopilina heterotoma* were quicker and more likely to locate microhabitats with hosts than naïve females of the species were (Papaj & Vet 1990).

Faccoli and Henry (2003) have reported that experienced *B. hylobii* wasps are more responsive to cues emitted by a feeding host than naïve wasps were, an effect they ascribed to associative learning of such cues during their first encounter with a host in its microhabitat (i.e. in bark). In the present study, the host microhabitat was represented by a bark patch covering a host in a chamber beneath it. If experienced wasps are more responsive to host cues, it should be expected that they will locate the bark patch microhabitat more rapidly than their naïve counterparts, thus increasing the likelihood that experienced wasps will be able to locate an infected host larva that will die of EPN infection during the trial within the time window during which it is still moving. As discussed above, experienced wasps were more responsive to artificial manual scratching, which indicates that they could also be more responsive to host vibrations, further aiding them in rapid host location and acceptance. Unfortunately, while associative learning of host volatiles has been studied extensively in parasitoid wasps, no studies on learning of vibrational stimuli have been published to date and any estimation of how learning might have affected wasp behaviour in this context will remain speculative until more in-depth research is conducted in this area.

Host type (e.g. EPN – infected), weight or species (*H. abietis* or *G. mellonella*) had no effect on the size of egg clutches laid by *B. hylobii* (with the exception of *R. bifasciatum*). The area of bark that was chewed also had no effect on egg clutch size, an indication that wasps were not adjusting the number of eggs they were laying in response to host activity and/ or volatiles being released. Egg clutch size is connected to the fecundity and therefore also the fitness of a species and has been the subject of extensive study in parasitoid ecology (Godfray et al. 1991). It is of particular interest in the context of gregarious parasitoid wasps that lay multiple eggs per host, as the clutch size and how it is adjusted in relation to host availability, quality and intraspecific competition will determine the reproductive success of the wasp and the fitness of its progeny (Charnov & Skinner 1984; Hardy et al. 1992; Henry & Day 2001; Pexton et al. 2005). The main factors influencing egg clutch size are thought to be the resources available to the mother, which will influence how many eggs she can produce, and the resources available to her progeny, which will influence her decision on how many eggs to allocate per host. The latter is heavily dependent on the size and/or quality of the target host and some parasitoids have been shown to alter their egg clutch size in response to host size (the larger the host, the more eggs are laid), and other factors influencing host quality (e.g. fewer eggs laid on

hosts infected with a pathogen) (Vinson 1980; Hardy et al. 1992). Henry and Day (2001) report that *B. hylobii* increased the handling time it spent per host as host weight increased and that the number of eggs allocated per host increased with host weight. However, they tested larvae up to 2000 mg in weight and do not indicate whether they found a significant difference in time or egg allocation within the host weight range of 100-400 mg. They also found that wasps showed no preference in attacking hosts of different sizes if host weight was above a threshold of 100 mg. Hosts below 100 mg were not parasitized and the authors report that wasps seemed to be unable to precisely locate them since they were seen repeatedly probing bark containing such hosts (Henry & Day 2001).

Everard et al. (2009) report that infection of *H. abietis* with *H. downesi* had no significant effect on egg clutch size and they suggest that female wasps commit to laying a full clutch once a host has been accepted (i.e. once it has produced stimuli sufficient to overcome a threshold level. This hypothesis is generally supported by the results of the present study. However, naïve wasps did lay significantly fewer eggs on *R. bifasciatum* than they did on other hosts. Since the venom used by *B. hylobii* to paralyse hosts seemed to be ineffective against this host and larval movements of *R. bifasciatum* were frequent and often quite violent (the author's experience is that *R. bifasciatum* will respond aggressively to handling and will often try to bite), the most likely explanation is that wasps aborted oviposition midway through the process, either because the host response did not match the expectations of the wasp or because the wasp was trying to protect its ovipositor from damage. By comparison, clutch size on *G. mellonella*, a host that also was not paralysed by wasp venom but that showed much less movement, was similar to that on controls.

Based on the results collected for *B. hylobii* in the present study, it must be assumed that the failure of *B. hylobii* to oviposit on its preferred host (*H. abietis*) when it is dead following either freeze-killing or infection with EPN is not due to the rejection by wasps once such a host has been located, but rather that it is the consequence of an inability of wasps to locate these hosts. *B. hylobii* females showed no indication of finding and subsequently rejecting hosts of poor quality that could not support their brood (i.e. dead from EPN infection). Host or substrate volatiles may play a role in initiating foraging behaviour or intensifying it, but host location and possibly host acceptance in this species appears to require the presence of vibrational stimuli. These findings stand in contrast to other studies on parasitoids of bark beetles. Freeze-killed larvae of the bark beetle *Dendroctonus pseudotsugae* were located and parasitized readily by the braconid parasitoid *Coeloides brunneri* (Richerson & Borden 1971). In another study, it was shown that volatiles released by feeding bark beetles alone were sufficient to elicit wasp probing and even host location (Mills et al. 1991). It should be noted that the cited studies examined wasp responses to hosts contained in their natural substrate (i.e. within frass galleries beneath the bark of infested trees or logs). It is possible that in

such a scenario, the volatile bouquet is more representative of natural conditions and emission rates are higher than in the oviposition arena used in the present study. Thus, the importance of volatile cues could be underestimated if relying only on results obtained using the arenas. The data presented here, however, also show that a high proportion of live hosts were parasitized within 24 h by both naïve and experienced wasps, proving that wasps were fully capable of locating and parasitizing hosts under the experimental conditions. Also, as discussed above, experienced wasps were especially prepared to oviposit on dead hosts if provided with an additional vibrational stimulus.

Several models and hypotheses have been advanced in an effort to gain a better understanding of parasitoid wasp fitness as it relates to foraging strategies and the development of offspring. The 'preference-performance hypothesis' stipulates that parasitoid wasp oviposition preference should correlate with the suitability of hosts to allow offspring development (Vinson & Iwantsch 1980; Charnov & Skinner 1984). Thus, evolution should produce foraging strategies that optimize the ability of wasps to locate the host habitat, pinpoint the position of the host and then make the best possible decision about whether or not to parasitize it and if so, what number of eggs to lay on it (Charnov & Skinner 1984). Since it is the female wasps that make these decisions, the hypothesis has also been referred to as the 'mother knows best' principle (Henry et al. 2005; Steiner et al. 2007). Other parameters such as the egg load in wasp ovaries and the age of the wasp may also affect foraging and oviposition. For example, parasitoid wasps with a high egg load are expected to search more intensively and therefore have higher encounter rates with hosts, accept hosts of lower quality and spend less time handling them (Minkenberg et al. 1992). Lawrence (1981) reports that experienced wasps were less responsive to vibrational cues than naïve wasps and demonstrated that this was most likely a consequence of the reduction of the ovarian egg load immediately after oviposition. As the number of eggs in their ovaries increased again when they were deprived of hosts after the first oviposition, the response of experienced wasps to vibrational cues did so as well (Lawrence 1981).

Experiments conducted in the present study were not controlled for egg load of wasps (eggs are already present in the ovaries of eclosing *B. hylobii* females) or age of wasps (experienced wasps were 2 days older than naïve wasps). The results obtained as well as those previously published, however, gave no indication that egg load or age of wasps alter foraging behavior and host acceptance in *B. hylobii*. In preliminary experiments conducted for the present study, naïve wasps seven days old were found to parasitize and forage at the same rate as five day old wasps. Lawrence (1981) made similar observations with *Biosteres longicaudatus* in her experiments (only 1.5 % difference in probing attempts with naïve wasps seven to five days old). Also, Henry and Day (2001) found that successive oviposition experience which is likely to correlate with increasing age and

reduced egg load had no discernable effect on host acceptance and handling time in *B. hylobii*, but that the size of egg clutches decreased with each host. They report that wasps parasitized up to four hosts per day. One prediction with respect to parasitoid host selection is that with decreasing egg load, wasps will become more selective when foraging for hosts (Heimpel & Rosenheim 1996). In the present study, experienced wasps showed a greater response to live and dead hosts as well as dead hosts augmented by artificial vibrational stimuli than naïve wasps did. It therefore seems unlikely that decreasing egg load or progressing age had any inhibitory effect on wasp responsiveness to host cues or that wasps were becoming more discriminatory for host quality as egg load decreased – in fact the opposite seems to be the case. Only wasps with a single oviposition experience were investigated and it is possible that such effects will only manifest after repeated oviposition when reduction egg load becomes more pressing for the female. If anything, reduced egg load seemed to make females less discerning and more responsive to host cues. Further research is necessary to gather more conclusive information on the relationship between female egg load, age and experience and foraging as well as host acceptance in *B. hylobii*.

The fact that experienced *B. hylobii* were more likely to oviposit on hosts that would not allow for development of the offspring (i.e. dead, heavily infected with EPN or of a different species) than naïve wasps were seems counterintuitive at first glance. After all, associative learning is thought to increase host specificity and thus wasp reproductive fitness (Vet et al. 1995; Steidle 1998). For example, Dukas & Duan (2000) report that reproductive success was significantly greater for fruit fly parasitoid *Biosteres arisanus* if wasps were permitted to learn substrate cues associated with the host. Most studies that have investigated learning in parasitoid wasps do not, however, include dead or pathogen-infected hosts. Different results for naïve and experienced wasps will therefore most likely reflect fitness increases due to enhanced location of hosts by wasps, but not necessarily changes in host acceptance as the result of host infection.

Bracon hylobii readily parasitized hosts it is not known to be associated with in the present study. The wasp is currently thought to have a very restricted host range (Kenis et al. 2004). All of the three host species that were offered besides *H. abietis* in this study were parasitized by either naïve wasps, experienced wasps or both, in some cases to a considerable degree (e.g. approximately 50 % of *G. mellonella* parasitized by experienced wasps). In the case of larvae of the longhorn beetle *A. striatum* and *R. bifasciatum* this could be due to the similar lifestyle and phylogeny these hosts share with *H. abietis* (all are the saproxylic larvae of coleopterans). Since these hosts have not been reported as hosts for *B. hylobii* to date, despite sharing the same habitat with *H. abietis* (forests and clearfell sites), foraging behaviour by *B. hylobii* appears to be restricted to an extremely specific microhabitat in its natural environment.

Rhagium bifasciatum is widely distributed in decomposing wood on sites with *B. hylobii* populations, though usually this wood is in stages of more advanced decomposition (often lacking a bark cover) in comparison to the tree stumps frequented by the parasitoid (Duffy 1953; Twinn & Harding 1999). This may segregate its habitat from that foraged by *B. hylobii*. *A. striatum* is, however, located in the upper region of tree stumps in very close proximity to *H. abietis* larvae. Two possible explanations could account for why *B. hylobii* will not parasitize this host in the field. First, *A. striatum* larvae bore deep into the stump once they have reached a size comparable to those of *H. abietis* that are parasitized by wasps, thus making them inaccessible to females (author's observation). Second, the larvae feed in the upper regions of the stump and are almost never found at or below the soil horizon where *H. abietis* densities are higher and foraging by *B. hylobii* is more intensive (Henry 1995; author's observation). *Galleria mellonella*, however, is both phylogenetically removed from the two longhorn beetle hosts and *H. abietis* (it is a lepidopteran species) and has a lifestyle not associated with live or dead wood.

The experimental setup did not include many of the physical and volatile characteristics of the natural *B. hylobii* habitat (e.g. bark texture and volatile bouquet of dead tree stumps, soil volatiles and texture, etc.). Parasitoid wasps locate host microhabitats by integrating multiple host and habitat cues and they may follow a sequence of cues to locate hosts, beginning with general volatile or visual cues associated with the host habitat and ending with more host-specific cues present in the host microhabitat (McAuslane et al. 1991; Fischer et al. 2001, van Alphen et al. 2003). In a natural setting, *B. hylobii* may use visual, volatile and textural cues associated with tree stumps and soil to locate a foraging microhabitat with high *H. abietis* density. Because these cues were mostly absent in arenas used for oviposition trials in the present study, wasps may have accepted host larvae for oviposition that it would usually parasitize (e.g. *R. bifasciatum* and *G. mellonella*). If, however, *B. hylobii* evaluates host identity based on volatile or tactile cues before or during oviposition once it is foraging for host on a tree stump, these cues would have to be of such a general nature so as to allow for a female wasp to mistake a lepidopteran larva for a coleopteran larva.

In the absence of a mechanism that will allow experienced wasps to detect hosts of low quality, their enhanced ability to locate such hosts may have adverse fitness effects. Based on the results presented here it appears that *B. hylobii* does not possess such a mechanism. Two factors must be taken into account in this context: Firstly, neither *S. carpocapsae* nor *H. downesi* are associated with the host (*H. abietis*) in its natural habitat (Kenis et al. 2004). The only EPN species that have been isolated on Irish clearfell sites to date are *S. feltiae* (Dillon 2003) and, based on a single tentative record, *S. kraussei* (see Chapter IV). Neither is known to infect *H. abietis* on a large scale in a natural setting, though there may be the occasional infection (Dillon et al. 2006 & 2008a). Thus, there seems

to be no strong selective pressure for *B. hylobii* to evolve a mechanism for evaluating hosts with respect to a possible EPN infection. It is worth contemplating that *B. hylobii* could reject hosts infected with a pathogen *H. abietis* is associated with in its natural habitat, for example the fungus *Beauveria caledonica* (Glare et al. 2008). Further research will be necessary to investigate this. Secondly, wasps were not given a choice in trials conducted for this study. In a natural setting, healthy, active host larvae in the close vicinity of infected hosts may be more attractive to *B. hylobii* and therefore reduce parasitism of infected hosts. Results recorded by Everard et al. (2009) appear to support this: only 10 % of *H. abietis* infected with *H. downesi* one day prior survived their choice trials and none of these moribund hosts was parasitized. In the present experiments, similarly moribund larvae were parasitized, possibly because they were offered to wasps without a healthy alternate host.

Bracon hylobii seems to require a very restricted set of cues to locate a host and accept it for oviposition. In the trials conducted for this study, wasps oviposited on hosts from a different phylogenetic order to their target host. They also deposited a full clutch of eggs on a dead host if it was supplemented with a scratching stimulus and was moved about manually. They are also known to oviposit on *H. abietis* in the absence of bark substrate (Henry, unpublished data). Naïve wasps show little response to host volatiles and experienced wasps are only attracted to volatiles emitted by a feeding host (Faccoli & Henry 2003). Given these observations, especially with regards to naïve wasps being largely unresponsive to volatile cues and the unspecific nature of vibrational cues in general, this parasitoid would be expected to have a broader host range in the habitat it occupies, one that contains at least two other potential hosts, *A. striatum* and *R. bifasciatum*, in relatively close proximity to *H. abietis*. The trials conducted for this study did not, however, include most of the structural and volatile complexity that *B. hylobii* would generally encounter while foraging in the field. It is reasonable to assume that females respond to several additional cues (visual, volatile or associated with the structure or texture of tree stumps) that either together or in succession guide them to the microhabitat with high *H. abietis* densities (i.e. recently created coniferous tree stumps) in the field.

Foraging behaviour that relies heavily on vibrational cues may also convey some fitness benefits. Since *B. hylobii* paralyses hosts at oviposition, wasps are much less likely to superparasitize a host when foraging in a host patch. Only one previously paralysed host was superparasitized in this study. Avoidance of superparsitism can increase fitness by reducing conspecific competition (Potting et al. 1997; Dorn & Beckage 2007). Some wasps mark parasitized hosts with pheromones, mainly to deter conspecific wasps or to avoid superparasitizing its own progeny (Nufio & Papaj 2001). Paralysed hosts were, however, washed and all eggs were removed from them in this study, thus reducing the

possibility of this factor influencing results (if at all relevant to *B. hylobii*). Wasps that are attracted primarily by vibrations will also be less likely to parasitize prepupal and pupal stages that move infrequently or not at all. These late life stages of the host may be of a lower quality, especially once pupae start sclerotizing and thus present a thicker cuticle for the mandibles of the freshly hatched wasp larvae to penetrate.

In summary, the fact that experienced *B. hylobii* are as likely to parasitize moribund EPN infected hosts as they are to parasitize healthy hosts is somewhat troubling as this implies a risk that *B. hylobii* populations will be affected indirectly due to progeny failing to develop on infected hosts. Also, both naïve and experienced wasps did not parasitize infected hosts any less frequently than they did healthy hosts if the infected host did not die during the trial. These results indicate that there is a possibility of intraguild predation occurring in the field when EPN are applied to control *H. abietis* – though the time window between EPN infection and the death of a host larvae in which an interaction can occur is relatively short (up to one week, but usually less than 48 h based on the results presented here and by Everard et al. [2009]). Everard et al. (2009) have already demonstrated that both eclosing wasps and larvae are highly susceptible to direct EPN infection, as are many other parasitoids (Kaya 1978; Georgis & Hague 1982; Mbata & Shapiro-Ilan 2010). The risk as estimated from the data presented in this study must, however, be interpreted in light of two previous findings: Firstly, field trials by Dillon et al. (2008b) have indicated that *B. hylobii* parasitism on clearfell sites is not adversely affected by application of *S. carpocapsae* or *H. downesi* to tree stumps and secondly, in a field situation wasps have multiple hosts to choose from, a fact that - as discussed above - should reduce the likelihood of wasps ovipositing on EPN infected hosts rather than healthy ones (Everard et al. 2009).

Nonetheless, on clearfell sites on which *B. hylobii* populations are present, it may be advisable to make allowances for them in the EPN application timing and extent. Henry (1995) found that over the course of a summer season (May to October), parasitism rates of *H. abietis* by *B. hylobii* were lowest in May. Most of the wasps that have overwintered in tree stumps will eclose in that month (author's observation). If EPN are applied late in May or early in June, infection of *B. hylobii* broods should therefore be comparatively low while adult wasps will most likely have eclosed in numbers sufficient to maintain the wasp population over the period immediately after EPN application when intraguild predation is likely to be at its highest. If the impact on the *B. hylobii* population on a clearfell site can be minimized in this way, it may complement pine weevil biocontrol efforts in the short-term over the summer season following EPN application as well as in the subsequent seasons when weevil populations may remain high.

Conclusions

The challenge that is facing the Irish forestry industry today is a formidable one. The current, effective method of controlling the pine weevil via the chemical pesticide permethrin no longer available, the need for alternative control methods is of the utmost urgency. Of course, the first criterion that any biological control replacement will have to fulfil is that of being effective in reducing pine weevil numbers to a level that represents a significant reduction in economic damage. The currently available data suggest that entomopathogenic nematodes (EPN) are capable of causing marked reductions in pine weevil emergence, but that these are not always sufficient to prevent unacceptable levels of damage to replanted seedlings on clearfell sites (Brixey et al. 2006; Dillon et al. 2006 ; Dillon et al. 2007; Aoife Dillon, personal communication). If the point of reducing the use of chemical pesticides is to be the reduction of the environmental impact of forestry, then any biocontrol agents that are to replace them must also qualify in this respect (van Lenteren et al. 2003; Dillon et al. 2008a). The results obtained for this thesis allow several predictions and recommendations regarding the risks associated with the use of EPN against the large pine weevil on a large scale. They also provide some valuable insights into the biology of EPN and the pine weevil parasitoid *Bracon hylobii*.

EPN numbers dropped off to low or undetectable levels only two years after they were applied against the pine weevil. In general EPN persistence was highest on sites with pine stumps and lowest on those with spruce stumps, irrespective of soil type (Chapter III). This effect seemed to be the result of EPN recycling in pine weevils within tree stumps as persistence was correlated with the number of weevils emerging from them. The risk of EPN persistence and spread can therefore be expected to be highest on sites with pine stumps. The spread and dispersal of the nematodes was low on all sampled sites, a result that corresponds with published literature for other setting in which EPN have been used for inundative biological control (Poinar & Hom 1986; Parkman et al. 1993; Smits 1996). Where spread of EPN off-site did occur, it appeared to be localised and off-site populations shrank or disappeared over time. Overall, the evidence indicates that the use of EPN against the large pine weevil will have little impact on the areas surrounding clearfell sites and that within four weeks of application, EPN persistence will be restricted mainly to areas in close proximity to treated stumps (i.e. 50 cm or closer). Moreover, the data presented here give a strong indication that EPN persist and reproduce in the bark of treated tree stumps for at least two years. Such localised persistence at relatively high densities should act as an

inoculative biological control measure against pine weevils within stumps over several seasons that is both temporally and spatially restricted by the natural population dynamics of the target species. Furthermore, even if localised populations of *S.carpocapsae* were to persist for more than three years after EPN application to clearfell sites, based on the published data on the risks associated with such persistence there is no reason to think that it would have a severe impact on the local insect fauna (Smits 1996, Dillon et al. 2008, Griffin et al. 2008) (Chapter III).

The intraspecific and intrastrain variation among *S. feltiae* isolates can be considerable (Yoshida 2003; Spiridonov et al. 2004), an observation further supported by what molecular analysis was possible in this study. The more diverse the genetics within this species, the more likely it is that indigenous populations will be affected by hybridisation and/or competition with applied strains from a different locality or country. The tentative identification of an EPN field isolate as *Steinernema kraussei* suggests that the diversity of EPN on Irish clearfell sites could be higher than previously thought. This could also increase the risk of competition and replacement of such indigenous species and strains by inundatively applied EPN. Evaluation of the persistence of exotic EPN species and strains after their application against the large pine weevil have, however, so far not produced any evidence to suggest that this is in fact happening (Torr et al. 2007; Dillon et al. 2008a). Since baseline inter- and intrastrain variation within local populations of *S. feltiae* strain 4cfmo appears to be high, it can be doubted that the introduction of an exotic strain such as EN02 will have any profound consequences, as competition among genetically distinct lines and the mixing of genetic material via hybridisation is most likely already occurring to a large degree in the natural populations (Chapter IV).

Steinernema carpocapsae fared considerably better at invading deadwood and infecting saproxylic longhorn beetles (*Rhagium bifasciatum*) within it than would have been expected judging by its commonly assigned attributes as an 'ambusher' EPN species. This supports previous observations in which this species performed well against the pine weevil and other wood boring insects despite their cryptic habitat (Lindegren 1981; Fallon et al. 2004; Brixey et al. 2006; Dillon et al. 2006). The somewhat troubling observation that in the laboratory both *S. carpocapsae* and *H. downesi* infected over 30 % of *R. bifasciatum* individuals within logs even at a low application dose of only 18,000 IJs was mitigated by results from the field, where infection rates were significantly reduced for *H. downesi* in particular. Since both EPN species were able to reproduce in *R. bifasciatum,* there is a risk that once infection within a piece of woody debris on a clearfell site occurs, IJs will be produced in numbers high enough to infect all insects within it over the summer season in a chain reaction. Field sampling of logs did not give rise to

concern in this respect, however, since even in decomposing logs in which high numbers of IJs per wood sample were found one year after EPN application to the site, not all *R. bifasciatum* individuals were infected. Why considerable numbers of *S. carpocapsae* IJs remained in cadavers even after eight weeks of emergence is unclear, but coupled with the protracted emergence trend of this species, it seems to pose a greater long-term risk to saproxylic insects than *H. downesi*, which is more likely to have a significant short-term impact. Since the clearfell sites that were surveyed contained a large amount of deadwood for saproxylic beetles to colonise and infection risk decreased significantly with distance from treated stumps, the overall risk to wood-decomposing insects like *R. bifasciatum* posed by EPN can be expected to be low, especially when application of EPN is as precise as possible (Chapter V).

The parasitoid *Bracon hylobii* did not parasitize pine weevil larvae that had been killed by either *S. carpocapsae* or *H. downesi* unless such hosts were augmented with artificially created vibrational cues (Chapter VI). The present study is the first to demonstrate that a parasitic wasp can be goaded into locating and ovipositing on a host it was previously not able to find, an observation that highlights the importance of vibrational cues have for some parasitoids that are specialised on cryptic hosts (Meyhofer & Casas 1999; Xiaoyi & Zhongqi 2008). While this provided some interesting insights into the processes guiding host location and acceptance in *B. hylobii*, it also showed that any pine weevil larvae that have been killed by EPN after field application are not attractive to wasps. This should reduce the effect EPN application has on this beneficial parasitoid. However, as was found in previous experiment by Everard et al. (2009), if the host larvae was infected with EPN but alive at the time it was offered to a wasp, only such host larvae that were close to death were significantly less likely to be parasitized than control hosts (an effect observed for naïve wasps only). Together with the observation that both naïve and experienced wasps respond primarily to vibrational cues, this implies that wasps are not capable of detecting EPN infection in hosts. Any drop in parasitism rate was most likely due to passive effects such as a reduction of host location success as a consequence of slackening host movement and feeding following EPN infection. Since these effects were less pronounced when wasps were given the choice between an infected and a healthy host (Everard et al. 2009), it is reasonable to assume that in a field situation in which wasps will be presented with multiple hosts in close proximity within their searching microhabitat, they will be more likely to parasitize such healthy hosts rather than heavily infected ones. This could especially apply to experienced wasps as they seem to be particularly sensitive toward vibrational cues. The results presented in Chapter VI also underline that in order to accurately estimate potential non-target effects, the behaviour not only of the nematodes, but also of the non-target insect must be taken into account.

In light of the problem that the immature and adult stages of *B. hylobii* are susceptible to potentially high infection rates with EPN (Everard et al. 2009), the most prudent approach to EPN application on clearfell sites with a presence of this parasitoid would be to time application in such a way as to allow the wasp population to build up after eclosion in early May for one to two generations (four to six weeks following eclosion) and then treat tree stumps with EPN. This would mean that adult wasps would be present on-site in sufficiently high numbers to locate any surviving pine weevil larvae not killed by EPN and to maintain a population of *B. hylobii* on a treated site while at the same time adding to the controlling effect of the EPN. However, to be most effective against the target pest, application of EPN must occur before pine weevil emergence commences. This is usually the case in early June, allowing for a one-month window in which EPN application should lead to an additive control effect of EPN and parasitoid.

The experiments and field sampling carried out for this thesis addressed at least four of the key parameters (establishment, dispersal, host range, direct non-target effects and indirect non-target effects) that van Lenteren et al. (2003) have pointed out are essential to making any informed predictions about the impact of inundative biological control using EPN. In the context of forestry application of EPN against pine weevil, no substantial risks emerged based on the results obtained. The only significant cause for concern was highlighted by the response of *B. hylobii* to EPN infected hosts. Field assessment of parasitation rates following EPN application against pine weevils in Ireland has, however, given no indication of EPN being responsible for any significant reduction in this respect (Dillon et al. 2008b).

In conclusion, risks associated with EPN use against the large pine weevil can be assumed to be low overall, though some non-target insects will be more at risk than others, especially if they are closely associated with this pest insect (e.g. *Bracon hylobii*). Taking this into consideration, it is clear that whether or not entomopathogenic nematodes will take a place as the main control method against the large pine weevil will hinge on their efficacy and econonomic viability as such.

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Appendix

A.1 Weevil emergence data collected in 2008 on sites treated with EPN

Table A.1: Mean number of pine weevils emerging from untreated tree stumps at four sites treated with *S. carpocapsae* in 2007. Weevils were collected in traps mounted at the top of tents that covered each stump. Data was used with the kind permission of the authors (Griffin et al. 2008). LP = lodgepole pine, SS = Sitka spruce.

			Mean number of adult weevils emerging from untreated stumps	
Site *(soil type)*	***N***	***Stump species***	**2007**	**2008**
Lackenrea (mineral)	**14**	**LP**	29.0	1.9
	13	**SS**	16.2	0.5
Glendine (peat)	**19**	**LP**	53.2	52.9
Knockeen (peat)	**15**	**SS**	0.5	1.7
Ballymacshaneboy (mineral)	**19**	**LP**	55.1	10.1

A.2 Log parameters

Logs were classified into ordinal or nominal groups for three parameters which were assessed visually:

a) Wood decay

Wood decay was graded on a scale from one to four based on the consistency of the wood and overall appearance. 1= log solid, no decay; 2 = outer layers (approximately 20 % of the total volume) of wood soft and spongy and/or loose, but central cylinder (remaining 80 % of log volume) solid with no signs of decay; 3 = at least half of the diameter of log soft and spongy and/or loose, with remainder being solid; 4 = logs soft and spongy and/or loose throughout, often crumbling when probed. A very small portion of the central cylinder (< 25 % of volume) of the log may remain intact, but often spongy as well.

b) Porosity

Porosity of logs was estimated by inspecting their outer surface. Porosity was recorded as the proportion in percent (to the closest 5 %) of surface area deemed to show clear and distinguishable cracks, holes or other porous features that would allow water and/or EPN to penetrate more easily than in non-porous areas. (This parameter was not recorded for logs collected at Lackenrea, Ballymacshaneboy and Deerpark).

c) Soil contact

The proportion of the surface area of the log that was in direct contact with the soil was estimated to the closest 5 %.

A.3 Standard curves for estimation of IJ numbers in wood samples

To estimate the number of IJs present in a given 10 g sample of wood as collected from logs in the field or from logs that were exposed to EPN in Laboratory and Field Exposure Experiments, wood samples were spiked with IJs and baited with waxmoth larvae. Two separate standard curves were created for each of the two nematode species longhorn beetle larvae were exposed to in Exposure Experiments (*S. carpocapsae* and *H. downesi*). All wood samples were spiked by adding the respective concentration of nematodes in 1 ml of tap water to a 9 cm Petri dish filled with 10 g of decomposing wood collected from logs colonized by *R. bifasciatum* (these logs were not used in experiments and were from sites not treated with EPN). Five Petri dish replicates were made per IJ concentration. After adding the IJ suspension, dishes were shaken for approximately 10 seconds to distribute the nematodes evenly. Six dishes of different replicates were stacked in random order. Dishes filled with 10 g of wood (no nematodes added) were placed at either end of a stack. The stacks were wrapped in parafilm and placed on plastic trays in random order, at least 2 cm apart. The dishes were then incubated for 48 h at to allow nematodes to acclimatise and spread in the wood sample. After 48 h, five waxmoth larvae were placed in each dish and baiting was conducted as described in 5.2.5.

Standard curve **ScA** was created using nematodes from the same batch of *S. carpocapsae* IJs applied to logs in Exposure Experiment I (5.2.6a) and ran in parallel to that experiment. IJ concentrations were: 0 (tap water control), 5, 10, 25, 50, 100, 200, 400, 800, 1200, 1600, 2400, 3200, 4000, 4800, 5600, 6400, 7200, 8000. This curve was used to estimate the number of *S. carpocapsae* IJs in wood samples collected at the end of Laboratory Exposure Experiment I and III.

Standard curve **ScB** was created using nematodes from the same batch of *S. carpocapsae* IJs applied to logs in Field Experiment I (see 5.2.8a) and ran in parallel to that experiment. IJ concentrations were: 0 (tap water control), 5, 10, 50, 200, 400, 800, 1600, 3200, 7200. This curve was used to estimate the number of *S. carpocapsae* IJs in wood samples collected at the end of Field Exposure Experiment I and II.

Sandard curve **HdA** was created using nematodes from the same batch of *H. downesi* IJs applied to logs in Exposure Experiment II (see) and ran in parallel to that experiment. IJ concentrations were: 0 (tap water control), 5, 10, 50, 200, 400, 800, 1600, 3200, 7200. This curve was used to estimate the number of *H. downesi* IJs in wood samples collected at the end of Laboratory Exposure Experiment II and III.

Standard curve **HdB** was created using nematodes from the same batch of *H. downesi* IJs applied to logs in Field Experiment II (see 5.2.8b) and ran in parallel to that experiment. IJ concentrations were: 0 (tap water control), 5, 10, 50, 200, 800, 1600, 3200, 4800, 7200. This curve was used to estimate the number of *S. carpocapsae* IJs in wood samples collected at the end of Field Exposure Experiment I and II.

a) Standard curves for estimation of IJ numbers in wood samples

To plot a standard curve, the IJ concentration data was log transformed (log base 10). The resulting data was plotted with the log base 10 of the IJ concentration (y) against the total number of bait insects infected for a given sample (x). This facilitated the derivation of the predicted number of IJ per sample as IJ concentration

stood alone in the resulting standard curve equation. All standard curves were cubic fits, as this produced the best match to the data based on R^2 values.

In all equations presented in the following sections, N_W is the total number of dead bait insects after repeated baitings and N_{logIJ} is the base 10 logarithm of the estimated number of IJs per 10 g wood sample. The estimated number N_{IJ} of IJs per 10 g wood sample was thus:

b) Standard curve ScA

A cubic curve provided a very good fit for the data (R^2 = 0.86) that was significant (DF = 3, F = 218.37, P < 0.001) (Fig A.1). The equation for the curve was

The curve dipped at the high end which indicated that the extrapolation of the curve was less reliable for high concentrations of IJs and would most likely lead to a slight underestimation of IJ numbers in wood samples in which 60 or more bait insects were infected. The intercept of this standard curve was 0.4744, corresponding to an estimated 2.98 IJs. All estimates derived from this curve were corrected by subtracting this number.

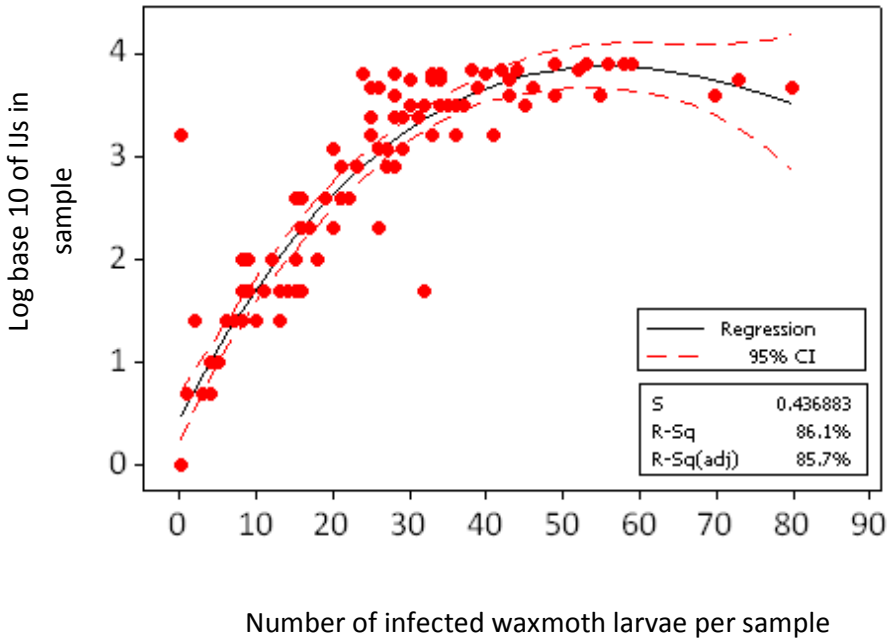

Fig A.1: Standard curve **ScA** (cubic fit) used to estimate the number of *S. carpocapsae* IJs in individual 10 g wood samples. Dashed red lines indicate 95 % confidence intervals. R^2 value is given. Line equation: $y = 0.4744 + 0.1410x - 0.001769x^2 + 0.000006x^3$. N = 5 samples per concentration, 8 concentrations included.

c) Standard curve ScB

A cubic curve provided an excellent fit for the data (R^2 = 0.97) that was significant (DF = 3, F = 436.97, P < 0.001) (Fig A.2). The equation for the curve was

Since the slope of the curve was positive and relatively steep for the highest numbers of dead bait insects (50 and beyond), IJ estimates for any wood samples producing more than 70 infected bait insects would most likely be overestimates. The curve intercept of 0.15 translated to approximately 1.41 IJs and all estimates derived from this curve were corrected by subtracting this amount.

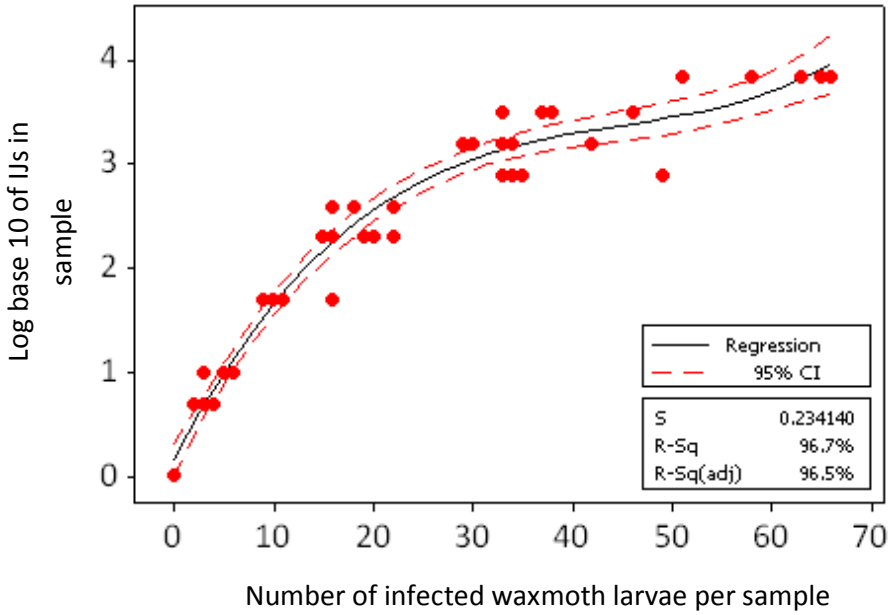

Fig A.2: Standard curve **ScB** (cubic fit) used to estimate the number of *S. carpocapsae* IJs in individual 10 g wood samples. Dashed red lines indicate 95 % confidence intervals. R^2 value is given. Line equation: $y = 0.1500 + 0.1855x - 0.003804x^2 + 0.000028x^3$. N = 5 samples per concentration, 9 concentrations included.

d) Standard curve HdA

A cubic curve provided an excellent fit for the data (R^2 = 0.92) that was significant (DF = 3, F = 351.23, P < 0.001) (Fig A.3). The equation for the curve was

As with standard curve ScB, this curve rose steeply for high number so f infected bait insects (approximately 60 to 70). Estimates for wood samples with similar or higher numbers of infected bait insects were expected to be overestimates. The intercept of 0.7456 corresponded to an estimate of 5.67 for zero value samples and was thus subtracted from all samples.

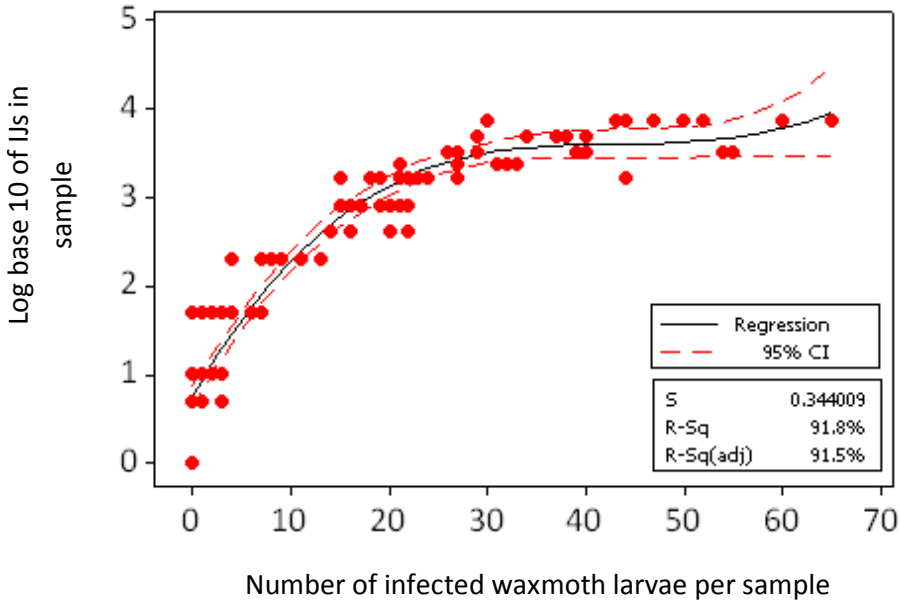

Fig A.3: Standard curve **HdA** (cubic fit) used to estimate the number of *H. downesi* IJs in individual 10 g wood samples. Dashed red lines indicate 95 % confidence intervals. R^2 value is given. Line equation: $y = 0.7456 + 0.1940x - 0.004423x^2 + 0.000034x^3$. N = 5 samples per concentration, 9 concentrations included.

e) Standard curve HdB

The cubic curve fit the data excellently ($R^2 = 0.94$) as well as significantly (DF = 3, F = 283.49, P < 0.001) (Fig A.4).

The equation for the curve was

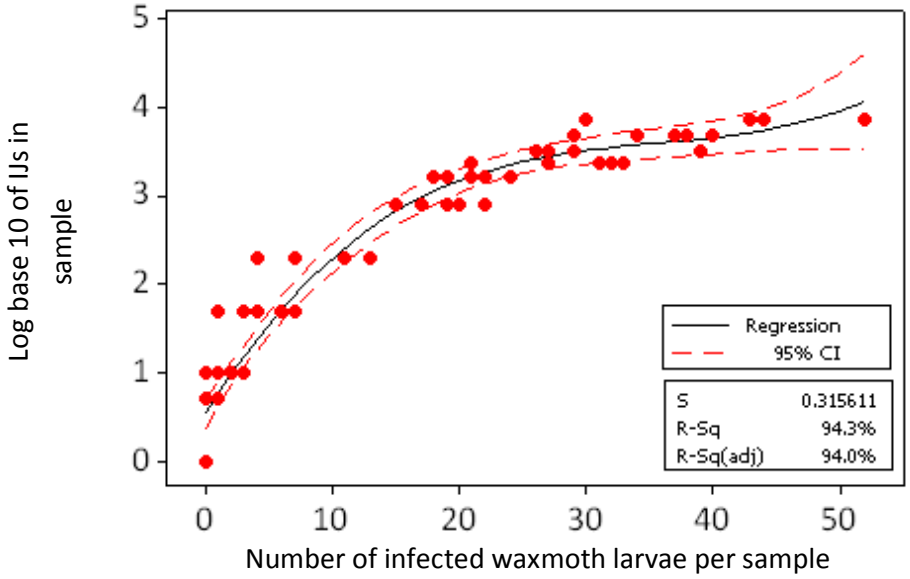

Fig A.4: Standard curve **HdB** (cubic fit) used to estimate the number of *H. downesi* IJs in individual 10 g wood samples. Dashed red lines indicate 95 % confidence intervals. R^2 value is given. Line equation: $y = 0.5482 + 0.2308x - 0.006124x^2 + 0.000057x^3$. N = 5 samples per concentration, 9 concentrations included.

The slope of this curve showed a slight incline in the range for high numbers of infected bait insects (approximately 40 to 50). Estimates for wood samples with similar or higher numbers of infected bait insects were expected to be overestimates. The intercept of 0.5482 translated to an estimate of 3.49 for zero value samples and was thus subtracted from all samples.

f) Comparison of standard curves

Since no statistical comparison of the cubic curves ScA and ScB was possible, the slope and intercept of the linear regressions of the data for ScA and ScB were compared. The data for both curves was combined in a General Linear Model in which the curve each sample belonged to (ScA or ScB) and the IJ concentration were crossed predictors and the number of dead bait insects per sample was the response (concentration data was log base 10 transformed). No significant effect was found for 'curve' as a predictor (DF = 1, F = 0.46, P = 0.500), nor was there a significant interaction between 'curve' and 'concentration' (DF = 1, F = 2.83, P = 0.95), indicating that there was no significant difference between the two linear regression fits of the two data sets.

Based on this result, the data from the two curves was combined and used to generate a third standard curve (ScAB) to be used to estimate the number of *S. carpocapsae* IJs in wood samples from logs collected on clearfell sites on which EPN had been applied to tree stumps (see 5.2.4) (Fig A.6).

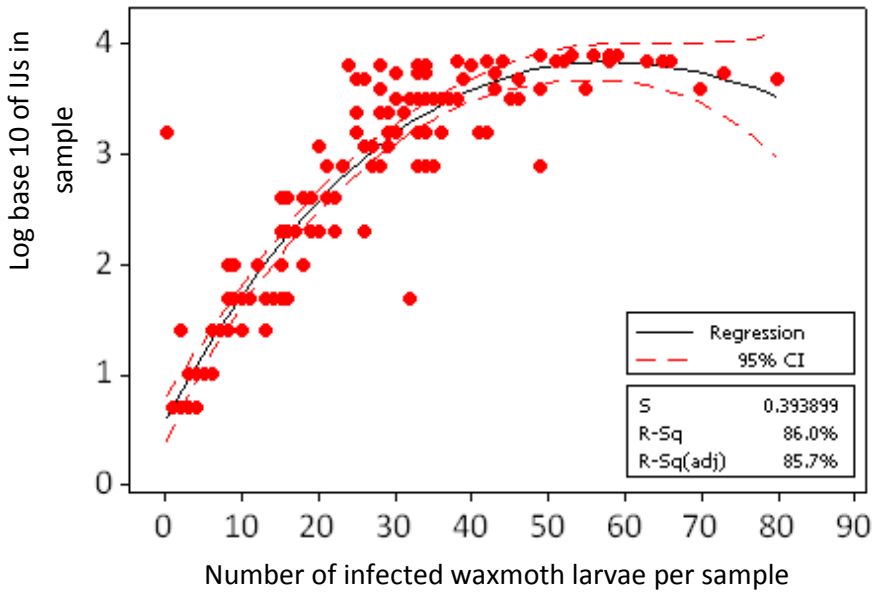

Fig A.5: Standard curve **ScAB** (cubic fit) used to estimate the number of *S. carpocapsae* IJs in individual 10 g wood samples. Dashed red lines indicate 95 % confidence intervals.. R^2 value is given. Line equation: $y = 0.6035 + 0.1261x - 0.001451x^2 + 0.000004x^3$. N = 10 samples per concentration, 8 concentrations included.

The cubic trend line once again provided the best fit (R^2 = 0.86) and this fit was significant (DF = 3, F = 297.66, P < 0.001). The equation of the curve was as follows:

The curve had a shape similar to the one of ScA and the same constraints for IJ estimates derived from samples with high numbers of dead bait insects (60 and more) apply as described for that standard curve (see A.3a). Estimates calculated from this standard curve were corrected by subtracting 4 IJs (due to the curve intercept).

A comparison of slope and intercept of a linear fit of the two *H. downesi* standard curve data sets using a General Linear Model showed that there was no significant difference in the intercepts (F = 2.22, DF = 1, P = 0.140) but that there was a significant difference in the slope (F = 6.19, DF = 1, P = 0.015). The slope for the linear fit of the HdA data set was 0.0579 and 0.0759 for the HdB data set (log base 10 of concentration vs total number of dead bait insects).

A.4 Weather data for field exposure experiments

Weather data for Field Exposure Experiment I (Featherbed, 5.2.8a) was collected at the weather station located at the Casement Aerodrome, South Dublin (53°18'N, 006°26'W, 93 m elevation). Weather data for field Exposure Experiment II (Kildalkey, 5.2.8b) was collected at the weather station located at Derrygreenagh (53°23'N, 007°15'W, 78 m elevation).

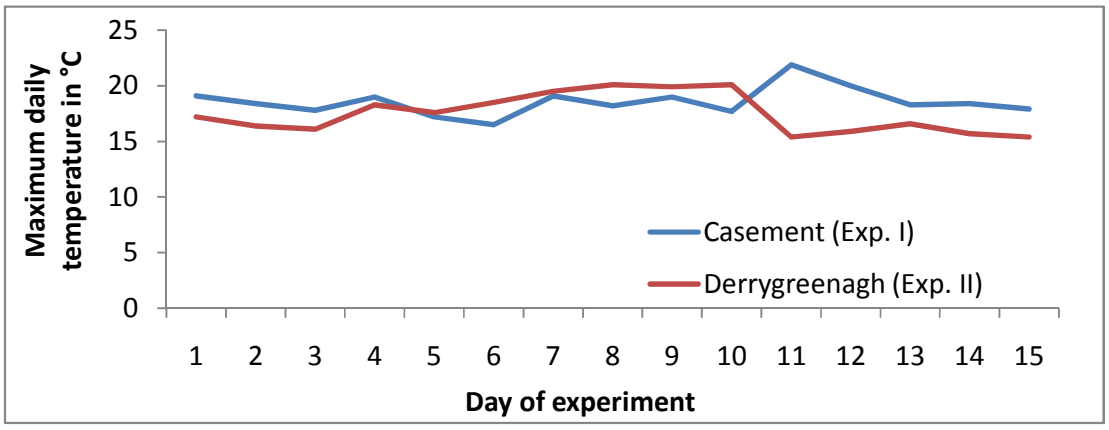

Fig A.6: Daily maximum temperature recorded at weather stations closest to the trial sites for field exposure experiment I (Casement) and II (Derrygreenagh).

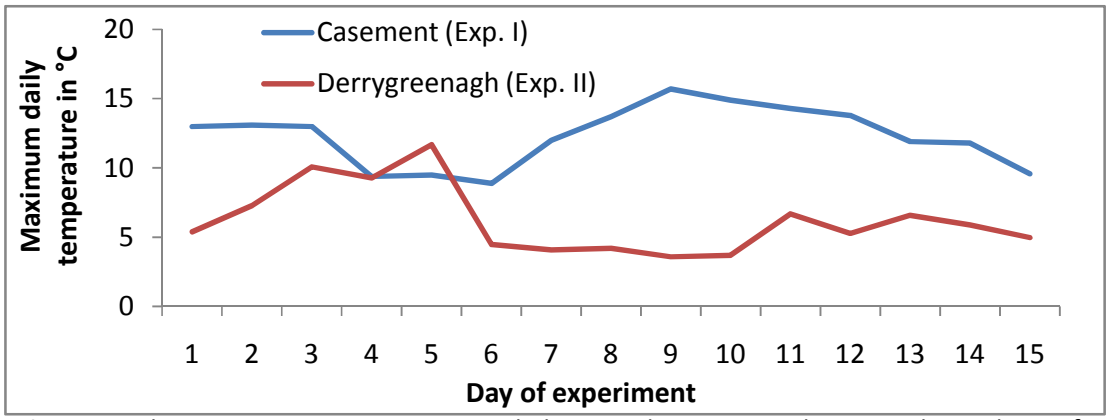

Fig A.7: Daily minimum temperature recorded at weather stations closest to the trial sites for field exposure experiment I (Casement) and II (Derrygreenagh).

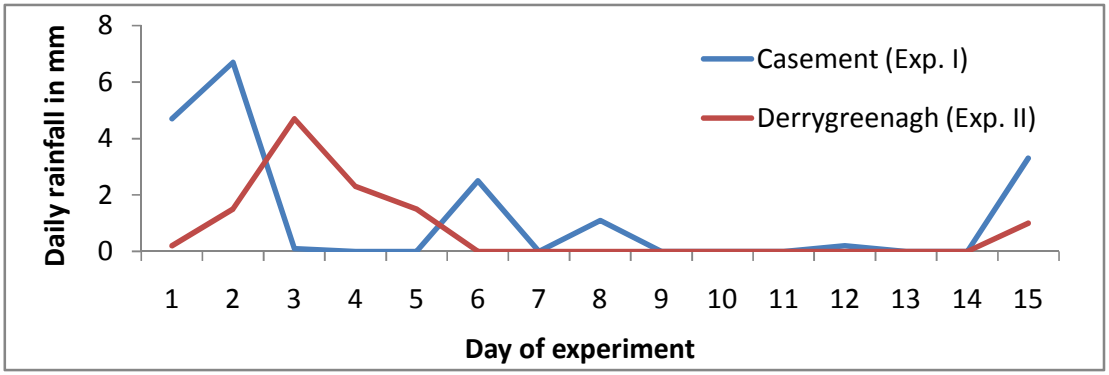

Fig A.8: Daily rainfall in mm recorded at weather stations closest to the trial sites for field exposure experiment I (Casement) and II (Derrygreenagh).

A.5 Statistical test results for length-corrected emergence from *R. bifasciatum* hosts infected with *S. carpocapsae* or *H. downesi* in logs

Table A.2: Results of a GLM comparing the slope and intercept of linear trend lines for the log base 10 +1 transformed emergence data from *R. bifasciatum* larvae corrected for length.

Comparison	***Factor***	***DF***	***F***	***P***
R. bifasciatum larvae infected with *S. carpocapsae* in high concentration logs vs. *R. bifasciatum* larvae infected with *H. downesi* in high concentration logs	Slope	7	18.87	**< 0.001**
	Intercept	1	156.63	**< 0.001**
R. bifasciatum larvae infected with *H. downesi* in high concentration logs vs. *R. bifasciatum* larvae infected with *H. downesi* in low concentration logs	Slope	7	1.86	**= 0.076**
	Intercept	1	91.95	**< 0.001**

Total emergence compared between *R. bifasciatum* larvae infected with *S. carpocapsae* and *H. downesi* in high concentration logs and with *H. downesi* in low concentration logs revealed a highly significant difference among these host groups after correction for host length (One-way ANOVA; DF = 2, F = 9.57, P < 0.001). Tukey's test (α = 0.05) showed that emergence was significantly higher from larvae infected with *H. downesi* in high concentration logs compared to the other two host groups – the same result that was obtained for raw data.

A.6 Calculations to estimate the amount of deadwood available to and colonised by saproxylic beetles on two clearfell sites (Lackenrea and Ballymachsaneboy)

It was assumed that the mean volume of logs found along CS transects was an adequate approximation of the mean volume of wood present in total in each 2 m long segment of a CS. It was also assumed that estimates of the proportion of logs suitable for saproxylic colonisation as well as LT pile height were adequate approximations valid for a section of LT pile extending 1 m to either side of the point where estimates and measurements were made. They were thus used as the basis for the estimation of the wood available in 2 m long LT segments. The following formulas were used to estimate the total amount of wood debris available to saproxylic beetles on each site:

$$(A) \quad V_{CS} = {}^{0.5}/_{m} * L * N_{CS} * M_{CS}$$

Formula *A* yielded the total volume of wood debris available to saproxylic insects located in cleared strips in litres (V_{CS}), where L is the length of surveyed area (400 m at Lackenrea, 160 m at Ballymacshaneboy), N_{CS} is the number of cleared strips in the surveyed area and M_{CS} is the mean volume of wood per 2 m segment of cleared strip as estimated from the mean volume of wood found along CS transects (see Table 5.32).

$$(B) \quad V_{LTs} = W_{LT} * D_{LT} * P_x * 0.5 * 200\ cm$$

Formula B yielded the estimated mean volume of wood debris available to saproxylic insects in litres for each 2 m segment of LT pile (V_{LTs}). For this calculation it was assumed that the cross-section of a LT pile was best approximated by a rectangle with a width corresponding to the mean width of LT piles on a site (W_{LT}), a height corresponding to the mean height of LT piles on a site (D_{LT}) and a length of 2 m. P_x was the mean estimated proportion of wood within LT piles available for colonisation with saproxylic beetles (Table 5.23). It was further assumed that due to the loose structure of LT piles, only about 50 % of the volume of a LT segment was occupied by wood.

$$(C) \quad V_{LT} = {}^{0.5}/_{m} * L * N_{LT} * V_{LTs}$$

Formula C yielded the total volume in litres of deadwood available to saproxylic insects on a site located in LT piles, where L is the length of the site and N_{LT} is the number of LT piles on the site.

Due to the irregular shape of the site at Lackenrea (Fig A.9), the site area as calculated from length and width measurements (400 m and 400 m) was estimated to be about twice as high as the actual site area. Total estimates of deadwood on this site were therefore corrected downward by 50 % (Table 5.24).

To estimate the number of individual logs available for colonisation by saproxylic insects in CS on a particular site (N_{logs}), the following formula was used:

$$(D) \quad N_{logs} = M_{logs} * 0.5 * L * N_{CS}$$

M_{logs} was the mean number of logs found per transect (N = 20 transects), L is the length of the site and N_{CS} is the number of CS on the site. A total of 25 logs were found along CS transects at Lackenrea and 14 logs were found at Ballymacshaneboy (N = 20 transects per site, see Table 5.23). The value calculated for Lackenrea was corrected downward by 57 % to account for the overestimation of site area.

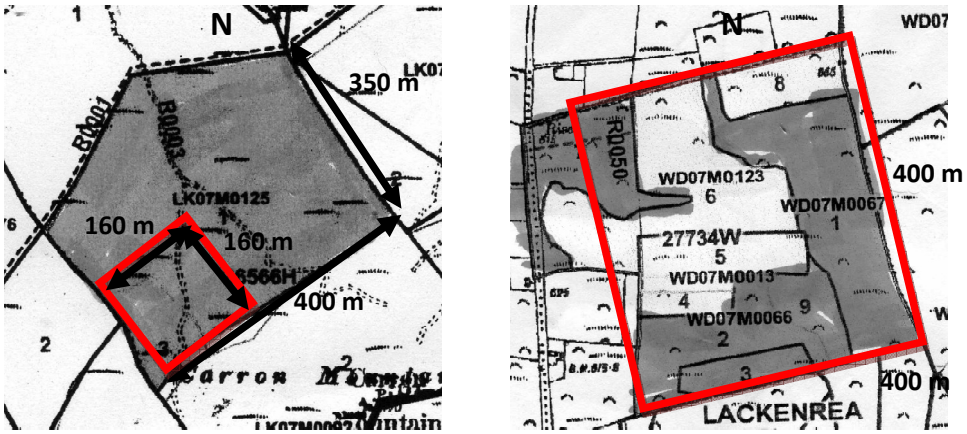

Fig A.9: Dimensions of the area surveyed for deadwood as taken from Coillte maps for Ballymachsaneboy (left) and Lackenrea (right). Grey area is total clearfelled area at each location, whereas only the clearfell area ecompassed by red lines was surveyed for deadwood. N = North.

A.7 Regression analysis results for field-sampled logs at Kilworth and Deerpark

Table A.3: Regression analysis results for Kilworth and Deerpark logs, infection rate or mean number of dead bait insects per 10 g wood sample vs. distance to closest treated stump (only logs with at least one infected *R. bifasciatum* individual included). Link function with Pearson Goodness-of-Fit P-value is given for binary logistic regression, equations are given for linear regression and test variables are listed (T for linear, Z for binary logistic regression). N = 9.

Type	*Regression*	*Equation*	R^2	*T or Z*	*DF*	*F*	*P*
Linear	Infection rate (Y) vs. distance from stump (X) logs with infected individuals only	y = 0.123+0.00209x	0.35	1.95	1	3.79	**0.092**
Linear	Mean number of dead bait insects in 10 g wood samples (Y) vs. distance from stump (X) logs with infected individuals only	y = 14.8-0.062x	0.04	-0.56	1	0.31	**0.593**

A.8 Detailed log data and parameters for all log segments sampled from field sites and used in Laboratory and Field Exposure Experiments

The tables on the following pages provide extensive data for infection rates of *R. bifasciatum* and all parameters that were recorded for logs collected in the field (Tables A.3 to A.7) and used in exposure experiments.

Table A.4: List of logs collected at **Kilworth** four weeks after EPN had been applied to site. Log measurements and parameters are given for each log. EPN species given refers to the EPN species applied to stumps in the area in which each log was collected. S.c. = *Steinernema carpocapsae*, H.d. = *Heterorhabditis downesi*. Distance to stump in cm gives the distance from the log to the closest stump in its vicinity to which EPN were applied. These distances were measured from the end of the log closest to the stump in question. A = area log was collected in (CS = cleared strip, LT = Lop and Top), P = porosity in %, L = length of log in cm, Ø = mean diameter of log in cm (N = 6 measurements, three at each end), V = volume of log calculated based on length and mean diameter, D = wood decay category (1= none to 4 = complete), M = moisture content of wood, SC = soil contact in %. The absolute number of live and infected larvae and pupae is given, numbers in brackets in these columns refer to the proportion of infected larvae or pupae in each log in %. W = number of infected bait insects per 750 g wood sample (N = 10 bait insects per log) and S = number of infected bait insects per soil sample (N= 10 bait insects per log). In the last column on the right the overall infection rate of *R. bifasciatum* individuals is given, including both larvae and pupae. In the last two rows, the mean values across all logs with standard deviation as well as the absolute numbers of live and infected *R. bifasciatum* larvae and pupae are given (median given for decay category).

Log No.	*EPN species*	*Distance to stump in cm*	*A*	*P (%)*	*L (cm)*	*Ø (cm)*	*V (L)*	*D*	*M*	*SC (%)*	*Larvae live/inf. (% inf.)*	*Pupae live/inf. (% inf.)*	*W*	*S*	*Total Inf. %*
K1	*S.c.*	97	CS	30	124	9.83	18.83	3	moist	20	62/0 *(0)*	18/0 *(0)*	3	0	**0**
K2	*S.c.*	37	CS	50	67	16.25	27.79	3	wet	20	19/0 *(0)*	9/0 *(0)*	0	0	**0**
K3	*S.c.*	52	CS	20	67	12.55	16.58	3	wet	30	31/0 *(0)*	9/0 *(0)*	0	0	**0**
K4	*S.c.*	22	CS	20	148	10.18	24.11	3	moist	5	61/20 *(24.69)*	18/8 *(30.77)*	10	0	**26.17**
K5	*S.c.*	70	CS	10	84	8.75	10.10	3	moist	50	7/0 *(0)*	0/0 *(0)*	0	0	**0**
K6	*S.c.*	41	CS	10	99	11.33	19.97	2	moist	20	19/0 *(0)*	12/0 *(0)*	0	0	**0**
K7	*S.c.*	27	CS	30	108	9.6	15.63	3	moist	20	9/0 *(0)*	5/0 *(0)*	8	0	**0**
K8	*S.c.*	45	CS	25	147	9.92	22.71	3	moist	5	37/1 *(2.63)*	3/0 *(0)*	0	7	**2.44**
K9	*S.c.*	56	CS	15	139	10.45	23.84	4	moist	40	1/0 *(0)*	2/0 *(0)*	0	0	**0**
K10	*S.c.*	15	CS	40	135	13.88	40.87	3	moist	50	38/8 *(17.39)*	20/0 *(0)*	4	10	**12.12**
K11	*S.c.*	29	CS	30	163	10.92	30.51	3	moist	10	42/1 *(2.33)*	13/0 *(0)*	6	1	**1.79**
K12	*S.c.*	58	CS	50	92	13.35	25.76	2	moist	20	28/0 *(0)*	11/0 *(0)*	0	0	**0**
K13	*H.d.*	54	CS	80	140	6.88	10.42	3	moist	30	7/0 *(0)*	1/0 *(0)*	0	0	**0**
K14	*H.d.*	62	CS	10	105	6.57	7.11	3	moist	10	1/0 *(0)*	2/0 *(0)*	0	0	**0**
K15	*H.d.*	27	CS	75	60	15.1	21.49	4	wet	20	9/0 *(0)*	8/0 *(0)*	0	0	**0**
K16	*H.d.*	358	LT	10	112	14.82	38.62	2	moist	50	11/0 *(0)*	6/0 *(0)*	0	0	**0**
Mean	-	65.63		31.56	111.88	11.27	22.15	3	-	25.00	23.88±19.79/	8.56±6.40/	1.94	1.13	**2.66**
±Stdev.		±20.17		± 2.26	±32.12	±20.17	± 9.64			±15.38	7.50±8.96	8.00±0	±3.32	±2.34	**±6.97**
Total							354.36				382/30 *(7.28)*	137/8 *(5.52)*			

Table A.5: List of logs collected at **Deerpark** in June of 2008, one year after EPN had been applied to site. Log measurements and parameters are given for each log. Note that log diameter was not recorded for logs sampled on this site (no volume calculation possible) and that not all parameters were determined for all logs. Wood decay category and moisture level of logs were not determined on this site. EPN species applied to the site was *Steinernema carpocapsae*. All logs were collected in the cleared areas of the site. Distance to stump in cm gives the distance from the log to the closest stump in its vicinity to which EPN were applied. These distances were measured from the end of the log closest to the stump in question. P = porosity in %, L = length of log in cm, SC = soil contact in %. The absolute number of live and infected larvae and pupae is given, numbers in brackets in these columns refer to the proportion of infected larvae or adults in each log in %. W = number of infected bait insects per 750 g wood sample (N=10 bait insects per log) and S = number of infected bait insects per soil sample (N = 10 bait insects per log). In the last column on the right the overall infection rate of *R. bifasciatum* individuals is given, including both larvae and adults. In the last two rows, the mean values across all logs with standard deviation as well as the absolute numbers of live and infected *R. bifasciatum* larvae and pupae are given.

Log	*Distance to stump in cm*	*P (%)*	*L (cm)*	*SC (%)*	*Larvae live/inf. (% inf.)*	*Adults live/inf. (% inf.)*	*W*	*S*	*Total Inf. %*
DP1	*N/A*	*N/A*	*N/A*	*N/A*	12/0 *(0)*	1/0 *(0)*	*N/A*	0	**0**
DP2	*N/A*	*N/A*	*N/A*	*N/A*	17/0 *(0)*	0/0 *(0)*	*N/A*	0	**0**
DP3	58	*N/A*	*N/A*	*N/A*	12/0 *(0)*	0/0 *(0)*	*N/A*	0	**0**
DP4	12	*N/A*	*N/A*	*N/A*	25/10 *(29.42)*	0/0 *(0)*	*N/A*	0	**29.42**
DP5	4	*N/A*	*N/A*	*N/A*	28/14 *(34.15)*	0/0 *(0)*	*N/A*	0	**34.15**
DP6	60	80%	72	25	8/0 *(0)*	0/0 *(0)*	0	3	**0**
DP7	295	80	74	30	3/0*(0)*	0/0 *(0)*	0	0	**0**
DP8	268	10	95	50	20/0 *(0)*	0/0 *(0)*	0	0	**0**
DP9	358	75	156	10	17/0 *(0)*	0/0 *(0)*	0	0	**0**
DP10	14	40	55	10	10/0 *(0)*	0/0 *(0)*	0	0	**0**
DP11	74	50	120	25	31/0 *(0)*	0/0 *(0)*	10	0	**0**
DP12	57	20	158	10	20/3 *(13.04)*	0/0 *(0)*	8	0	**13.04**
DP13	20	10	90	0	47/6 *(11.32)*	0/0 *(0)*	10	0	**11.32**
DP14	75	20	170	30	23/0 *(0)*	0/0 *(0)*	0	0	**0**
DP15	49	100	137	50	61/0 *(0)*	0/0 *(0)*	0	0	**0**
DP16	170	*N/A*	75	20	6/5 *(54.55)*	0/1 *(100)*	10	1	**50.00**
Mean ±Stdev.	108.14 ±116.57	40.58 ±34.48	109.27 ±40.57	23.64 ±16.14	21.13±15.19/ 2.38±4.29	0.063±0.25/ 0.063±0.25	8.13 ±4.82	0.25 ±0.83	**8.90 ±17.22**
Total no.					338/42 *(11.05)*	1/1 *(50.00)*			

Table A.6: List of logs collected at **Raheenkyle** in July of 2008,four weeks after EPN had been applied to site. Log measurements and parameters are given for each log. EPN species applied to stumps was *Steinernema carpocapsae*. Distance to stump in cm gives the distance from the log to the closest stump in its vicinity to which EPN had been applied. These distances were measured from the end of the log closest to the stump in question. A = area log was collected in (CS = cleared strip, LT = Lop and Top), P = porosity in %, L = length of log in cm, Ø = mean diameter of log in cm (N = 6 measurements, three at each end), V = volume of log calculated based on length and mean diameter, D = wood decay category (1= none to 4 = complete), M = moisture content of wood, SC = soil contact in %. The absolute number of live larvae, pupae and adults is given, no infected individuals were found. W = number of infected bait insects per 750 g wood sample (N=10 bait insects per log) and S = number of infected bait insects per soil sample (N= 10 bait insects per log). In the last two rows, the mean values across all logs with standard deviation as well as the absolute numbers of live *R. bifasciatum* larvae, pupae and adults are given (median given for decay category).

Log	*Distance to stump in cm*	*P (%)*	*A*	*L (cm)*	*Ø (cm)*	*V (L)*	*D*	*M*	*SC (%)*	*Larvae*	*Pupae*	*Adults*	*W*	*S*
R1	79	40	CS	210	5.85	11.30	3	moist	5	11	7	0	0	0
R2	124	20	CS	125	8.05	12.74	3	moist	5	18	10	0	0	0
R3	48	10	CS	126	9.63	18.39	3	moist	25	34	6	0	0	0
R4	54	10	CS	146	9.12	19.09	2	dry	1	23	5	1	0	0
R5	131	10	CS	161	8.67	19.02	2	dry	5	21	1	0	0	0
R6	0	80	CS	231	5.90	12.65	2	moist	80	16	5	0	0	0
R7	22	100	CS	120	8.07	12.28	3	wet	60	6	0	0	0	9
R8	44	20	CS	87	6.83	6.39	3	moist	1	2	0	0	0	0
R9	0	30	CS	54	10.57	9.48	2	dry	0	11	0	1	0	2
R10	67	40	CS	77	6.98	5.91	3	dry	50	15	0	4	0	0
R11	0	15	CS	227	6.07	13.14	3	dry	0	35	16	0	0	0
R12	12	80	LT	196	11.80	42.93	3	moist	0	10	1	0	0	0
R13	169	60	CS	172	10.57	30.21	3	moist	40	36	13	0	0	0
R14	76	75	CS	103	6.90	7.71	4	moist	50	12	2	0	0	0
R15	0	40	CS	132	8.07	13.51	2	moist	2	17	5	0	0	0
Mean	55.07	42.00		144.47	8.20	15.65	3	-	21.6	18.13	6.45	0.40		0.73
±Stdev.	±53.39	±29.99		±54.46	±1.85	±9.74			±27.13	±11.08	±4.82	±1.06		±2.34
Total no.						234.75				272	71	6		

Table A.7: List of logs collected at **Featherbed** in July of 2008, four weeks after EPN had been applied to site. Log measurements and parameters are given for each log. EPN species applied to stumps was *Steinernema carpocapsae*. Distance to stump in cm gives the distance from the log to the closest stump in its vicinity to which EPN had been applied. These distances were measured from the end of the log closest to the stump in question. A = area log was collected in (CS = cleared strip, LT = Lop and Top), P = porosity in %, L = length of log in cm, Ø = mean diameter of log in cm (N = 6 measurements, three at each end), V = volume of log calculated based on length and mean diameter, D = wood decay category (1= none to 4 = complete), M = moisture content of wood, SC = soil contact in %. The absolute number of live larvae is given, no infected individuals were found. W = number of infected bait insects per 750 g wood sample (N=10 bait insects per log) and S = number of infected bait insects per soil sample (N= 10 bait insects per log). In the last two rows, the mean values across all logs with standard deviation as well as the absolute numbers of live and infected *R. bifasciatum* larvae (median given for decay category).

Log	*Distance to stump in cm*	*A*	*P (%)*	*L (cm)*	*Ø (cm)*	*V (L)*	*D*	*M*	*SC (%)*	*Larvae*	*W*	*S*
F1	24	CS	10	166	10.15	26.90	2	moist	20.00	29	0	2
F2	35	LT	5	92	9.30	12.52	3	wet	25.00	47	0	0
F3	67	CS	80	77	7.20	6.28	4	dry	5.00	8	0	0
F4	150	CS	50	156	9.15	20.54	2	dry	0.00	44	0	0
F5	63	CS	40	96	15.05	34.20	3	dry	1.00	52	0	0
F6	10	CS	50	139	9.55	19.94	3	dry	20.00	42	0	0
F7	73	CS	25	106	11.30	21.29	2	wet	50.00	21	0	0
F8	12	CS	15	142	7.40	12.23	3	dry	2.00	32	0	0
F9	0	CS	5	117	11.35	23.71	2	dry	0.00	6	0	1
F10	88	CS	30	138	8.55	15.87	2	dry	10.00	7	0	0
F11	17	LT	40	124	12.95	32.71	3	moist	15.00	35	0	0
F12	75	CS	10	112	8.65	13.18	4	moist	15.00	4	0	0
F13	76	LT	50	80	7.20	6.52	4	dry	0.00	17	0	0
F14	15	CS	10	153	11.45	31.55	3	moist	15.00	45	0	1
F15	26	LT	10	141	9.30	19.18	3	moist	50.00	24	0	0
Mean	48.73±4		28.67	22.6	9.90	119.77	3	-	15.20	27.53	0	0.27
±Stdev.	0.57		±22.32	±28.16	±2.20	±8.90			±16.40	±16.54		±0.59
Total no.						296.62				413		

Table A.8: List of logs collected at **Featherbed** in August of 2009, one year after EPN had been applied to site. Log measurements and parameters are given for each log. EPN species applied to stumps was *S. carpocapsae*. Distance to stump in cm gives the distance from the log to the closest stump in its vicinity to which EPN had been applied. These distances were measured from the end of the log closest to the stump in question. A = area log was collected in (CS = cleared strip, LT = Lop and Top), P = porosity in %, L = length of log in cm, Ø = mean diameter of log in cm (N = 6 measurements, three at each end), V = volume of log calculated based on length and mean diameter, D = wood decay category (1= none to 4 = complete), M = moisture content of wood, SC = soil contact in %. The absolute number of live larvae is given, with the number of infected larvae indicated after the slash. Proportion of infected individuals in brackets. W = number of infected bait insects per 750 g wood sample (N=10 bait insects per log) and S = number of infected bait insects per soil sample (N= 10 bait insects per log). In the last two rows, the mean values across all logs with standard deviation as well as the absolute numbers of live and infected *R. bifasciatum* larvae is given(proportion of infected larvae in brackets; median given for decay category).

Log	*Distance to stump in cm*	*A*	*P (%)*	*L (cm)*	*Ø (cm)*	*V (L)*	*D*	*M*	*SC (%)*	*Larvae*	*Pupae*	*Adults*	*W*	*S*
F16	15	CS	15	121	11.47	25.02	4	dry	5.00	19/0 *(0)*	9	0	0	0
F17	16	CS	80	136	13.37	38.22	3	moist	5.00	7/0 *(0)*	6	0	1	0
F18	158	CS	5	166	10.18	27.08	2	dry	10.00	7/0 *(0)*	1	0	0	0
F19	112	CS	2	67	9.05	8.63	4	dry	20.00	8/0 *(0)*	11	0	0	0
F20	16	CS	60	124	11.42	25.42	3	dry	5.00	4/0 *(0)*	16	0	0	0
F21	15	CS	1	110	8.73	13.20	1	dry	5.00	13/1 *(7.7)*	3	0	0	0
F22	103	CS	15	112	10.28	18.63	3	dry	1.00	13/0 *(0)*	6	0	0	0
F23	51	CS	5	87	9.42	12.13	3	moist	50.00	0/0 *(0)*	0	0	0	0
F24	128	LT	30	97	11.70	20.89	2	dry	1.00	9/0 *(0)*	3	0	0	0
F25	61	LT	40	107	7.75	10.11	2	dry	15.00	2/0 *(0)*	7	0	0	0
F26	46	CS	80	55	16.40	23.27	4	moist	20.00	17/0 *(0)*	4	1	0	0
F27	22	CS	10	102	12.18	23.81	2	dry	20.00	4/0 *(0)*	1	0	0	0
F28	44	CS	60	147	8.90	18.31	2	dry	10.00	28/0 *(0)*	14	0	0	0
F29	82	CS	75	73	10.30	12.18	3	moidt	50.00	6/0 *(0)*	5	0	0	0
F30	63	CS	40	71	14.90	24.79	2	moist	25.00	9/0 *(0)*	4	0	0	0
Mean ±Stdev.	62.13 ±45.60	-	34.53 ±29.81	105.00 ±31.24	11.04 ±2.39	19.76 ±7.96	3	-	16.13 ±15.69	9.73±7.30 /0.07±0.26	6.00 ±4.72	0.07 ±0.26	0.07 ±0.26	0
Total no.						301.69				146/1 *(0.68)*	90	1		

Table A.9: List of log segments (50 cm in length each) to which **1.8 million *S. carpocapsae*** IJs were applied in **Laboratory Exposure Experiment I**. Log measurements and parameters are given for each log. P = porosity in %, W = weight of log in kg, Ø = mean diameter of log in cm (N = 6 measurements, three at each end), V = volume of log calculated based on length and mean diameter, D = wood decay category (1= none to 4 = complete), M = moisture content of wood. The absolute number of live and infected larvae and adults is given, numbers in brackets in these columns refer to the proportion of infected larvae or pupae in each log in %. The density of *R. bifasciatum* individuals per log is given in the second to last column on the right. This was calculated by dividing the number of individuals collected from a log by the volume of the log. In the last column on the right the overall infection rate of *R. bifasciatum* individuals is given. No pupae or adults were recovered from this set of logs. In the bottom two rows, the mean values across all logs with standard deviation as well as the absolute numbers of live and infected *R. bifasciatum* larvae and adults are given (median given for decay category).

Log	*EPN species and concentration*	*P (%)*	*W (kg)*	*Ø (cm)*	*V (L)*	*D*	*M*	*Larvae live/inf. (% inf.)*	*Adults live/inf. (% inf.)*	*R. bifasciatum density (individuals per L)*	*Total Inf. %*
A1	*S.c. 1,800,000*	90	1.59	9.43	6.99	4	moist	0/3 *(100)*	0/1 *(100)*	0.57	**100.00**
A2	*S.c. 1,800,000*	20	1.82	8.70	5.94	4	wet	0/4 *(100)*	0/3 *(100)*	1.18	**100.00**
A3	*S.c. 1,800,000*	100	2.95	9.87	7.65	4	moist	2/1 *(66.67)*	0/2 *(100)*	0.65	**60.00**
A4	*S.c. 1,800,000*	10	2.03	7.13	4.00	4	moist	0/7 *(100)*	0/2 *(100)*	2.25	**100.00**
A5	*S.c. 1,800,000*	100	1.42	6.40	3.22	4	moist	0/3 *(100)*	0/0 *(0)*	0.93	**100.00**
A6	*S.c. 1,800,000*	10	1.44	5.73	2.58	2	wet	3/11 *(27.27)*	0/2 *(100)*	6.20	**81.25**
A7	*S.c. 1,800,000*	100	1.12	5.73	2.58	4	wet	0/0 *(0)*	0/0 *(0)*	0.00	**0.00**
A8	*S.c. 1,800,000*	90	1.20	5.53	2.40	2	wet	0/4 *(100)*	0/0 *(0)*	1.66	**100.00**
A9	*S.c. 1,800,000*	10	0.96	5.90	2.73	2	dry	1/5 *(16.67)*	0/0 *(0)*	2.19	**83.33**
A10	*S.c. 1,800,000*	100	1.96	10.30	8.33	4	moist	0/0 *(0)*	0/0 *(0)*	0.00	**0.00**
A11	*S.c. 1,800,000*	100	2.18	9.57	7.19	4	wet	1/9 *(11.11)*	0/2 *(100)*	1.67	**91.67**
A12	*S.c. 1,800,000*	60	1.89	9.27	6.74	4	wet	0/3 *(100)*	0/3 *(100)*	0.89	**100.00**
A13	*S.c. 1,800,000*	50	1.72	8.27	5.37	3	moist	0/3 *(100)*	0/1 *(100)*	0.75	**100.00**
A14	*S.c. 1,800,000*	100	2.48	7.93	4.94	3	moist	0/2 *(100)*	0/0 *(0)*	0.40	**100.00**
A15	*S.c. 1,800,000*	30	2.27	7.10	3.96	3	wet	1/12 *(8.33)*	0/2 *(100)*	3.79	**93.33**
Mean		65.00	1.72	7.53	4.68	4		0.53±0.89/	0±0/	1.54	**80.64**
±Stdev.		±38.08	±0.52	±1.79	±2.18			4.47±3.67	1.20±1.15	±1.63	**±34.56**
Total amount					**74.63**			**8/67** ***(89.33)***	**18** ***(100)***		

Table A.10: List of log segments (50 cm in length each) to which **18,000 *S. carpocapsae*** IJs were applied in **Laboratory Exposure Experiment I**. Log measurements and parameters are given for each log. P = porosity in %, W = weight of log in kg, Ø = mean diameter of log in cm (N = 6 measurements, three at each end), V = volume of log calculated based on length and mean diameter, D = wood decay category (1= none to 4 = complete), M = moisture content of wood. The absolute number of live and infected larvae is given, numbers in brackets in these columns refer to the proportion of infected larvae or pupae in each log in %. The density of *R. bifasciatum* individuals per log is given in the second to last column on the right. This was calculated by dividing the number of individuals collected from a log by the volume of the log. In the last column on the right the overall infection rate of *R. bifasciatum* individuals is given. No pupae or adults were recovered from this set of logs. In the bottom two rows, the mean values across all logs with standard deviation as well as the absolute numbers of live and infected *R. bifasciatum* larvae are given (median given for decay category).

Log	*EPN species and concentration*	*P (%)*	*W (kg)*	*Ø (cm)*	*V (L)*	*D*	*M*	*Larvae live/inf. (% inf.)*	*R. bifasciatum density (individuals per L)*	*Total Inf. %*
B1	*S.c. 18,000*	10	1.54	8.30	5.41	2	dry	0/0 *(0)*	0.00	**0.00**
B2	*S.c. 18,000*	100	3.78	9.57	7.19	3	wet	1/0 *(0)*	0.14	**0.00**
B3	*S.c. 18,000*	90	1.19	8.83	6.13	3	moist	2/1 *(33.33)*	0.49	**33.33**
B4	*S.c. 18,000*	40	1.14	5.90	2.73	3	moist	1/0 *(0)*	0.37	**0.00**
B5	*S.c. 18,000*	90	2.86	11.50	10.39	3	moist	2/0 *(0)*	0.19	**0.00**
B6	*S.c. 18,000*	100	1.35	6.73	3.56	3	moist	2/0 *(0)*	0.56	**0.00**
B7	*S.c. 18,000*	90	1.62	7.50	4.42	3	moist	2/0 *(0)*	0.45	**0.00**
B8	*S.c. 18,000*	100	2.27	8.93	6.27	4	moist	1/1 *(50.00)*	0.32	**50.00**
B9	*S.c. 18,000*	100	3.18	9.90	7.70	3	moist	6/1 *(14.29)*	0.91	**14.29**
B10	*S.c. 18,000*	100	1.36	6.63	3.46	3	wet	0/0 *(0)*	0.00	**0.00**
B11	*S.c. 18,000*	100	3.17	9.50	7.09	3	wet	5/3 *(37.50)*	1.13	**37.50**
B12	*S.c. 18,000*	100	1.00	6.77	3.60	4	wet	0/0 *(0)*	0.00	**0.00**
B13	*S.c. 18,000*	100	2.10	9.17	6.60	4	wet	2/0 *(0)*	0.30	**0.00**
B14	*S.c. 18,000*	100	1.28	8.20	5.28	3	moist	1/0 *(0)*	0.19	**0.00**
B15	*S.c. 18,000*	80	0.95	6.10	2.92	3	moist	0/0 *(0)*	0.00	**0.00**
Mean	-	97.00	1.83	1.83	5.09	3	-	2.07±2.40/	0.34	**9.01**
±Stdev.		±6.75		±0.83	±1.73			0.4±0.83	±0.34	**±16.91**
Total amount					**82.74**			**25/6 *(19.35)***		

Table A.11: List of log segments (50 cm in length each) to which **tap water** was applied in **Laboratory Exposure Experiment I**. Log measurements and parameters are given for each log. P = porosity in %, W = weight of log in kg, Ø = mean diameter of log in cm (N = 6 measurements, three at each end), V = volume of log calculated based on length and mean diameter, D = wood decay category (1= none to 4 = complete), M = moisture content of wood. The absolute number of live and infected larvae and pupae is given, numbers in brackets in these columns refer to the proportion of infected larvae or pupae in each log in %. The density of *R. bifasciatum* individuals per log (larvae and adults combined) is given in the second to last column on the right. This was calculated by dividing the number of individuals collected from a log by the volume of the log. In the last column on the right the overall infection rate of *R. bifasciatum* individuals is given. No pupae or adults were recovered from this set of logs. In the bottom two rows, the mean values across all logs with standard deviation as well as the absolute numbers of live and infected *R. bifasciatum* larvae and pupae are given (median given for decay category).

Log	*P (%)*	*W (kg)*	*Ø (cm)*	*V (L)*	*D*	*M*	*Larvae live/inf. (% inf.)*	*Adults live/inf. (% inf.)*	*R. bifasciatum density (individuals per L)*
C1	20	2.26	9.33	6.84	4	moist	2	3	0.73
C2	60	2.65	8.67	5.90	4	wet	9	2	1.86
C3	100	2.28	8.53	5.72	4	moist	6	0	1.05
C4	30	1.54	7.20	4.07	4	moist	4	1	1.23
C5	100	1.14	6.43	3.25	4	moist	2	0	0.62
C6	30	1.04	5.77	2.61	2	wet	8	2	3.83
C7	80	1.03	5.50	2.38	4	wet	0	0	0.00
C8	100	0.92	5.50	2.38	2	wet	1	0	0.42
C9	10	0.77	5.07	2.02	2	dry	3	0	1.49
C10	100	2.62	10.30	8.33	4	moist	4	0	0.48
C11	100	2.41	9.80	7.54	4	wet	4	1	0.66
C12	30	1.35	9.20	6.65	4	wet	0	1	0.15
C13	40	1.78	8.30	5.41	3	moist	3	0	0.55
C14	100	1.88	8.67	5.90	3	moist	4	0	0.68
C15	20	1.67	7.13	4.00	3	wet	5	2	1.75
Mean	61.00	1.69	7.69	4.87	4	-	3.67	0.80	1.03
±Stdev.	±38.14	±0.65	±1.97	±2.35			±2.61	±1.01	±0.95
Total amount				**72.99**			**55**	**12**	

Table A.12: List of log segments (50 cm in length each) to which **1.8 million *H. downesi*** IJs were applied in **Laboratory Exposure Experiment II**. Log measurements and parameters are given for each log. P = porosity in %, W = weight of log in kg, Ø = mean diameter of log in cm (N = 6 measurements, three at each end), V = volume of log calculated based on length and mean diameter, D = wood decay category (1= none to 4 = complete), M = moisture content of wood. The absolute number of live and infected larvae, pupae and adults is given, numbers in brackets in these columns refer to the proportion of infected larvae or pupae in each log in %. The density of *R. bifasciatum* individuals (larvae, pupae and adults combined) per log is given in the second to last column on the right. This was calculated by dividing the number of individuals collected from a log by the volume of the log. In the last column on the right the overall infection rate of *R. bifasciatum* individuals is given. No pupae or adults were recovered from this set of logs. In the bottom two rows, the mean values across all logs with standard deviation as well as the absolute numbers of live and infected *R. bifasciatum* larvae, pupae and adults are given (median given for decay category).

Log	*EPN species and concentration*	*P (%)*	*W (kg)*	*Ø (cm)*	*V (L)*	*D*	*M*	*Larvae live/inf. (% inf.)*	*Pupae live/inf. (% inf.)*	*Adults live/inf. (% inf.)*	*R. bifasciatum density (individuals per L)*	*Total Inf. %*
X1	*H.d. 1,800,000*	5	1.54	9.20	6.65	3	dry	2/9 *(22.22)*	0/1 *(100)*	0/0 *(0)*	1.81	**83.33**
X2	*H.d. 1,800,000*	5	1.6	10.55	8.74	3	dry	1/3 *(25.00)*	1/0 *(0)*	0/0 *(0)*	0.57	**60.00**
X3	*H.d. 1,800,000*	30	4.06	14.55	16.63	2	dry	0/18 *(100)*	0/0 *(0)*	0/6 *(100)*	1.44	**100.00**
X4	*H.d. 1,800,000*	15	0.64	8.68	5.92	4	dry	0/4 *(100)*	0/0 *(0)*	0/1 *(100)*	0.84	**100.00**
X5	*H.d. 1,800,000*	40	1.07	7.42	4.32	2	dry	3/0 *(0)*	0/0 *(0)*	0/0 *(0)*	0.69	**0.00**
X6	*H.d. 1,800,000*	70	2.5	11.67	10.69	3	moist	0/3 *(100)*	0/0 *(0)*	0/0 *(0)*	0.28	**100.00**
X7	*H.d. 1,800,000*	40	1.46	8.30	5.41	3	wet	0/3 *(100)*	0/0 *(0)*	1/2 *(33.33)*	1.11	**83.33**
X8	*H.d. 1,800,000*	20	1.58	8.10	5.15	2	moist	0/0 *(0)*	0/0 *(0)*	0/0 *(0)*	0.00	**0.00**
X9	*H.d. 1,800,000*	5	5.56	30.53	73.22	3	moist	1/5 *(16.67)*	0/0 *(0)*	0/1 *(100)*	0.10	**85.71**
X10	*H.d. 1,800,000*	1	1.5	8.82	6.11	3	wet	0/5 *(100)*	0/0 *(0)*	0/2 *(100)*	1.15	**100.00**
X11	*H.d. 1,800,000*	30	1.51	8.72	5.97	2	dry	1/1 *(50.00)*	0/0 *(0)*	0/0 *(0)*	0.34	**50.00**
X12	*H.d. 1,800,000*	20	1.42	8.77	6.04	3	moist	0/1 *(100)*	0/0 *(0)*	0/1 *(100)*	0.33	**100.00**
X13	*H.d. 1,800,000*	10	0.81	7.70	4.66	4	dry	2/2 *(50.00)*	0/5 *(100)*	1/4 *(20.00)*	3.01	**78.57**
X14	*H.d. 1,800,000*	30	3.54	9.85	7.62	3	wet	0/11 *(100)*	0/0 *(0)*	1/3 *(25.00)*	1.97	**93.33**
X15	*H.d. 1,800,000*	10	5.47	13.10	13.48	3	wet	1/2 *(66.67)*	0/0 *(0)*	0/3 *(100)*	0.45	**83.33**
Mean		22.07	2.28	10.94	11.69	3	-	0.73±0.96/	0.0713±0.26/	0.20±0.41/	0.94	**75.41**
±Stdev.		±18.51	±1.61	±5.58	±16.75			4.47±4.85	0.40±1.30	1.53±1.81	±0.83	**±33.69**
Total amount					180.60			11/67 *(91.78)*	1/6 *(14.29)*	3/23 *(13.04)*		

Table A.13: List of log segments (50 cm in length each) to which **18,000 *H. downesi*** IJs were applied in **Laboratory Exposure Experiment II**. Log measurements and parameters are given for each log. P = porosity in %, W = weight of log in kg, Ø = mean diameter of log in cm (N = 6 measurements, three at each end), V = volume of log calculated based on length and mean diameter, D = wood decay category (1= none to 4 = complete), M = moisture content of wood. The absolute number of live and infected larvae, pupae and adults is given, numbers in brackets in these columns refer to the proportion of infected larvae or pupae in each log in %. The density of *R. bifasciatum* individuals (larvae, pupae and adults combined) per log is given in the second to last column on the right. This was calculated by dividing the number of individuals collected from a log by the volume of the log. In the last column on the right the overall infection rate of *R. bifasciatum* individuals is given. No pupae or adults were recovered from this set of logs. In the bottom two rows, the mean values across all logs with standard deviation as well as the absolute numbers of live and infected *R. bifasciatum* larvae, pupae and adults are given (median given for decay category).

Log	*EPN species and concentration*	*P (%)*	*W (kg)*	*Ø (cm)*	*V (L)*	*D*	*M*	*Larvae live/inf. (% inf.)*	*Pupae live/inf. (% inf.)*	*Adults live/inf. (% inf.)*	*R. bifasciatum density (individuals per L)*	*Total Inf. %*
Y1	*H.d. 18,000*	2	2.12	8.90	6.22	4	moist	1/1 *(50.00)*	0/0 *(0)*	0/0 *(0)*	0.32	**50.00**
Y2	*H.d. 18,000*	30	1.50	8.07	5.11	3	dry	0/2 *(100)*	0/0 *(0)*	1/0 *(0)*	0.59	**66.67**
Y3	*H.d. 18,000*	20	2.68	9.05	6.43	4	wet	0/4 *(100)*	0/0 *(0)*	0/3 *(100)*	1.09	**100.00**
Y4	*H.d. 18,000*	50	0.52	6.37	3.18	4	moist	3/3 *(50.00)*	0/ *(100)*	0/1 *(100)*	2.51	**62.50**
Y5	*H.d. 18,000*	2	3.83	13.55	14.42	2	dry	6/2 *(25.00)*	0/0 *(0)*	0/0 *(0)*	0.55	**25.00**
Y6	*H.d. 18,000*	50	0.97	10.32	8.36	4	dry	3/1 *(75.00)*	0/0 *(0)*	0/0 *(0)*	0.48	**25.00**
Y7	*H.d. 18,000*	50	1.10	9.32	6.82	2	dry	0/0 *(0)*	0/0 *(0)*	0/1 *(100)*	0.15	**100.00**
Y8	*H.d. 18,000*	20	1.33	7.35	4.24	3	dry	0/0 *(0)*	0/0 *(0)*	0/0 *(0)*	0.00	**0.00**
Y9	*H.d. 18,000*	15	4.66	11.88	11.09	3	moist	12/9 *(42.86)*	0/0 *(0)*	0/2 *(100)*	2.07	**47.83**
Y10	*H.d. 18,000*	5	2.41	9.03	6.41	4	moist	2/13 *(15.38)*	0/0 *(0)*	0/2 *(100)*	2.65	**88.24**
Y11	*H.d. 18,000*	20	1.78	9.87	7.65	4	wet	1/0 *(100)*	0/0 *(0)*	0/2 *(100)*	0.39	**66.67**
Y12	*H.d. 18,000*	10	2.94	9.95	7.78	4	wet	4/1 *(20.00)*	0/0 *(0)*	1/3 *(75.00)*	1.16	**44.44**
Y13	*H.d. 18,000*	70	3.45	13.28	13.86	3	dry	1/2 *(66.67)*	0/2 *(100)*	0/0 *(0)*	0.36	**80.00**
Y14	*H.d. 18,000*	1	2.09	10.35	8.41	3	dry	8/1 *(11.11)*	0/1 *(100)*	0/0 *(0)*	1.19	**20.00**
Y15	*H.d. 18,000*	30	1.31	8.23	5.32	4	moist	0/2 *(100)*	0/0 *(0)*	0/4 *(100)*	1.13	**100.00**
Mean		25.00	2.18	9.70	7.69	4		2.93±3.56/	0±0/	0.14±0.36/	0.98	**5.71**
±Stdev.		± 21.36	±1.16	±2.01	±3.23			2.79±3.75	0.29±0.61	1.00±1.18	±0.84	**±5.98**
Total amount					109.98			41/41 *(50.00)*	0/4 *(100)*	2/18 *(11.11)*		

Table A.14: List of log segments (50 cm in length each) to which **18,000 *H. downesi*** IJs were applied in **Laboratory Exposure Experiment III**. Log measurements and parameters are given for each log. P = porosity in %, W = weight of log in kg, Ø = mean diameter of log in cm (N = 6 measurements, three at each end), V = volume of log calculated based on length and mean diameter, D = wood decay category (1= none to 4 = complete), M = moisture content of wood. The absolute number of live and infected larvae and pupae is given, numbers in brackets in these columns refer to the proportion of infected larvae or pupae in each log in %. The density of *R. bifasciatum* individuals (larvae and pupae combined) per log is given in the second to last column on the right. This was calculated by dividing the number of individuals collected from a log by the volume of the log. In the last column on the right the overall infection rate of *R. bifasciatum* individuals is given. No pupae or adults were recovered from this set of logs. In the bottom two rows, the mean values across all logs with standard deviation as well as the absolute numbers of live and infected *R. bifasciatum* larvae and adults are given (median given for decay category).

Log	*EPN species and concentration*	*P (%)*	*W (kg)*	*Ø (cm)*	*V (L)*	*D*	*M*	*Larvae live/inf. (% inf.)*	*Adults live/inf. (% inf.)*	*R. bifasciatum density (individuals per L)*	*Total Inf. %*
ZHd1	*H.d. 18,000*	5	3.06	12.07	11.44	4	moist	16/1 *(5.83)*	0/0 *(0)*	1.486563	**5.88**
ZHd2	*H.d. 18,000*	50	0.48	5.65	2.51	4	moist	0/3 *(100)*	0/1 *(100)*	1.59541	**100.00**
ZHd3	*H.d. 18,000*	20	0.58	8.27	5.37	2	dry	1/3 *(75.00)*	0/0 *(0)*	0.745261	**75.00**
ZHd4	*H.d. 18,000*	5	1.91	9.75	7.47	3	dry	9/0 *(0)*	0/0 *(0)*	1.339368	**0.00**
ZHd5	*H.d. 18,000*	5	1.37	9.52	7.11	2	dry	1/1 *(50.00)*	0/0 *(0)*	0.28117	**50.00**
ZHd6	*H.d. 18,000*	0	1.83	9.75	7.47	4	moist	4/8 *(66.66)*	0/0 *(0)*	1.607241	**66.67**
ZHd7	*H.d. 18,000*	2	2.68	10.83	9.22	4	moist	3/0 *(0)*	0/0 *(0)*	0.325466	**0.00**
Mean		12.43	1.70	9.40	7.22	4	-	4.86±2.29/	0±0/	1.05	**42.51**
±Stdev.		±19.15	±0.14	±1.82	±2.32			5.76±2.81	0.14±0.36	±0.61	**±40.97**
Total amount					50.57			34/16 *(32.00)*	0/1 *(100)*		

Table A.15: List of log segments (50 cm in length each) to which **18,000 *S. carpocapsae*** IJs were applied in **Laboratory Exposure Experiment III**. Log measurements and parameters are given for each log. P = porosity in %, W = weight of log in kg, Ø = mean diameter of log in cm (N = 6 measurements, three at each end), V = volume of log calculated based on length and mean diameter, D = wood decay category (1= none to 4 = complete), M = moisture content of wood. The absolute number of live and infected larvae and pupae is given, numbers in brackets in these columns refer to the proportion of infected larvae or pupae in each log in %. The density of *R. bifasciatum* individuals (larvae and pupae combined) per log is given in the second to last column on the right. This was calculated by dividing the number of individuals collected from a log by the volume of the log. In the last column on the right the overall infection rate of *R. bifasciatum* individuals is given. No pupae or adults were recovered from this set of logs. In the bottom two rows, the mean values across all logs with standard deviation as well as the absolute numbers of live and infected *R. bifasciatum* larvae and adults are given (median given for decay category).

Log No.	*EPN species and concentration*	*P (%)*	*W (kg)*	*Ø (cm)*	*V (L)*	*D*	*M*	*Larvae live/inf. (% inf.)*	*Pupae live/inf. (% inf.)*	*Adults live/inf. (% inf.)*	*R. bifasciatum density (individuals per L)*	*Total Inf. %*
ZSc1	*S.c. 18,000*	5	4.06	13.68	14.71	4	moist	10/4 (28.57)	0/0 *(0)*	0/0 *(0)*	0.95	**28.57**
ZSc2	*S.c. 18,000*	10	0.89	8.10	5.15	4	dry	0/0 *(0)*	1/1 *(50.00)*	1/0 *(0)*	0.58	**33.33**
ZSc3	*S.c. 18,000*	0	0.73	7.22	4.09	4	dry	3/3 *(0)*	2/0 *(100)*	0/0 *(0)*	1.22	**0.00**
ZSc4	*S.c. 18,000*	2	3.67	10.30	8.33	4	wet	2/5 *(28.57)*	0/0 *(0)*	0/0 *(0)*	3.12	**19.23**
ZSc5	*S.c. 18,000*	40	1.47	10.08	7.99	3	dry	1/9 *(10.00)*	0/0 *(0)*	0/0 *(0)*	1.25	**90.00**
ZSc6	*S.c. 18,000*	2	1.66	8.10	5.15	4	moist	0/0 *(0)*	0/0 *(0)*	0/0 *(0)*	0.00	**0.00**
ZSc7	*S.c. 18,000*	20	1.75	8.75	6.01	4	wet	4/1 *(20.00)*	0/0 *(0)*	0/0 *(0)*	0.83	**20.00**
Mean ±Stdev.		11.29 ± 15.46	2.01 ±1.02	9.46 ±1.21	7.35 ±1.70	4	-	5.37±7.63/ 2.71±3.45	0.43±0.14/ 0.79±0.38	0.14±0.38/ 0±0	1.14 ±1.06	**31.86 ±32.46**
Total amount					51.43			39/19 *(32.76)*	3/1 *(75.00)*	1/101 *(0)*		

Table A.16: List of log segments (50 cm in length each) to which **1.8 million *S.carpocapsae*** IJs were applied in **Field Exposure Experiment I**. Log measurements and parameters are given for each log. P = porosity in %, W = weight of log in kg, Ø = mean diameter of log in cm (N = 6 measurements, three at each end), V = volume of log calculated based on length and mean diameter, D = wood decay category (1= none to 4 = complete), M = moisture content of wood. The absolute number of live and infected larvae and pupae is given, numbers in brackets in these columns refer to the proportion of infected larvae or pupae in each log in %. The density of *R. bifasciatum* individuals (larvae and pupae combined) per log is given in the second to last column on the right. This was calculated by dividing the number of individuals collected from a log by the volume of the log. In the last column on the right the overall infection rate of *R. bifasciatum* individuals is given. No pupae or adults were recovered from this set of logs. In the bottom two rows, the mean values across all logs with standard deviation as well as the absolute numbers of live and infected *R. bifasciatum* larvae and adults are given (median given for decay category).

Log No.	*EPN species and concentration*	*P (%)*	*W (kg)*	*Ø (cm)*	*V (L)*	*D*	*M*	*Larvae live/inf. (% inf.)*	*Pupae live/inf. (% inf.)*	*R. bifasciatum density (individuals per L)*	*Total Inf. %*
FA1	*S.c. 1,800,000*	30	1.88	9.87	7.65	3	dry	5/3 *(37.5)*	3/4 *(57.14)*	1.96	**46.67**
FA2	*S.c. 1,800,000*	5	0.50	6.55	3.37	2	dry	0/1 *(100)*	0/0 *(0)*	0.30	**100.00**
FA3	*S.c. 1,800,000*	30	2.22	10.28	8.31	3	dry	4/17 *(80.95)*	5/3 *(32.5)*	3.49	**68.97**
FA4	*S.c. 1,800,000*	5	1.80	8.35	5.48	4	wet	2/15 *(88.24)*	4/6 *(60.00)*	4.93	**77.78**
FA5	*S.c. 1,800,000*	5	2.27	7.67	4.62	3	dry	1/3 (*75.00)*	2/3 *(60.00)*	1.95	**66.67**
FA6	*S.c. 1,800,000*	20	2.30	9.67	7.34	4	wet	4/6 *(60.00)*	4/3 *(42.86)*	2.32	**52.94**
FA7	*S.c. 1,800,000*	100	3.05	10.32	8.36	3	wet	2/13 *(86.67)*	0/5 *(100)*	2.39	**90.00**
FA8	*S.c. 1,800,000*	10	1.30	6.70	3.53	3	dry	1/4 *(80.00)*	2/1 *(33.33)*	2.27	**62.50**
FA9	*S.c. 1,800,000*	30	6.54	14.50	16.51	4	wet	2/3 *(60.00)*	6/1 *(14.29)*	0.73	**33.33**
FA10	*S.c. 1,800,000*	70	3.11	10.28	8.31	3	wet	7/20 *(74.07)*	1/2 *(66.67)*	3.61	**73.33**
Mean		30.50	2.50	9.42	7.35	3	-	2.80±2.15/	2.70±2.06/	0.94	**75.41**
±Stdev.		±33.30	±1.70	±2.45	±4.00			8.50±7.00	2.80±1.87	±0.83	**±33.69**
Total amount					73.48			28/85 *(75.22)*	27/28 *(50.91)*		

Table A.16: List of log segments (50 cm in length each) to which **180,000 *S. carpocapsae*** IJs were applied in **Field Exposure Experiment I**. Log measurements and parameters are given for each log. P = porosity in %, W = weight of log in kg, Ø = mean diameter of log in cm (N = 6 measurements, three at each end), V = volume of log calculated based on length and mean diameter, D = wood decay category (1= none to 4 = complete), M = moisture content of wood. The absolute number of live and infected larvae and pupae is given, numbers in brackets in these columns refer to the proportion of infected larvae or pupae in each log in %. The density of *R. bifasciatum* individuals (larvae and pupae combined) per log is given in the second to last column on the right. This was calculated by dividing the number of individuals collected from a log by the volume of the log. In the last column on the right the overall infection rate of *R. bifasciatum* individuals is given. No adults were recovered from this set of logs. In the bottom two rows, the mean values across all logs with standard deviation as well as the absolute numbers of live and infected *R. bifasciatum* larvae and pupae are given (median given for decay category).

Log	*EPN species and concentration*	*P (%)*	*W (kg)*	*Ø (cm)*	*V (L)*	*D*	*M*	*Larvae live/inf. (% inf.)*	*Pupae live/inf. (% inf.)*	*Adults live/inf. (% inf.)*	*R. bifasciatum density (individuals per L)*	*Total Inf. %*
FB1	S.c. 180,000	10	2.40	9.03	6.41	3	dry	8/1 *(11.11)*	2/0 *(0)*	0/0 *(0)*	1.72	**9.09**
FB2	S.c. 180,000	30	1.98	9.22	6.67	3	dry	6/5 *(45.45)*	0/0 *(0)*	0/0 *(0)*	1.95	**38.46**
FB3	S.c. 180,000	10	1.53	8.77	6.04	3	dry	10/1 *(9.09)*	1/0 *(0)*	1/0 *(0)*	2.81	**47.06**
FB4	S.c. 180,000	10	2.21	10.32	8.36	3	wet	3/4 *(57.14)*	4/2 *(33.33)*	2/2 *(50.00)*	3.35	**32.14**
FB5	S.c. 180,000	30	1.48	6.62	3.44	3	dry	17/9 *(34.62)*	1/1 *(50.00)*	0/0 *(0)*	3.49	**41.67**
FB6	S.c. 180,000	50	2.64	10.73	9.05	2	dry	6/6 *(50.00)*	0/0 *(0)*	0/0 *(0)*	2.65	**41.67**
FB7	S.c. 180,000	30	1.87	8.28	5.39	3	dry	14/10 *(41.67)*	0/0 *(0)*	0/0 *(0)*	4.64	**56.00**
FB8	S.c. 180,000	30	1.65	8.05	5.09	4	dry	9/11 *(55.00)*	2/3 *(60.00)*	0/0 *(0)*	4.91	**28.00**
FB9	S.c. 180,000	30	2.79	10.35	8.41	2	dry	17/6 *(26.09)*	0/1 *(100)*	1/0 *(0)*	4.64	**25.64**
FB10	S.c. 180,000	40	5.03	15.68	19.32	3	dry	26/9 *(25.71)*	2/0 *(0)*	1/1 *(50.00)*	0.57	**45.45**
Mean		27.00	2.36	9.71	78.18	3	-	11.66±6.92/	1.20±1.32/	0.50±0.70/	3.07	**36.52**
±Stdev.		±13.37	±1.10	±2.17	±4.63			6.2±3.56	0.70±1.06	0.30±0.67	±1.42	**±13.29**
Total amount					180.60			116/62 *(34.48)*	12/7 *(37.00)*	5/3 *(37.50)*		

Table A.17: List of log segments (50 cm in length each) to which **18,000 *S. carpocapsae*** IJs were applied in **Field Exposure Experiment I**. Log measurements and parameters are given for each log. P = porosity in %, W = weight of log in kg, Ø = mean diameter of log in cm (N = 6 measurements, three at each end), V = volume of log calculated based on length and mean diameter, D = wood decay category (1= none to 4 = complete), M = moisture content of wood. The absolute number of live and infected larvae and pupae is given, numbers in brackets in these columns refer to the proportion of infected larvae or pupae in each log in %. The density of *R. bifasciatum* individuals (larvae, pupae and adults combined) per log is given in the second to last column on the right. This was calculated by dividing the number of individuals collected from a log by the volume of the log. In the last column on the right the overall infection rate of *R. bifasciatum* individuals is given. No In the bottom two rows, the mean values across all logs with standard deviation as well as the absolute numbers of live and infected *R. bifasciatum* larvae, pupae and adults are given (median given for decay category).

Log	*EPN species and concentration*	*P (%)*	*W (kg)*	*Ø (cm)*	*V (L)*	*D*	*M*	*Larvae live/inf. (% inf.)*	*Pupae live/inf. (% inf.)*	*Adults live/inf. (% inf.)*	*R. bifasciatum density (individuals per L)*	*Total Inf. %*
FC1	S.c. 18,000	70	3.24	11.28	10.00	3	dry	31/1 *(3.12)*	2/0 *(0)*	0/0 *(0)*	3.40	2.94
FC2	S.c. 18,000	80	1.48	7.03	3.89	2	dry	3/0 *(0)*	0/0 *(0)*	0/0 *(0)*	2.06	0.00
FC3	S.c. 18,000	20	1.45	7.58	4.52	3	dry	8/0 *(0)*	0/0 *(0)*	0/0 *(0)*	0.44	0.00
FC4	S.c. 18,000	40	1.28	8.52	5.70	3	dry	2/0 *(0)*	3/0 *(0)*	0/0 *(0)*	5.09	3.45
FC5	S.c. 18,000	20	3.84	11.50	10.39	3	dry	22/0 *(0)*	2/1 *(33.33)*	3/1 *(25.00)*	0.48	20.00
FC6	S.c. 18,000	50	1.42	7.18	4.05	3	dry	2/0 *(0)*	0/0 *(0)*	0/0 *(0)*	1.48	16.67
FC7	S.c. 18,000	20	1.55	8.80	6.08	3	dry	4/0 *(0)*	2/1 *(33.33)*	1/1 *(50.00)*	4.44	3.70
FC8	S.c. 18,000	10	2.97	9.75	7.47	2	dry	22/0 *(0)*	1/0 *(0)*	2/0 *(0)*	3.61	3.70
FC9	S.c. 18,000	30	2.37	9.25	6.72	3	dry	19/1 *(5.00)*	0/0 *(0)*	6/0 (*0)*	0.89	50.00
FC10	S.c. 18,000	10	1.98	8.27	5.37	2	dry	3/0 *(0)*	0/3 *(100)*	0/0 *(0)*	0.56	0.00
Mean		35.00	2.16	8.92	64.19	3	-	11.60±10.80/	1.00±1.16/	1.20±1.94/	2.25	**10.05**
±Stdev.		±24.61	±0.91	±1.56	±2.29			0.20±0.42	0.50±0.97	0.20±0.42	±1.76	**±15.69**
Total amount					64.19			116/2 *(1.70)*	10/5 *(33.33)*	12/2 *(14.29)*		

Table A.18: List of log segments (50 cm in length each) to which **1.8 million *S. carpocapsae*** IJs were applied in **Field Exposure Experiment II**. Log measurements and parameters are given for each log. P = porosity in %, W = weight of log in kg, Ø = mean diameter of log in cm (N = 6 measurements, three at each end), V = volume of log calculated based on length and mean diameter, D = wood decay category (1= none to 4 = complete), M = moisture content of wood. The absolute number of live and infected larvae and pupae is given, numbers in brackets in these columns refer to the proportion of infected larvae or pupae in each log in %. The density of *R. bifasciatum* individuals (larvae, pupae and adults combined) per log is given in the second to last column on the right. This was calculated by dividing the number of individuals collected from a log by the volume of the log. In the last column on the right the overall infection rate of *R. bifasciatum* individuals is given. In the bottom two rows, the mean values across all logs with standard deviation as well as the absolute numbers of live and infected *R. bifasciatum* larvae, pupae and adults are given (median given for decay category).

Log	*EPN species and concentration*	*P (%)*	*W (kg)*	*Ø (cm)*	*V (L)*	*D*	*M*	*Larvae live/inf. (% inf.)*	*Pupae live/inf. (% inf.)*	*Adults live/inf. (% inf.)*	*R. bifasciatum density (individuals per L)*	*Total Inf. %*
SCA1	*S.c. 1,800,000*	2	2.16	11	8.33	2	moist	9/20*(69.60)*	0/1 *(100)*	0/3 *(100)*	1.68	**72.73**
SCA2	*S.c. 1,800,000*	50	3.17	10.5	9.94	4	moist	2/8 *(80.00)*	1/0 *(0)*	0/4 *(100)*	1.51	**80.00**
SCA3	*S.c. 1,800,000*	20	6.03	15.3	20.02	3	moist	4/18 *(81.82)*	2/18 *(10.00)*	2/0 *(0)*	2.20	**81.82**
SCA4	*S.c. 1,800,000*	2	2.62	12	10.51	3	dry	4/8 *(66.67)*	1/0 *(0)*	1/1 *(50.00)*	1.43	**60.00**
SCA5	*S.c. 1,800,000*	5	2.15	8.2	5.85	2	dry	4/2 (33.33*)*	0/0 *(0)*	1/1 *(50.00)*	1.37	**37.50**
SCA6	*S.c. 1,800,000*	15	0.78	9.2	5.88	4	dry	0/1 *(100)*	0/0 *(0)*	0/0 *(0)*	0.17	**100**
SCA7	*S.c. 1,800,000*	10	3.4	11.3	10.69	2	dry	5/8 *(61.54)*	0/0 *(0)*	0/0 *(0)*	1.22	**61.54**
SCA8	*S.c. 1,800,000*	15	3.46	14.2	12.54	4	moist	1/16 *(94.12)*	0/0 *(0)*	2/2 *(50.00)*	1.67	**85.71**
SCA1	*S.c. 1,800,000*	5	1.62	8.7	6.08	3	wet	4/3 *(42.86)*	1/0 *(0)*	2/1 *(33.33)*	1.81	**36.36**
SCA2	*S.c. 1,800,000*	10	4.26	12.2	14.49	3	moist	9/1 *(10.00)*	0/0 *(0)*	2/0 *(0)*	0.83	**8.33**
Mean		13.40	2.97	11.26	10.43	3	-	4.20±2.97/	0.50±0.71/	2.70±2.06/	1.39	**62.40**
±Stdev.		±14.19	±1.47	±2.29	±4.45			8.50±7.18	1.90±5.67	2.80±1.87	±0.56	**±27.85**
Total amount					104.33			42/85 *(6.93)*	5/19 *(79.17)*	10/12 *(54.55)*		

Table A.19: List of log segments (50 cm in length each) to which **18,000 *S. carpocapsae*** IJs were applied in **Field Exposure Experiment II**. Log measurements and parameters are given for each log. P = porosity in %, W = weight of log in kg, Ø = mean diameter of log in cm (N = 6 measurements, three at each end), V = volume of log calculated based on length and mean diameter, D = wood decay category (1= none to 4 = complete), M = moisture content of wood. The absolute number of live and infected larvae and pupae is given, numbers in brackets in these columns refer to the proportion of infected larvae or pupae in each log in %. The density of *R. bifasciatum* individuals (larvae, pupae and adults combined) per log is given in the second to last column on the right. This was calculated by dividing the number of individuals collected from a log by the volume of the log. In the last column on the right the overall infection rate of *R. bifasciatum* individuals is given. In the bottom two rows, the mean values across all logs with standard deviation as well as the absolute numbers of live and infected *R. bifasciatum* larvae, pupae and adults are given (median given for decay category).

Log	*EPN species and concentration*	*P (%)*	*W (kg)*	*Ø (cm)*	*V (L)*	*D*	*M*	*Larvae live/inf. (% inf.)*	*Adults live/inf. (% inf.)*	*R. bifasciatum density (individuals per L)*	*Total Inf. %*
SCB1	S.c. 18,000	30	2.82	12.2	12.21	3	dry	3/0 *(0)*	1/0 *(0)*	0.33	0.00
SCB2	S.c. 18,000	15	1.58	10.8	8.28	4	moist	14/3 *(17.85)*	1/3 *(75.00)*	2.29	28.57
SCB3	S.c. 18,000	80	1.4	11.3	7.13	4	moist	0/0 *(0)*	4/0 *(0)*	0.56	0.00
SCB4	S.c. 18,000	5	4.41	13.5	16.36	3	moist	12/1 *(7.69)*	0/0 *(0)*	0.79	7.69
SCB5	S.c. 18,000	30	1.96	10	7.96	3	moist	1/0 *(0)*	0/0 *(0)*	0.13	0.00
SCB6	S.c. 18,000	40	3.41	18	16.67	3	dry	7/0 *(0)*	2/0 *(0)*	0.54	0.00
SCB7	S.c. 18,000	10	3.98	12.8	16.29	2	moist	11/1 *(8.33)*	0/0 *(0)*	0.74	8.33
SCB8	S.c. 18,000	30	3.46	11	12.70	3	dry	6/0 *(0)*	1/0 *(0)*	0.55	0.00
Mean		30.00	2.88	12.45	12.20	3	-	6.75±5.23/	1.14±1.36/	0.74	**5.57**
±Stdev.		±23.45	±1.13	±2.51	±4.02			0.63±1.06	0.38±1.06	±0.66	**±9.97**
Total amount					97.58			54/5 *(8.47)*	9/3 *(25.00)*		

Table A.20: List of log segments (50 cm in length each) to which **1.8 million *H. downesi*** IJs were applied in **Field Exposure Experiment II**. Log measurements and parameters are given for each log. P = porosity in %, W = weight of log in kg, Ø = mean diameter of log in cm (N = 6 measurements, three at each end), V = volume of log calculated based on length and mean diameter, D = wood decay category (1= none to 4 = complete), M = moisture content of wood. The absolute number of live and infected larvae and pupae is given, numbers in brackets in these columns refer to the proportion of infected larvae or pupae in each log in %. The density of *R. bifasciatum* individuals (larvae, pupae and adults combined) per log is given in the second to last column on the right. This was calculated by dividing the number of individuals collected from a log by the volume of the log. In the last column on the right the overall infection rate of *R. bifasciatum* individuals is given. In the bottom two rows, the mean values across all logs with standard deviation as well as the absolute numbers of live and infected *R. bifasciatum* larvae, pupae and adults are given (median given for decay category).

Log	*EPN species and concentration*	*P (%)*	*W (kg)*	*Ø (cm)*	*V (L)*	*D*	*M*	*Larvae live/inf. (% inf.)*	*Pupae live/inf. (% inf.)*	*Adults live/inf. (% inf.)*	*R. bifasciatum density (individuals per L)*	*Total Inf. %*
HDA1	*H.d. 1,800,000*	30	3.79	11.6	11.50	3	moist	5/6 *(54.55)*	0/0 *(0)*	3/0 *(0)*	1.22	**42.86**
HDA2	*H.d. 1,800,000*	20	10	10.4	7.42	4	moist	5/10 *(66.67)*	0/0 *(0)*	2/5 *(71.43)*	2.96	**68.18**
HDA3	*H.d. 1,800,000*	50	5.42	16.5	19.52	3	moist	2/7 *(77.78)*	0/0 *(0)*	0/5 *(100)*	0.72	**85.71**
HDA4	*H.d. 1,800,000*	90	3.64	12.9	13.24	2	dry	0/1 *(100)*	1/0 *(0)*	0/0 *(0)*	0.15	**50.00**
HDA5	*H.d. 1,800,000*	2	3.21	11.6	11.88	2	dry	2/5 *(71.40)*	0/0 *(0)*	0/5 *(100)*	1.01	**83.33**
HDA6	*H.d. 1,800,000*	15	1.36	8.6	5.54	4	dry	1/1 *(50.00)*	0/0 *(0)*	0/1 *(100)*	0.54	**66.67**
HDA7	*H.d. 1,800,000*	40	3.97	11.2	13.04	3	dry	3/8 *(72.73)*	0/0 *(0)*	1/1 *(50.00)*	1.00	**69.23**
HDA8	*H.d. 1,800,000*	40	3.46	12.6	14.21	3	dry	0/0 *(0)*	0/0 *(0)*	0/0 *(0)*	0.00	**0.00**
HDA9	*H.d. 1,800,000*	20	2.73	11.1	10.15	4	moist	4/14 *(77.78)*	0/1 *(100)*	3/0 (*0)*	2.17	**68.18**
HDA10	*H.d. 1,800,000*	20	4.35	11.8	13.44	3	moist	0/0 *(0)*	0/0 *(0)*	0/0 *(0)*	0.00	**0.00**
Mean		52.70	4.19	11.83	11.99	3	-	2.20±1.99/	0.1±0.32/	0.90±1.29/	0.98	**53.42**
±Stdev.		±24.58	±2.30	±2.03	±3.84			5.20±4.73	0.1±0.32	1.70±2.31	±0.96	**±30.97**
Total amount					119.94			22/52 *(70.27)*	1/1 *(50.00)*	9/17 *(65.38)*		

Table A.21: List of log segments (50 cm in length each) to which **18,000 *H. downesi*** IJs were applied in **Field Exposure Experiment II**. Log measurements and parameters are given for each log. P = porosity in %, W = weight of log in kg, Ø = mean diameter of log in cm (N = 6 measurements, three at each end), V = volume of log calculated based on length and mean diameter, D = wood decay category (1= none to 4 = complete), M = moisture content of wood. The absolute number of live and infected larvae and pupae is given, numbers in brackets in these columns refer to the proportion of infected larvae or pupae in each log in %. The density of *R. bifasciatum* individuals (larvae, pupae and adults combined) per log is given in the second to last column on the right. This was calculated by dividing the number of individuals collected from a log by the volume of the log. In the last column on the right the overall infection rate of *R. bifasciatum* individuals is given. In the bottom two rows, the mean values across all logs with standard deviation as well as the absolute numbers of live and infected *R. bifasciatum* larvae, pupae and adults are given (median given for decay category).

Log No.	*EPN species and concentration*	*P (%)*	*W (kg)*	*Ø (cm)*	*V (L)*	*D*	*M*	*Larvae live/inf. (% inf.)*	*Adults live/inf. (% inf.)*	*R. bifasciatum density (individuals per L)*	*Total Inf. %*
HDB1	*H.d. 18,000*	2	4.42	13.5	15.80	2	moist	3/0 *(0)*	2/0 *(0)*	0.63	**0**
HDB2	*H.d. 18,000*	20	1.21	8.2	5.90	3	dry	1/0 *(0)*	2/0 *(0)*	0.51	**0**
HDB3	*H.d. 18,000*	20	4.13	12.8	14.56	2	dry	3/0 *(0)*	0/0 *(0)*	0.21	**0**
HDB4	*H.d. 18,000*	15	1.86	9.3	11.03	4	dry	5/0 *(0)*	2/0 *(0)*	0.45	**0**
HDB5	*H.d. 18,000*	10	1.88	10.7	8.88	4	moist	0/0 *(0)*	2/0 *(0)*	1.01	**0**
HDB6	*H.d. 18,000*	20	3.73	16.4	19.77	3	moist	9/0 *(0)*	4/0 *(0)*	0.00	**0**
HDB7	*H.d. 18,000*	5	2.94	14.5	14.46	2	dry	2/0 *(0)*	0/0 *(0)*	0.69	**0**
HDB8	*H.d. 18,000*	10	1.85	9.3	6.72	3	dry	0/0 *(0)*	1/0 *(0)*	0.45	**0**
Mean		12.75	2.75	11.84	12.14	3	-	3.88±3.23/	1.50±1.31/	0.49	**0**
±Stdev.		±17.11	±1.22	±2.90	±4.83			0±0	0±0	±0.31	**±0**
Total amount					97.12			31/0 *(0)*	12/0 *(0)*		

A.9 Linear regression analysis results for *R. bifasciatum* density regressed against the infection rate per log segment.

Table A.22: Linear regression analysis results for *R. bifasciatum* density (x) regressed against the arcsin of the infection rate (y) per log segment. Logs containing no *R. bifasciatum* excluded.

Log segment set	**N**	**Equation**	**T**	**R^2**	**DF**	**F**	**P**
Field exposure experiment I (high concentration)	10	y = 0.857- 0.0259x	- 0.29	0.01	1	0.08	**0.781**
Field exposure experiment I (medium concentration)	10	y = 0.674 - 0.399x	1.16	0.14	1	1.35	**0.279**
Laboratory exposure experiment II (*H. downesi*, low concentration)	15	y = 0.658 + 0.080x	0.47	0.02	1	0.22	**0.645**
Field exposure experiment II (high concentration, *S. carpocapsae*)	10	y = 0.925 - 0.134x	- 0.93	0.10	1	0.86	**0.380**
Field exposure experiment II (high concentration, *H. downesi*)	8	y = 0.700 - 0.083x	0.36	0.02	1	0.13	**0.734**
Laboratory exposure experiment III (*S. carpocapsae)*	6	y = 0.420 + 0.047x	- 0.22	0.01	1	0.05	**0.835**
Laboratory exposure experiment III (*H. downesi)*	7	y = 0.256 + 0.037x	0.21	0.01	1	0.04	**0.843**

A.10 Scratching platform design for *B. hylobii* scratching stimulus oviposition trials

Arenas were similar to standard arenas (see 6.2.2) in dimension and design. However, the base was glued to the lid of a Petri dish that in turn was glued to a wooden platform. Underneath the platform ran an eccentric driveshaft that was driven at 22 rpm by an electric motor (Geared motor, 11 rpm, 24V DC, Crouzet, Basingstoke, England). The driveshaft in turn moved a series of ten articulated wire arms. Galvanised steel wire was used (1 mm diameter) (B&Q, Eastliegh, England). The wire arms ran through a hole in the platform underneath each arena and into the host chamber within it (slits had been melted through the Petri dish lid and arena base that were glued over the hole to allow the wire to pass through and move freely along an axis at a right angle to the driveshaft (see Fig A.10). Perspex slides as described in 6.2.2 but lacking the glass slide were stuck directly to the Petri dish base of each arena with plasticine (BluTack™, Barker Inc, Fort Wayne, USA) in such a way that the tip of a wire arm protruded into one of the two host chambers (Fig A.10). Ten arenas were arranged along the length of the platform (the platform was 117 cm in length and arenas were spaced approximately 1 cm apart long its length). The bark patch in ten arenas arranged along the wood platform could thus be scratched from below simultaneously.

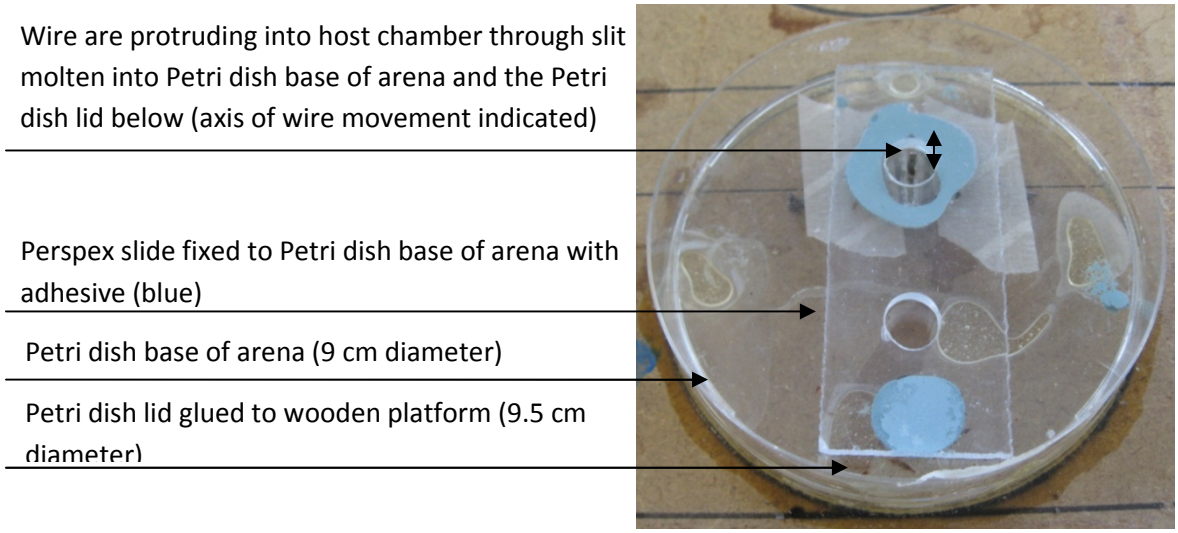

Fig A.10: View from above of arena glued to wooden platform that had a wire arm in one chamber for artificial automated scratching of the bark from below. Arrows beside wire tip in host chamber indicate movement of wire.

The wire arms were clipped to a length causing them to protrude only slightly (approximately 1 mm) over the lip if the chamber, thus creating a scratching path of two to three mm in amplitude on the underside of a bark patch taped over this chamber. To increase contact between wire and bark throughout the experiment, a stainless steel washer (inner diameter: 1.3 cm, outer diameter 2.4 cm, 2 mm thick) was taped down over the bark, leaving a bark window of 1.3 cm in its centre directly over the host chamber (see Plate A.1). There was enough space in each of

these chambers with wire arms to also accommodate a pine weevil host larva. Hosts were placed into the chambers in such a way that they would not be squashed or squeezed overly by the movement of the wire arm. Arena size was decreased in this series of trials to increase the likelihood of wasps coming in contact with bark. To this end, arena enclosures were created by placing the base of a 3.5 cm Petri dish over each metal washer upside down. The base was fixed in place with adhesive and a hole was melted in its top to allow introduction of the wasp. This hole was sealed with a piece of adhesive once a wasp was inside (see Plate A.1). A small piece of filter paper (Whatman No 1) soaked in 50:50 honey/tap water solution was placed inside the 3.5 cm Petri dish base as a food supply for wasps.

To reduce moisture loss, an inverted 50 ml plastic tub (8.5 cm diameter; 'Econo' by Hutamaki) was placed over each arena when running an experiment and a moistened piece of tissue paper was placed beside the arena (this was also covered by the plastic tub). New 3.5 cm Petri dish bases were used for each experiment and the bases including the wires inside the chambers were washed with 70 % ethanol to remove any scent from the previous experiment. A space for a control arena (no scratching) was marked out beside each 'scratching' arena (Plate A.1). A schematic view of the platform setup with all essential measurements and parts is shown in Fig A.11 and photographs of the platform and arenas are included as well.

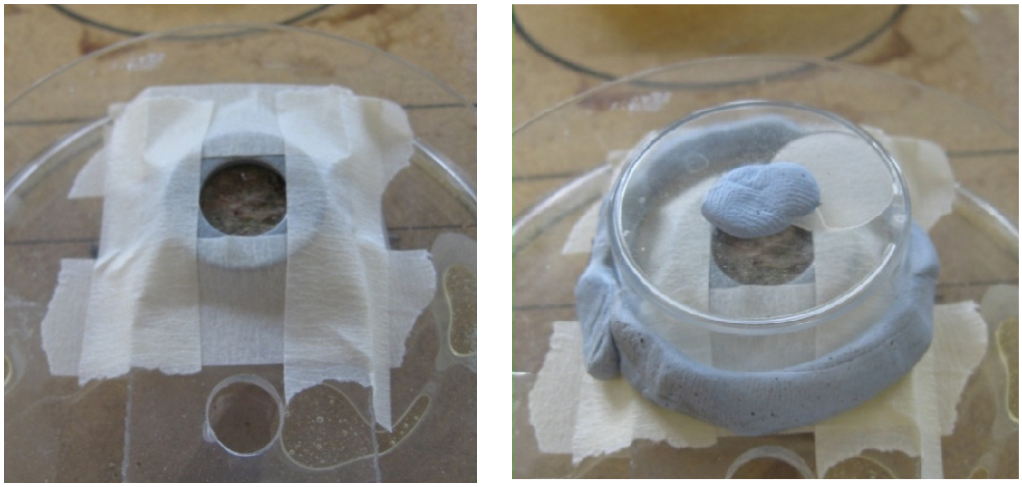

Plate A.1: View from above of bark patch with stainless steel washer secured in place over host chamber with wire arm for scratching (left) and finished scratching arena with 3.5 cm Petri dish base placed over bark and sealed hole for wasp introduction (right).

A custom built power supply (24 V, DC) (created in-house at NUI Maynooth) drove the motor and was in turn plugged into the mains power supply. A timer was placed in between the custom power supply and the mains. It was set to 15 minutes of alternating on and off for the first 12 h after the experiment began and 30 minute alternating on and off for the remaining 12 h. This not only protected the motor from overheating, but was also intended to simulate an intermittently moving host as well as allow wasps a window without scratching to oviposit (thus simulating a paralyzed host).

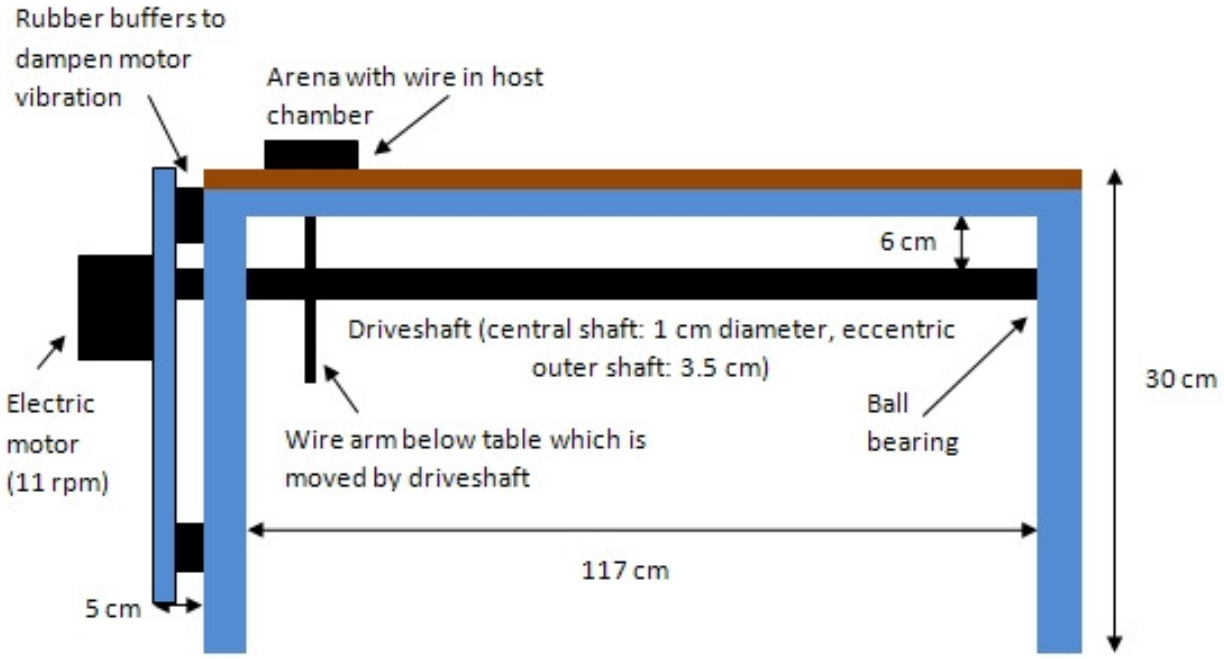

Fig A.11: Schematic side view of overall scratching platform setup and measurements. The driveshaft was fitted into a ball bearing at the end opposite the motor to minimize stress on the motor. The driveshaft consisted of a central shaft (plexiglass) running through a length of plastic tubing, creating an eccentric driveshaft that would move the wire arms touching it back and forth (see Fig A.12 through A.14). Blue components were made of stainless steel and welded or screwed together, brown components were made of wood.

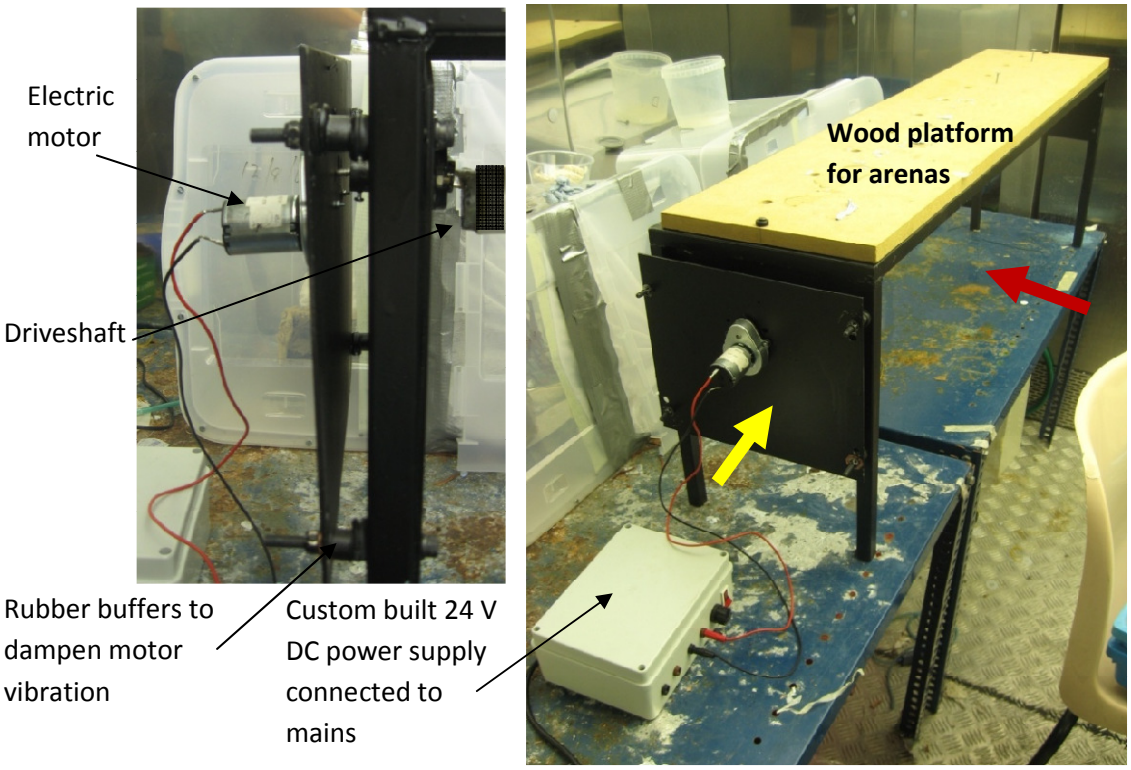

Fig A.12: Photographs of motor mounted on wire frame for scratching platform (left) and finished platform. Red arrow shows angle of view for Fig A.11 and yellow arrow shows angle of view for Fig A.12.

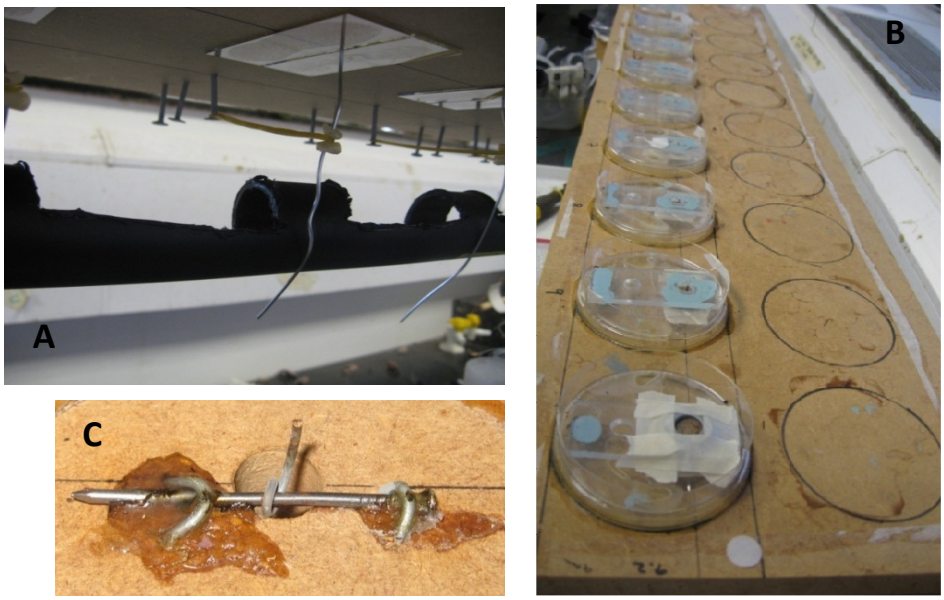

Fig A.13: (A) Scratching wires as they were positioned in relation to the driveshaft below the platform, with rubber bands holding them in place. Sections of the outer driveshaft were cut away to reduce stress on the electric motor. Glass slides glued to the underside of the platform helped guide the wire in a straight path. The latter two features were not essential design components. **(B)** Scratching arenas arranged along scratching platform with black circles indicating space for no-scratch control arenas. **(C)** Wire arm wrapped around a nail glued across the hole in the wood through which it ran, allowing for wire articulation. The glass slides glued to the underside of the platform in **(A)** helped guide the wire in a straight path but were not an essential design component.

A.11 Poking stimulus platform design for *B. hylobii* poking stimulus oviposition trials

In the summer of 2009, experiments with a simplified setup which allowed for the poking (rather than scratching) of bark from below were conducted. Standard arenas with a small hole (3 mm diameter) melted in their base to allow a wire access to the arena from below were used. A perspex slide was taped directly inside the base of the Petri with one of its holes placed over the hole molten for wire access. The arenas were then placed on a wooden platform (30 cm by 20 cm) with the hole in the base of each arena aligned with a hole drilled through the platform (3 mm diameter). A stainless steel washer (inner diameter: 2 mm, outer diameter: 2.5 cm) was taped to the underside of the platform to circle each of the holes drilled through it. The arenas were fastened to the platform with three strips of masking tape each. The wooden platform with the arenas was then placed level on a metal stand 20 cm high and attached to it with masking tape. A PS 3D rotating plate (Grant-bio; Shepreth, England) was placed under the wooden platform. A piece of Styrofoam (approximately 15 cm by 15 cm, 5 cm thick) was fastened on top of the rotating plate with masking tape. Six lengths of gardening wire (1 mm diameter) were stuck into the Styrofoam layer in a position roughly below each of the holes in the wooden platform. Each length of wire was approximately 10 cm long and bent so as to allow wires to flex in response to pressure from the rotating platform

(this was done to reduce the risk of wires puncturing the bark. The end of each wire was threaded through the corresponding hole in the wooden platform so that its tip entered the host chamber (Fig A.14). Finally, each wire was twisted one at its top end (the one in the host chamber) to create a stopper that would prevent the wire from slipping out through the hole in the metal washer taped on the underside of the wooden platform. Each wire was allowed to protrude only slightly (approximately 1 mm) above the lip of each chamber when at its highest point. The host was positioned in the chamber so as to allow free movement of the wire. After the bark patch had been taped over the chamber, it was confirmed that the wire was in fact poking the bark. This was done by touching the bark with a fingertip and by looking at the bark, since the poking created a slight, but visually perceptible 'bulging' of the bark patch. The rotating plate was set to 10 rotations per minute for all experiments and a timer switched on and off the mains power at 15 minute alternating intervals for the first 12 h after the experiment began and 30 minute intervals for the remaining 12 h. This was intended to stimulated an intermittently moving host more realistically as well as allow wasps a window without poking to oviposit (thus simulating a paralyzed host).

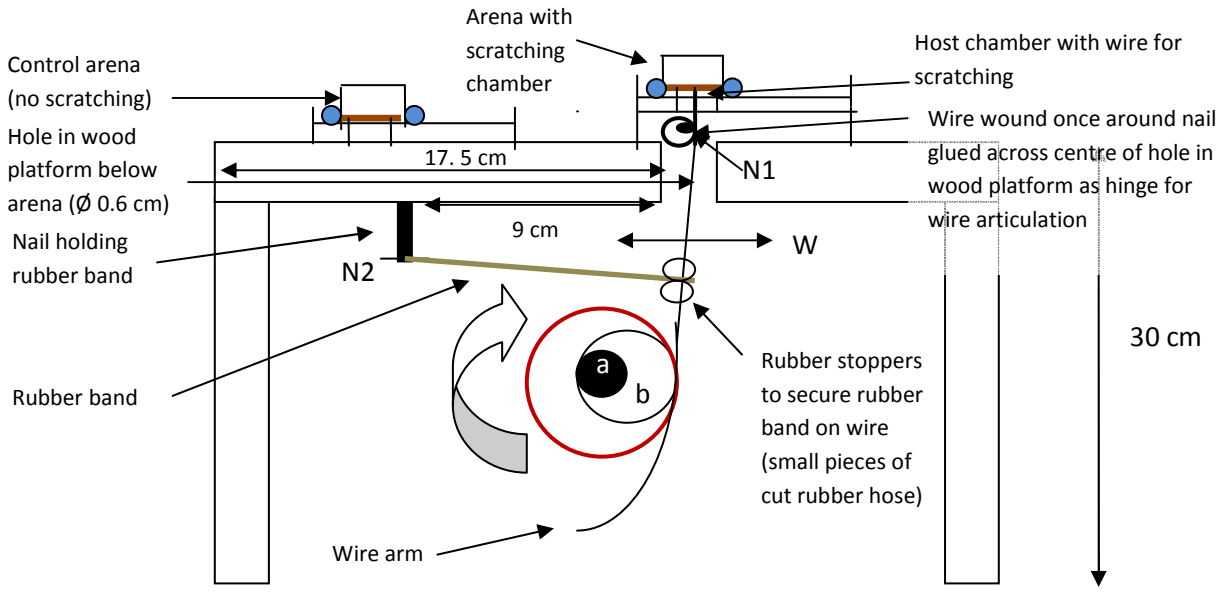

Fig A.13: Schematic side view (for angle see Fig A.11) of overall scratching platform setup and measurements. The central driveshaft (a) was attached to the motor and made of plexiglass (1 cm diameter). The outer driveshaft (b) was attached to the central driveshaft and was 3.5 cm in diameter (plastic tubing). Its eccentric rotation resulted in the rotational diameter indicated by the red circle (5 cm in diameter). Wire arms were wound once around a nail that was glued across the holes drilled into the platform through which the wires ran (N, see Fig A.12). This allowed wires to articulate. Wires were pulled back and kept in contact with driveshaft by a rubber band running from a nail driven into the underside of the platform (N2) to the wire arm, where it was secured in place by two rubber stoppers (pieces of cut rubber hosing pushed onto wire). The wire was thus moved back and forth by the motion of the driveshaft as it turned (W) and scratched the bark (brown) in the scratching arenas on the platform. Blue = adhesive with which arena covers (3.5 cm Petri dish bases) were held in place. Wires were bent and positioned on the driveshaft individually and in such a way that wire arms in scratching chambers had a movement amplitude of approximately 2 mm.

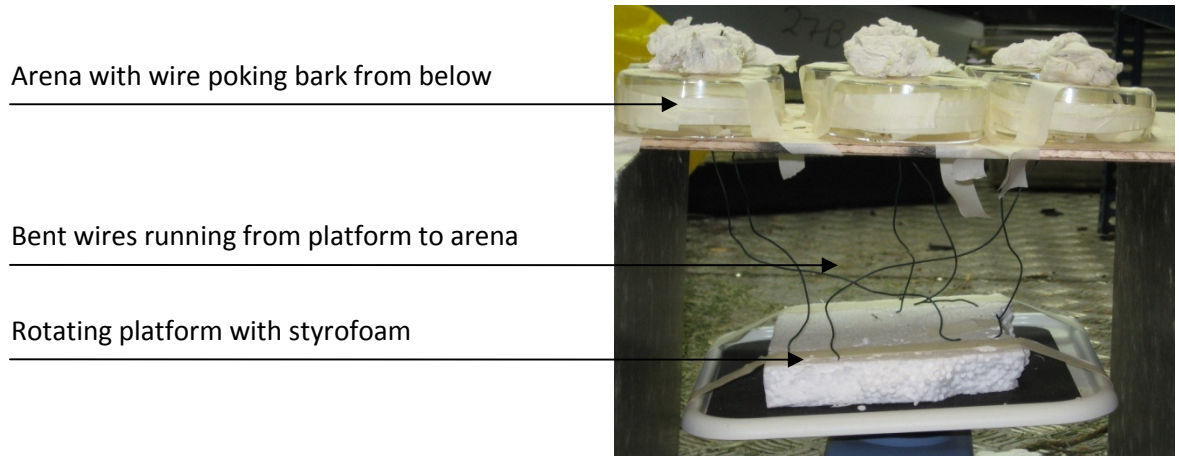

Fig A.14: Photograph of the experimental setup used to create an artificial vibrational stimulus by poking the bark from below with wires.

A.12 Host weight in oviposition trials

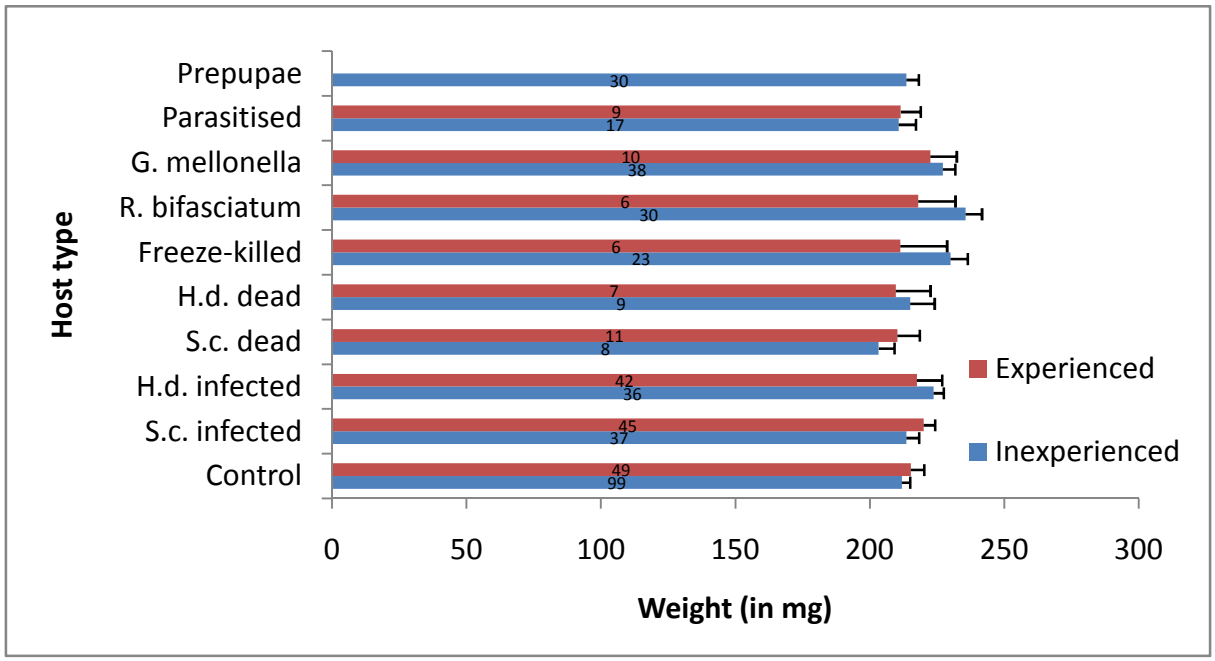

Fig A.15: Mean host weight for each of the host types used in trials with *B. hylobii*. Error bars represent standard error. Numbers inside bars give N.

A.13 Occurence of chewed bark

Table A.23: Statistical cross-comparison of number of trials with naïve wasps in which bark was chewed on by *H. abietis* hosts depending on host condition at the end of the trial (live or dead due to infection) (χ^2 – test or Fisher's exact test, 10 cross-comparisons, Bonferroni-adjusted significance level of $\alpha = 0.005$). DF = 1 for all comparisons, for N see Fig 6.11.

	S.c. dead after 24h	*S.c.* live after 24h	*H.d.* dead after 24h	*H.d.* live after 24h
Control	χ2 = 22.873, **P < 0.001**	χ2 = 0.041, **P = 0.840**	χ2 = 43.600, **P < 0.001**	χ2 = 0.617, **P = 0.432**
S.c. dead after 24h		χ2 = 13.200, **P < 0.001**	**P = 0.416**	χ2 = 8.869, **P = 0.003**
S.c. live after 24h			χ2 = 23.704, **P < 0.001**	χ2 = 0.537, **P = 0.464**
H.d. dead after 24h				χ2 = 17.698, **P < 0.001**

Table A.24: Statistical cross-comparison of number of trials with experienced wasps in which bark was chewed on by *H. abietis* hosts depending on host condition at the end of the trial (live or dead due to infection) (χ^2 – test or Fisher's exact test, 10 cross-comparisons, Bonferroni-adjusted significance level of $\alpha = 0.005$). DF = 1 for all comparisons, for N see Fig 6.12.

	S.c. dead after 24h	*S.c.* live after 24h	*H.d.* dead after 24h	*H.d.* live after 24h
Control	χ2 = 4.158, **P = 0.041**	χ2 = 15.835, **P < 0.001**	χ2 = 11.929, **P = 0.001**	χ2 = 0.152, **P = 0.696**
S.c. dead after 24h		**P = 0.281**	**P = 0.418**	χ2 = 1.990, **P = 0.158**
S.c. live after 24h			**P = 1**	χ2 = 8.174, **P = 0.004**
H.d. dead after 24h				χ2 = 6.709, **P = 0.010**

A.14 Pairwise comparison of duraryion of behaviour bouts

Table A.25: Results of pairwise comparison of the duration of behavioural bouts in response to host movement for each of the three behaviours distinguished when wasps were on the bark patch with respect to the location.

Wasp location and behaviour	**Searching close**	**Pausing close**	**Searching away**	**Pausing away**	**Probing away**
Mann-Whitney U-test result	W = 207.0 **P = 0.131**	W = 254.0 **P = 0.267**	W = 450.5 **P = 0.250**	W = 446.0 **P = 0.279**	W = 92.0 **P = 0.085**

A.15 Continuous observation trials: Pairwise comparisons between duration of searching behaviour and pausing behaviour

Table A.26: Statistical comparison of the duration of wasp behaviours with respect to host movement when the wasp was close to or away from the host

Wasp location and host behaviour	**Close, host moving**	**Close, host not moving**	**Away, host moving**	**Away, host not moving**
Mann-Whitney U-test result	W = 121.0 **P < 0.001**	W = 162.0 **P < 0.001**	W = 193.0 **P < 0.001**	W = 602.0 **P < 0.001**

A.16 Statistical comparison for wasp oviposition after first probing bout in relation to host movement and abortion of searching and probing bouts in relation to host movement

Table A.27: Results of statistical tests comparing data sets for which means values and absolute numbers are presented in Tables 6.18 and 6.19. Tests used were Fisher's exact test or X^2 – test (oviposition) and X^2 – Goodness-of-Fit tests against the mean proportion of records of host movement (41% for naÏve and experienced wasp controls, 39 % for total naÏve wasps and 42 % for total experienced wasps).

Host type (*H.abietis*)	Oviposition after first probing bout (+ vs. -)	Abortion of searching bout	Abortion of probing bout
NaÏve Control	P = 0.061	X^2 = 5.372, **P = 0.020**	N/A
Exp. Control	P = 0.004	X^2 = 2.937, **P = 0.087**	N/A
NaÏve Total	P = 0.010	X^2 = 10.326, **P < 0.001**	X^2 = 3.526, **P = 0.060**
Exp.Total	X^2 = 16.429, P < 0.001	X^2 = 9.492, **P = 0.002**	X^2 = 3.936, **P = 0.047**

A.17 Continuous observation trials: Wasps behaviour close and away from hosts in relation to host movement or scratching

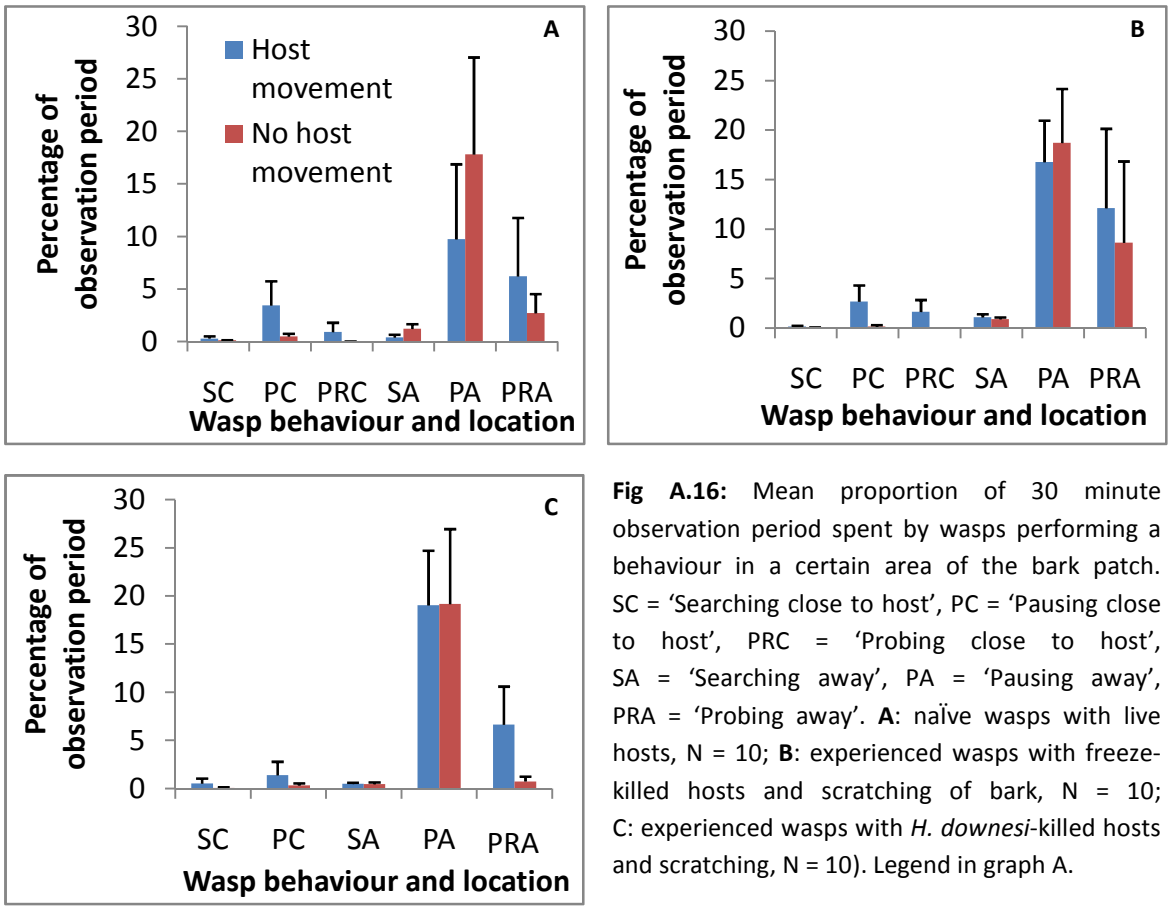

Fig A.16: Mean proportion of 30 minute observation period spent by wasps performing a behaviour in a certain area of the bark patch. SC = 'Searching close to host', PC = 'Pausing close to host', PRC = 'Probing close to host', SA = 'Searching away', PA = 'Pausing away', PRA = 'Probing away'. **A**: naïve wasps with live hosts, N = 10; **B**: experienced wasps with freeze-killed hosts and scratching of bark, N = 10; C: experienced wasps with *H. downesi*-killed hosts and scratching, N = 10). Legend in graph A.

A.18 Continuous observation trials: Behavioural transitions of wasps

Tables A.28 through A.31 give the number of times wasps transitioned between the behaviours that were distinguished (searching, pausing and probing) for each of the four trial groups (i.e. trials with experienced wasps and live *H.abietis*, experienced wasps and freeze-killed *H.abietis*, experienced wasps with EPN-killed *H.abietis* and as well as naïve wasps with live *H.abietis.*

Table A.28: Number of transitions of **experienced wasps** in continuous observation trials with **live hosts**. SC = 'Searching close to host', PC = 'Pausing close to host', PRC = 'Probing close to host', SA = 'Searching away', PA = 'Pausing away', PRA = 'Probing away'. + = host is moving, - = host is not moving. Each number in a cell gives the number of times a wasp transitioned from a behaviour/location combination with relation to host behaviour in the 'From' column to a behaviour/location combination with relation to host behaviour in the 'To' column. N = 39 trials.

From: \ To:		Other	SC +	SC -	PC +	PC -	PRC +	PRC -	SA +	SA -	PA +	PA -	PRA +	PRA -
Other			*0*	*1*	*0*	*0*	*0*	*0*	*0*	*7*	*63*	*4*	*5*	*0*
SC	**+**	*0*	*0*	*0*	*121*	*0*	*8*	*0*	*2*	*0*	*0*	*0*	*0*	*0*
SC	**-**	*0*	*0*	*0*	*0*	*36*	*0*	*1*	*0*	*8*	*0*	*1*	*0*	*1*
PC	**+**	*0*	*102*	*0*	*0*	*0*	*137*	*1*	*1*	*1*	*0*	*0*	*1*	*0*
PC	**-**	*0*	*0*	*20*	*0*	*0*	*4*	*3*	*0*	*17*	*0*	*0*	*0*	*0*
PRC	**+**	*0*	*12*	*1*	*123*	*5*	*0*	*0*	*0*	*0*	*0*	*1*	*0*	*0*
PRC	**-**	*0*	*0*	*2*	*0*	*2*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
SA	**+**	*0*	*2*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*151*	*2*	*4*	*0*
SA	**-**	*40*	*0*	*9*	*0*	*2*	*0*	*0*	*0*	*0*	*2*	*488*	*0*	*39*
PA	**+**	*3*	*13*	*1*	*0*	*0*	*1*	*0*	*130*	*11*	*0*	*0*	*17*	*2*
PA	**-**	*25*	*1*	*14*	*0*	*0*	*2*	*0*	*18*	*417*	*0*	*0*	*2*	*53*
PRA	**+**	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*0*	*20*	*2*	*0*	*0*
PRA	**-**	*3*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*60*	*2*	*28*	*0*	*0*

Table A.29: Number of transitions of **experienced wasps** in continuous observation trials with **freeze-killed hosts and scratching added after 15 minutes**. For legend see Table A.27.

From: \ To:		Other	SC +	SC -	PC +	PC -	PRC +	PRC -	SA +	SA -	PA +	PA -	PRA +	PRA -
Other			*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*11*	*0*	*3*	*0*
SC	**+**	*0*	*0*	*0*	*24*	*0*	*6*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
SC	**-**	*0*	*0*	*0*	*0*	*4*	*0*	*1*	*0*	*3*	*0*	*1*	*0*	*0*
PC	**+**	*0*	*23*	*1*	*0*	*0*	*2*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
PC	**-**	*1*	*1*	*2*	*0*	*0*	*0*	*0*	*0*	*2*	*0*	*0*	*0*	*0*
PRC	**+**	*0*	*4*	*0*	*4*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
PRC	**-**	*0*	*0*	*0*	*0*	*1*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
SA	**+**	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*27*	*1*	*2*	*0*
SA	**-**	*5*	*0*	*4*	*0*	*1*	*0*	*0*	*0*	*0*	*0*	*66*	*0*	*9*
PA	**+**	*1*	*1*	*0*	*1*	*0*	*0*	*0*	*24*	*2*	*0*	*0*	*4*	*0*
PA	**-**	*5*	*0*	*2*	*0*	*0*	*0*	*0*	*3*	*57*	*0*	*0*	*1*	*6*
PRA	**+**	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*2*	*0*	*4*	*0*	*0*	*0*
PRA	**-**	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*10*	*1*	*4*	*0*	*0*

Table A.28: Number of transitions of **naïve wasps** in continuous observation trials with **live hosts**. For legend see Table A.27.

To: / From:		Other	SC +	SC -	PC +	PC -	PRC +	PRC -	SA +	SA -	PA +	PA -	PRA +	PRA -
Other			*0*	*0*	*0*	*0*	*0*	*0*	*0*	*6*	*13*	*0*	*0*	*0*
SC	**+**	*0*	*0*	*0*	*12*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
SC	**-**	*0*	*0*	*0*	*0*	*3*	*0*	*0*	*0*	*3*	*0*	*0*	*0*	*0*
PC	**+**	*0*	*8*	*0*	*0*	*0*	*26*	*0*	*1*	*0*	*0*	*0*	*0*	*0*
PC	**-**	*0*	*0*	*2*	*0*	*0*	*0*	*0*	*0*	*1*	*0*	*0*	*0*	*0*
PRC	**+**	*0*	*1*	*0*	*24*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
PRC	**-**	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
SA	**+**	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*99*	*1*	*0*	*0*
SA	**-**	*11*	*0*	*2*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*84*	*0*	*4*
PA	**+**	*2*	*2*	*0*	*0*	*0*	*1*	*0*	*89*	*0*	*0*	*0*	*46*	*0*
PA	**-**	*2*	*0*	*2*	*0*	*0*	*0*	*0*	*4*	*76*	*0*	*0*	*1*	*12*
PRA	**+**	*1*	*1*	*0*	*0*	*0*	*0*	*0*	*1*	*0*	*40*	*0*	*0*	*0*
PRA	**-**	*1*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*9*	*1*	*5*	*0*	*0*

Table A.30: Number of transitions of **experienced wasps** in continuous observation trials with ***H. downesi* killed hosts and scratching added after 15 minutes**. For legend see Table A.27.

To: / From:		Other	SC +	SC -	PC +	PC -	PRC +	PRC -	SA +	SA -	PA +	PA -	PRA +	PRA -
Other			*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*8*	*0*	*2*	*0*
SC	**+**	*0*	*0*	*0*	*8*	*0*	*0*	*0*	*0*	*0*	*1*	*0*	*0*	*0*
SC	**-**	*0*	*0*	*0*	*0*	*3*	*0*	*0*	*0*	*0*	*0*	*2*	*0*	*0*
PC	**+**	*0*	*6*	*0*	*0*	*0*	*0*	*0*	*1*	*0*	*0*	*0*	*0*	*0*
PC	**-**	*0*	*0*	*1*	*0*	*0*	*0*	*0*	*0*	*2*	*0*	*0*	*0*	*0*
PRC	**+**	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
PRC	**-**	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
SA	**+**	*0*	*1*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*61*	*0*	*1*	*0*
SA	**-**	*4*	*0*	*1*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*43*	*0*	*2*
PA	**+**	*1*	*2*	*0*	*0*	*0*	*0*	*0*	*53*	*0*	*2*	*0*	*34*	*0*
PA	**+**	*3*	*0*	*3*	*0*	*0*	*0*	*0*	*5*	*38*	*0*	*0*	*0*	*4*
PRA	**M**	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*3*	*0*	*31*	*0*	*0*	*0*
PRA	**X**	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*2*	*0*	*4*	*0*	*0*

A.19 Statistical pairwise comparison of length and depth in wood of live and infected *R. bifasciatum* larvae in Laboratory and Field Exposure Experiments

Variable tested	EPN species	Treatment	Statistical test	Result
Length	***S. carpocapsae***	LEE I high	**T-test**	**T = - 0.78, DF = 8, P = 0.458**
		LEE III low	**M.-W. U-test**	**W = 929.0, P = 0.291**
		FEE I high	**M.-W. U-test**	**W = 958.0, P = 0.396**
		FEE II high	**M.-W. U-test**	**W = 1503.0, P = 0.529**
	H. downesi	LEE I high	**M.-W. U-test**	**W = 1163.0, P = 0.023**
		LEE III low	**T-test**	**T = 1.20, DF = 23, P = 0.243**
		FEE I high	**T-test**	**T = - 0.42, DF = 26, P = 0.679**
Depth in wood	***S. carpocapsae***	LEE I high	**T-test**	**T = 0.60, DF = 7, P = 0.569**
		LEE III low	**M.-W. U-test**	**W = 949.5, P = 0.788**
		FEE I high	**M.-W. U-test**	**W = 3679.0, P = 0.143**
		FEE II high	**T-test**	**T = 1.07, DF = 51, P = 0.290**
	H. downesi	LEE I high	**T-test**	**T = - 0.19, DF = 15, P = 0.855**
		LEE III low	**M.-W. U-test**	**W = 1807.0, P = 0.873**
		FEE I high	**M.-W. U-test**	**W = 648.5, P = 0.659**
		FEE II high	**T-test**	**T = 1.86, DF = 48, P = 0.569**