Management of adult and immature large pine weevil (*Hylobius abietis* [L.]) using novel local-provenance entomopathogenic fungi and commercial entomopathogenic nematodes.

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DECLARATION

All the work referred to in this thesis has not been submitted in support of any application for another degree or qualification of this or any other university or other institute of learning.

ABSTRACT

The large pine weevil (*Hylobius abietis*) is one of the most economically significant pests in European and Asian coniferous forests, particularly in restocking areas where clear-felling creates optimal breeding conditions. Current control strategies heavily rely on synthetic insecticides, which, despite their efficacy, raise environmental concerns and face increasing regulatory restrictions. As a result, there is a growing interest in biological control agents (BCAs) such as entomopathogenic fungi (EPFs) and entomopathogenic nematodes (EPNs) as sustainable alternatives.

This thesis investigates the potential of native EPFs and their interactions with commercial EPNs to improve *H. abietis* management. A large-scale bioprospecting study was conducted to isolate novel EPF strains from different habitats, using a dual baiting technique to maximize diversity. The most promising isolates were tested under controlled laboratory conditions to assess their virulence, infection effects, and impact on *H. abietis* feeding behaviour. *Beauveria bassiana* 35G emerged as a particularly effective strain, significantly reducing weevil feeding activity and inducing high mortality rates. Subsequent field trials evaluated its efficacy in standalone applications and in combination with *Steinernema carpocapsae*, demonstrating additive effects when applied at reduced operational doses. Additionally, an assessment of non-target impacts showed no significant effects on overall invertebrate abundance or taxon richness, supporting the environmental safety of the tested BCAs.

Efforts to target adult weevils proved challenging, as they exhibited high resilience to fungal infection, and EPF delivery method was not sufficiently effective. However, billets successfully attracted emerging adults, suggesting potential for future refinements in attract-and-infect strategies. While field experiments confirmed the potential of EPF-EPN combinations to suppress *H. abietis*, site-specific variations in efficacy highlighted the complexity of multi-agent biocontrol strategies. Practical constraints on EPN application, such as geographical limitations and variable site conditions, indicate that biological control may not be universally applicable across all restocking sites.

Future research should focus on refining formulation technologies, such as encapsulation and gel-based carriers, to enhance persistence and efficacy under diverse forestry conditions. Long-term ecological studies are also needed to assess the sustainability of EPF applications and their interactions with native soil microbial communities.

These findings provide a foundation for integrating EPFs and EPNs into sustainable forestry pest management programs, offering a viable pathway to reducing reliance on synthetic insecticides. While further optimization and large-scale validation are necessary, the results of this research reinforce the potential of biological control as a key component in the future of environmentally responsible forest pest management.

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CHAPTER 1:
GENERAL INTRODUCTION

The problem with the forest pest *Hylobius abietis*.

Hylobius abietis (L.) (Coleoptera: Curculionidae), commonly known as the large pine weevil (LPW), is a major forest pest of coniferous plantations, posing a substantial threat to restocking efforts in northern Europe and Asia, where it is native and widespread (Leather *et al.*, 1999). This weevil is highly attracted to freshly cut conifer stumps that release volatiles such as alpha-pinene, signalling the availability of wood material suitable for oviposition (Skrzecz and Moore, 1997, Moore, 2001, Inward *et al.*, 2012). In natural woodlands, the scarcity of fallen tree stumps naturally limits *H. abietis* populations. However, over the last century, the establishment of large-scale coniferous plantations, predominantly managed by clear-felling of vast areas, has significantly increased *H. abietis* populations, negatively impacting forest regeneration (Munro, 1927, Evans, 1997, Inward *et al.*, 2012). The clear-felling method provides hundreds or even thousands of stumps, leading to a sharp increase in *H. abietis* populations and damage to newly planted trees (Leather *et al.*, 1999, Inward *et al.*, 2012).

Newly emerged adults primarily feed on the bark of young conifer saplings, often girdling them and leading to their death (Willoughby *et al.*, 2017). A single adult *H. abietis* can kill or severely damage multiple saplings up to five years old, which is when young trees begin to withstand debarking damage. Consequently, even small number of adults can cause substantial economic losses in replanted sites (Wainhouse *et al.*, 2004, Dillon *et al.*, 2006). Without protective measures, restocking sapling loses can reach 90% to 100% of new plantations, with an average loss of 50% reported in the UK and Ireland during first few years post-planting (Heritage and Moore, 2001, Kapranas *et al.*, 2016, Fedderwitz *et al.*, 2022). In Europe, traditional pest control and protective measures on susceptible trees usually rely on application of potentially harmful insecticides either before or after planting (Långström and Day, 2007, Wainhouse *et al*., 2007).

The overpopulation of *H. abietis* threatens 3.4 million hectares of European forest, with estimated losses of approximately €140 million per year in the absence of insecticidal protection (Långström and Day, 2007). In the UK alone, public and private forestry suffered estimated losses of €4.6 million in 1998 (Leather *et al.*, 1999). Research efforts are increasingly focused on developing alternative strategies to reduce the dependence on synthetic insecticides (Kapranas *et al.*, 2016, McNamara *et al.*, 2018a). In the UK, an integrated pest management approach is recommended (Willoughby *et al.*, 2017).

In recent years, the potential impact of *H. abietis* has gained increasing attention due to higher temperatures linked to climate change, which is expected to shorten its lifecycle, favour its dispersal, and also lead to shifts on its suitable areas of distribution (Inward *et al.*, 2012, Kapranas *et al.*, 2016, McNamara *et al.*, 2018a).

Biology and lifecycle

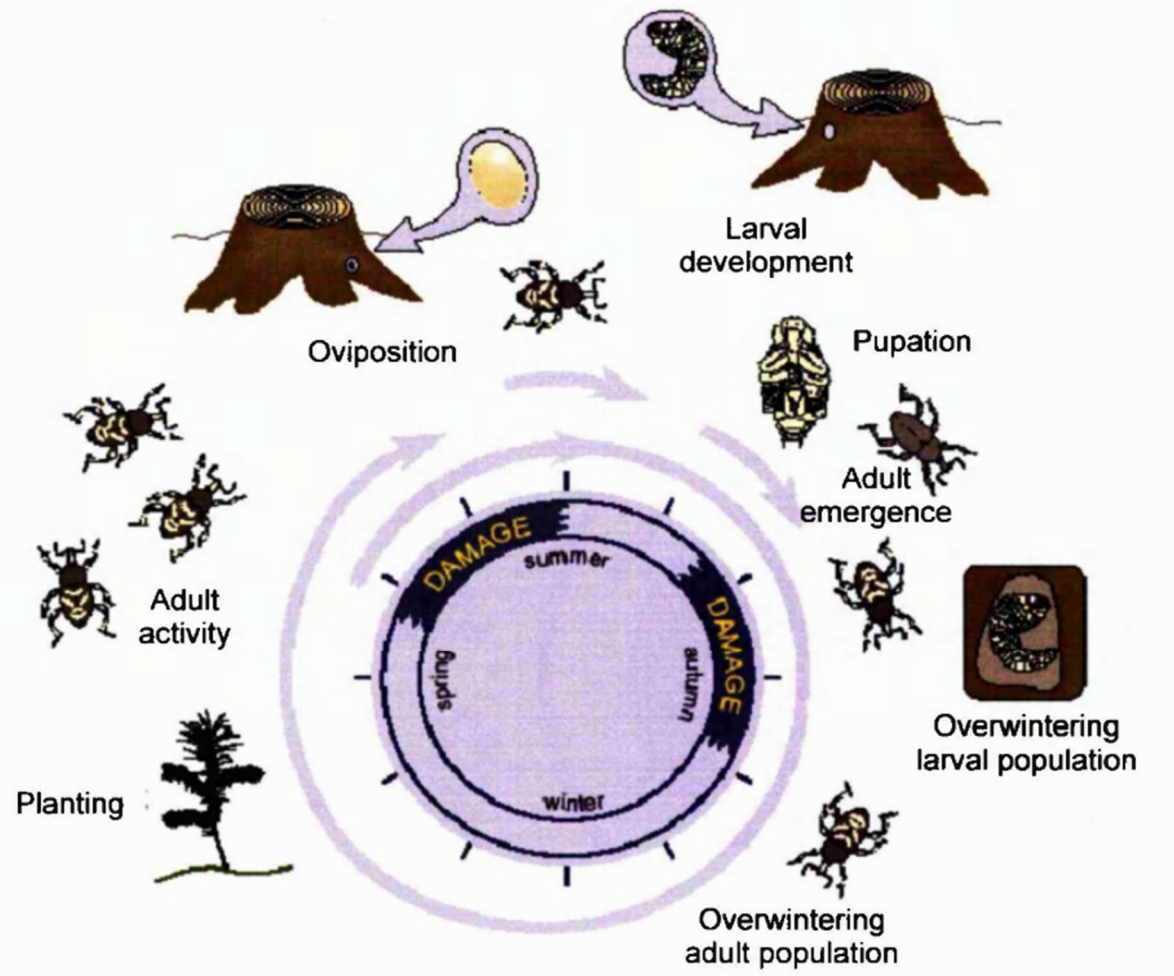


Figure 1.1. Generalised lifecycle of *H. abietis* in managed forest systems: Adult weevils colonise recently felled sites and oviposit in or near conifer stumps. Eggs hatch into larvae that develop under the bark, feeding on phloem tissues and progressing through multiple instars. Larvae may overwinter within the stump before pupating, while some individuals complete development without overwintering. Adult emergence occurs from both overwintered and non-overwintered cohorts, typically resulting in two peaks of adult activity. Newly emerged adults disperse and feed on the bark of replanted seedlings, causing economically significant damage. Adapted from Torr *et al.*, (2005).

The lifecycle of *H. abietis* is predominantly semi-voltine (longer than one year), although it ranges from 12 months (univoltine) in its southernmost range of distribution to up to four years in northern latitudes (Leather *et al.*, 1999, Moore *et al.*, 2004). Larval development varies based on temperature, host tree species, on the host tree, seasonal daylight duration, and site-specific factors (Figure 1.1). Development is faster on Scots pine, *Pinus sylvestris* L. 1753 than on Norway spruce, *Picea abies* (L.) Karsten 1881 (Von Sydow, 1997, Wainhouse *et al*., 2007).

Female *H. abietis* can lay several hundreds of eggs during their lifespan. Oviposition occurs primarily in spring and summer, peaking in May and June (Nordlander *et al.*, 1997, Leather *et al.*, 1999). Eggs are deposited mainly below ground by the base of freshly cut conifer stumps, which remain suitable for oviposition for up to five years after felling, depending on the tree species. Eggs are also laid in bark crevices, where they are protected from desiccation and predation (Nordlander *et al.*, 1997, Örlander *et al.*, 1997, Willoughby *et al.*, 2017). Hatch rates vary, with eggs generally hatching within two to four weeks, depending on environmental temperature and humidity (Day *et al.*, 2004).

Hatched larvae feed in the cambial layer of the stumps for one to three years, depending on environmental conditions, development accelerates in warmer conditions and extends in colder temperatures (Willoughby *et al.*, 2017). Larvae are protected beneath the bark, passing through multiple moults before pupation. In colder latitudes, *H. abietis* larvae can overwinter at the third or fourth instar stage, delaying development until conditions become favourable (Leather *et al.*, 1999, Torr *et al.*, 2005, Wainhouse *et al*., 2007).

Mature larvae pupate within the stump, constructing protective chambers beneath the bark. The timing of pupation and adult emergence depends on the microclimatic conditions and stump quality. Emergence can occur within a year of egg-laying, or it can be delayed until late summer of the following year (Leather *et al.*, 1999, Moore, 2001). In colder climates, larval development can extend up to two years, and in extreme cases, up to five years. However, approximately 75% of *H. abietis* larvae in Europe complete their development within one year being ready to emerge from the protective chambers (Nordenhem, 1989, Leather *et al.*, 1999).

Adults can live up to 4 years. However, their emergence from pupal chambers can be postponed, with individuals over-wintering until spring when temperatures exceed 8-9 °C

(Leather *et al.*, 1999, Torr *et al*, 2005). Newly emerged adults need a period of maturation feeding for reproductive development primarily feeding on the bark of young conifer and broadleaf trees saplings (Wainhouse *et al.*, 2004, Willoughby *et al.*, 2017). While adults also feed on the bark of mature conifers, and their new branches at the top of their canopies, they do not pose a significant threat to established trees. However, a single adult *H. abietis* can kill multiple saplings up to five years old before they develop resistance to debarking damage (Dillon *et al.*, 2006).

Implications of Climate Change

The lifecycle of *H. abietis* is strongly influenced by temperature fluctuations. Climate change is expected to shorten its developmental time, increase its dispersal capacity, and potentially alter its natural distribution. Warmer conditions may favour a shift toward univoltine (one-year) development in regions where the species was previously semi-voltine (Inward *et al.*, 2012, Wainhouse *et al.*, 2014, Fedderwitz *et al.*, 2022). Such changes could exacerbate the damage caused by *H. abietis* in forestry areas, necessitating a reassessment of current management strategies. A shift to a univoltine life cycle could, however, also be beneficial. Fallow periods could be a feasible management strategy with a univoltine species, which is not feasible with a semi-voltine life cycle.

Control of *Hylobius abietis* (L.)

Current control strategies for *H. abietis* primarily involve chemical insecticides, cultural practices, mechanical site preparation, and biological control agents (Willoughby *et al.*, 2020). These approaches are increasingly supported by GIS integrated decision-support systems that integrate pest monitoring, site characteristics, and management thresholds to guide treatment choice and timing (Fedderwitz *et al*., 2022).

Decision-support systems are particularly important in *H. abietis* management because the appropriateness of remedial measures depends strongly on pest pressure, site history, and timing relative to weevil development (Willoughby *et al.*, 2017). The In UK forestry, the Hylobius management support system (HMSS) was developed by Forest Research as a web-based tool that use billet counts together with a population-dynamics model to predict site-level damage risk and inform actions such as fallow strategy, planting stock choice, nematode use, and the likely need for top-up insecticide applications (Kapranas *et al.*, 2016, Willoughby *et al.*, 2017). In Ireland, related decision-support approaches based on stump

hacking, site factors, and crop monitoring are already used operationally (Fedderwitz *et al*., 2022).

The UK HMSS was specifically designed to reduce unnecessary pesticide use by predicting in advance when and where intervention may be needed, rather than relying solely on post-damage monitoring (Willoughby *et al.*, 2017). In Ireland, similarly it is emphasised that future *H. abietis* management should combine biological and silvicultural options with improved forecasting tools, centralised field knowledge, and nationally adapted decision frameworks (Fedderwitz *et al*., 2022)

Chemical Control

On many sites in the UK and Ireland, integrated control management of *H. abietis* may include the application of synthetic chemicals under derogation by the Forest Stewardship Council (FSC, 2005). Acetamiprid is currently the primary insecticide used in nurseries to protect seedlings before planting and is often supplemented with additional top-up spraying on-site (Kapranas *et al.*, 2016, Willoughby *et al.*, 2017, Moore *et al.*, 2021). However, both the effectiveness and environmental impact of pesticides vary. While Acetamiprid is not classified as "highly hazardous", many other neonicotinoids pose a significant risk to non-target organisms, such as honeybees (*Apis mellifera* Linnaeus 1758), and do not effectively supress *H. abietis* populations, as they exhibit a repellent rather than a lethal effect on weevils (Rose *et al.*, 2005, Torr *et al*, 2005, van der Sluijs *et al.*, 2013).

Concerns remain about the environmental effects of certain pesticides, particularly those classified as "highly hazardous chemicals" by the Forest Stewardship Council (FSC) (Kapranas *et al.*, 2016, McNamara *et al.*, 2018a). For example, cypermethrin has been shown to be highly toxic to non-target freshwater organisms, including ground beetles (Carabidae) and pollinators (Bendahou *et al.*, 1999, Desneux *et al.*, 2007, Saha and Kaviraj, 2008, Merivee *et al.*, 2015). Consequently, the use if these chemicals is being progressively phased out across Europe, in line with sustainable forest management principles and the EU Directive 2009/128/EC, which mandates the reduction of chemical pesticide use (Kapranas *et al.*, 2016, McNamara *et al.*, 2018a).

Given these limitations, alternative control methods, such as biological and cultural strategies, are increasingly being explored and implemented to minimise reliance on synthetic insecticides. These approaches aim to provide effective pest control while reducing the ecological risk associated with conventional chemical treatments.

Cultural Control

Cultural control methods provide an alternative to chemical insecticides by modifying the environment to make it less suitable for *H. abietis* reproduction and survival. Altering planting schedules and tree species composition can influence *H. abietis* population dynamics, mitigating the impact on regenerating forests. One strategy is delaying restocking by at least two years following clear-felling, allowing existing stumps to decompose, and become unsuitable for larval development. This strategy is effective only in the absence of nearby clear-felled areas, as adult *H. abietis* can disperse over long distances (Leather *et al.*, 1999, Kapranas *et al.*, 2016). However, prolonged delays can have negative consequences, including increased competition from vegetation, reduced seedling growth, and heightened vulnerability to vole damage (Luoranen and Viiri, 2012).

Another cultural technique involves the application of wood-decay fungi, such as *Phlebiopsis gigantea* (Fr.), to stumps to make them unsuitable for oviposition. *P. gigantea* has proven effective in controlling the forest pest *Heterobasidium annosum* (Fr.) Bref, and has been shown to reduce the attractiveness of stumps to *H. abietis* females, though it does not significantly reduce local weevil populations (Skrzecz and Moore, 1997, Dillon *et al.*, 2008a).

Mechanical Control

Mechanical site preparation techniques, such as stump harvesting and soil mounding—where soil is heaped around planted seedlings to improve drainage and reduce weed competition—, help reduce *H. abietis* attacks on newly planted saplings, especially when surrounded by pure mineral soil (Willoughby *et al.*, 2020). Stump harvesting may partially limit breeding grounds and *H. abietis* abundance (Rahman *et al.*, 2018). Piri *et al.*, (2020) found that combining stump removal with mineral soil mounding decreased damage from weevil to saplings reducing the need for supplementary planting. However, stump removal has potential drawbacks, including soil erosion and compaction, loss nutrient stocks and carbon stores, and loss of valuable habitats (Walmsley and Godbold, 2009). Habitat loss directly affects non-target biodiversity such as the saproxylic beetle *Rhagium bidasciatum* Fabricius 1775 (Coleoptera: Cerambycidae), which primarily inhabits brash but also feeds for at least part of their lifecycle on decomposed wood for larval development, such as tree stumps three to four years after felling (Twinn and Harding, 1999). This species is considered beneficial in forest management of clear-fell sites in Europe (Harvey *et al.*, 2016). Transient

impacts have also been reported on ground dwelling beetles (specially ground beetles), as result of stump harvesting used as source of bioenergy (Shevlin *et al.*, 2017).

Physical Barriers

Physical protection methods, such as collar guards and protective coatings, have been used to prevent and mitigate *H. abietis* damage. In Swedish trials, collard guards proved as efficient as permethrin over three years (Petersson *et al.*, 2004, Petersson *et al.*, 2006). Several guards are available in the UK, including Multipro Sleeves, plastic Biosleeves and Weenets (Willoughby *et al.*, 2017, Hardy *et al.*, 2020b). Additional physical protection methods of the lower part of the stem of seedling include sand-and-glue-based coating (Conniflex) and polysaccharide coatings (Flexcoat). Conniflex has proven efficient protecting Norway spruce and Scots pine against *H. abietis* in Swedish field trials (Hardy *et al.*, 2020b). However, in the UK and Ireland, where *H. abietis* populations are at least seven times higher than in heavily infested Swedish sites, these methods have proven less effective (Willoughby *et al.*, 2017, Willoughby *et al.*, 2020).

Silvicultural practices incorporating risk management strategies and using Geographic Information Systems (GIS), can significantly enhance *H. abietis* control efforts (Wainhouse *et al*., 2007, Kapranas *et al.*, 2016, Willoughby *et al.*, 2020). GIS enables the identification of high-risk areas, allowing targeted interventions such as mounding to improve sapling survival and reduce damage (Von Sydow, 1997, Örlander and Nilsson, 1999, Örlander and Nordlander, 2003) .

Biological Control and its application to control *H. abietis*.

Biological control has been used for millennia to regulate pest populations, but its modern applications were established in the 19th century. There are four main types of biological control: natural, conservation, classical and augmentative biological control (ABC) (van Lenteren *et al.*, 2017).

Natural biological control relies on naturally occurring predators, parasitoids, or pathogens that regulate pest populations as an ecosystem service without human intervention. For example, the parasitoid *Bracon hylobii* Ratzerburg 1848 can parasitise *H. abietis* larvae at rates ranging from 15% to 90% (Harvey *et al.*, 2016).

Conservation biological control involves human intervention to enhance the presence and efficiency of natural enemies (parasitoids, predators, or microorganisms) in the environment. A well-documented example is the establishment of "beetle banks", created by splitting tillage field systems with strips of perennial grasses that provide overwintering habitats for natural enemies of agricultural pests (Thomas *et al.*, 2002).

Classical biological control consists of the release of non-native natural enemies to control invasive pests. While effective when properly assessed, this method has sometimes led to unintended major ecological consequences, such as the displacement of native ladybirds in Europe and North America following the introduction of the escape of the Asian coccinellid *Harmonia axyridis* Pallas 1773 from glasshouses (Roy and Wajnberg, 2007). However, when adequately screened, classical control has proven effective, as seen in the control of the non-native psyllid pest *Ctenarytaina eucalypti* Maskell 1890 in Irish eucalyptus plantations by releasing the exotic parasitoid wasp *Psyllaephagus pilosus* Noyes 1988 that resulted in both effective and environmentally benign control (Chauzat *et al.*, 2002).

ABC is the most actively managed form of biological control, involving the mass production and release of natural enemies or microbial biopesticides in large numbers to supress pest populations (Sporleder and Lacey, 2013). ABC is classified into two approaches: inundative biological control, where natural enemies achieve immediate control of pests on crops of short production cycle, and seasonal inoculative biological control, that is used for control of pests during several generations in crops with a long production cycle (van Lenteren *et al.*, 2017).

A key feature of ABC is the non-persistence of the biological control agents, meaning that once they have supressed the pest population, they do not establish long-term populations in the environment, even though they are applied in vast numbers to knock-down the target. This reduces the risk of unintended ecological impacts and minimises disruption to beneficial native species. Additionally, ABC poses fewer health risks to farm, and forestry workers compared to synthetic pesticides, as biopesticides do not require harvesting interval or a waiting period before re-entering treated areas (Sporleder and Lacey, 2013, Chiew *et al.*, 2022).

Biopesticides and biological control agents offer multiple advantages compared to chemical pesticides. They are more sustainable, as pests do not develop resistance to them, present negligible effects on non-targets and they do not cause phytotoxic damage to plants,

leading to better yields and healthier crops (Dillon *et al.*, 2012, van Lenteren *et al.*, 2017). A very successful example is the control of thrips and whiteflies in Spanish greenhouse sweet pepper production, where predatory mites and hemipterans have almost entirely replaced chemical pesticides (Calvo *et al.*, 2012).

The inundative use of entomopathogenic nematodes (EPN) and fungi (EPF) has emerged as a highly effective strategy for managing *H. abietis* and other forest and agricultural pests. Over recent decades, both successful applications and practical limitations have contributed to the development of increasingly optimised recommendations for use and deployment of EPN as biological control agents (Campos-Herrera, 2015, Lacey *et al.*, 2015, Kapranas *et al.*, 2017). The development of mycoinsecticides has led to the commercialisation and broader operational use of several EPF species and varieties of anamorphic hypocrealean fungi such as *Beauveria* and *Metarhizium*, against a range of insect pests in greenhouse, horticultural, agricultural and seminatural environments (Faria and Wraight, 2007, Altinok *et al.*, 2019), including EPF species that show important pathogenicity against a range of coleopteran pests (Ansari *et al.*, 2008, Mayerhofer *et al.*, 2015, 2016, Wu *et al.*, 2016).

. Laboratory studies have demonstrated the efficacy of both EPNs and EPFs against all developmental stages of *H. abietis* (Ansari and Butt, 2012, Williams *et al.*, 2015), and this potential has also been evaluated under field conditions (Torr *et al.*, 2007, Kapranas *et al.*,2016, Barta *et al.*, 2019). Importantly, positive interactions have been reported when both certain combinations of EPFs and EPNs are applied together in field trials (Williams *et al.*, 2013b, McNamara *et al.*, 2018a). Research in this area continues advancing rapidly, driven by the technical advances in isolating, identifying, and applying new promising and efficient strains altogether with commercially available biological control agents.

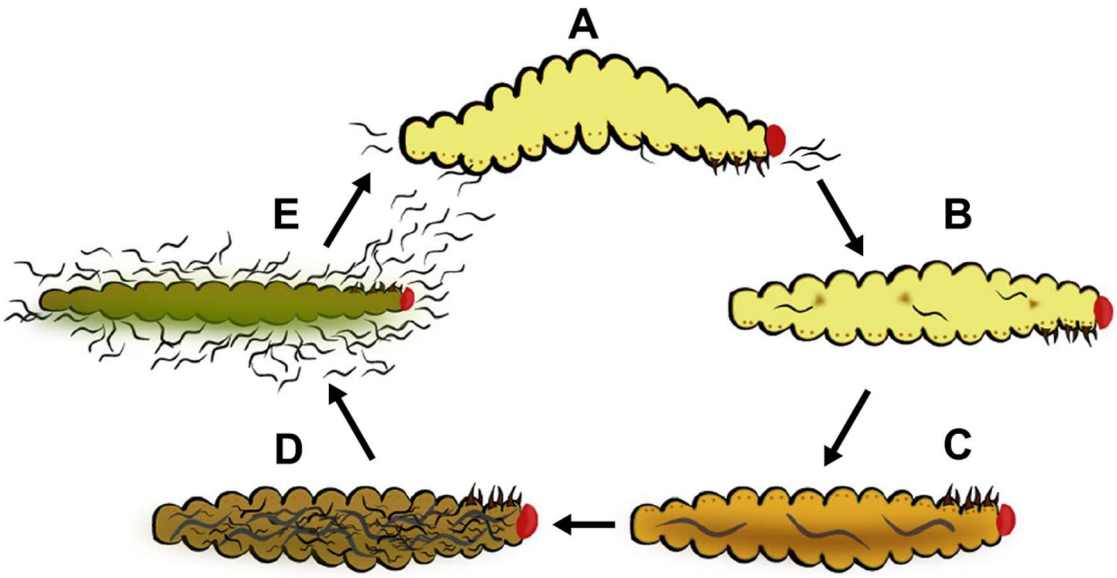


Figure 1.2. Generalised life cycle of entomopathogenic nematodes (EPNs) and their symbiotic bacteria: (A) Infective juveniles (IJs), the free-living and infective stage, locate and penetrate a susceptible insect host. (B) Once inside the host haemocoel, IJs release their symbiotic bacteria. (C) The bacteria proliferate rapidly, causing host mortality and creating suitable conditions for nematode development; IJs then recover and develop into adults. (D) Adult nematodes reproduce within the host cadaver, producing one or more generations. (E) As resources are depleted, new infective juveniles are formed, re-associate with symbiotic bacteria, and emerge from the cadaver to seek new hosts. Adapted from Baiocchi *et al.*,(2017), content available via license CC by 4.0 (http://creativecommons.org/licenses/by/4.0/).

EPN are soil-dwelling parasites that actively seek out insect hosts, guided by carbon dioxide and heat cues, employing a combination of ambush and cruising foraging strategies (Gaugler and Campbell, 1993, Girling *et al.*, 2010). Upon locating the host, EPNs enter through natural openings or directly penetrate the cuticle, releasing symbiotic bacteria (*Xenorhabdus* spp. in Steinernematidae and *Photorhabdus* spp. in Heterorhabditidae) (Kaya and Gaugler, 1993, Forst *et al.*, 1997). These bacteria rapidly kill the host providing a nutrient-rich environment in which nematodes develop, reproduce, and complete their lifecycle within the cadaver (Figure 1.2). Once resources are depleted, thousands of new non-feeding infective juveniles (IJs) disperse into the soil, seeking new hosts (Kaya and Gaugler, 1993, Torr *et al*, 2005, Dillon *et al.*, 2006).

The efficacy of EPNs as biological control agents is influenced by multiple factors, including soil type, moisture, temperature, ultraviolet (UV) radiation, predation by soil organisms (such as collembolans, mites and nematophagous fungi), metabolic starvation in absence of suitable hosts, and the availability of hosts for long term recycling (Williams *et al.*, 2013a, Campos-Herrera, 2015, Kapranas *et al.*, 2016). Infective activity of nematodes appears to be enhanced by the root-routeways that firstly, attracts EPN by the chemicals and vibrations produced by the host feeding underground and secondly, facilitates nematode deep penetration into soil (Ennis *et al.*, 2010). However, field studies suggest that *H. abietis* infestation levels and spatial distribution significantly influence the efficacy of EPN applications, as density-dependent parasitism can reduce control efficiency when pest populations are excessively high (Kapranas *et al.*, 2016). Despite extensive laboratory studies on EPN host-finding and dispersal, their behaviour in natural forest environments remains insufficiently understood as foraging behaviour observed in laboratory conditions do not match EPNs field activity (Kapranas *et al.*, 2017).

EPN of the genera *Steinernema* and *Heterorhabditis* include species that are efficacious against *H. abietis* primarily targeting its larval stages. Pupae and callow adults are also susceptible, though with lower infection rates, while fully mature adults are the least affected (Torr *et al*, 2005, Dillon *et al.*, 2006, Williams *et al.*, 2015). In the UK and Ireland, several EPN species have shown high infection rates in *H. abietis* larvae, with *Steinernema carpocapsae* Weiser 1955, *Heterorhabditis downesi* Stock, Griffin & Burnell 2002*, Steinernema feltiae* Filipjev 1934, and *Steinernema kraussei* Steiner 1923 presenting also different persistence after field applications (Torr *et al.*, 2007, Dillon *et al.*, 2008b). *Heterorhabditis downesi* stands out as the most effective in reducing stump emergence of adults close to an acceptable commercial threshold of six adults per stump (Dillon *et al.*, 2007, Dillon *et al.*, 2008a, Williams *et al.*, 2013a). Field trials have demonstrated that optimal application timing coincides with late-instar larvae and pupae presence within tree stumps, as these stages are more susceptible to EPN infection (Dillon *et al.*, 2006, Kapranas *et al.*, 2017).

Among commercially available nematodes, *S. carpocapsae* is the easiest and most cost-effective to mass-produce and has demonstrated reliable field efficacy, consistently reducing weevil emergence below the economic damage threshold of 20 adults per stump when applied at recommended doses (Kapranas *et al.*, 2017). Operational applications in the UK and Ireland involve pressure hose drenching around tree stumps approximately 12 months post-clear-felling, between mid-May and early July, using a concentration of 3.5 million IJs in 500 ml of water per stump (Torr *et al*, 2005). However, climate change is altering *H.*

abietis phenology, advancing developmental stages and increasing fecundity, which may necessitate adjustments in the timing and frequency of EPN applications (Inward *et al.*, 2012).

Although other commercial EPN strains and native species, such as *H. downesi*, provide superior weevil control, their large-scale production remains expensive and technically challenging (Dillon *et al.*, 2006, Dillon *et al.*, 2007, Dillon *et al.*, 2008b). Ideally, a successful biological control agent should both reduce *H. abietis* populations and mitigate damage to replanted forests. While EPN applications significantly suppress weevil populations, residual overwintering adults may still threaten the 2,500 seedlings per hectare typically present in a restocked site (Girling *et al.*, 2010).

Current research aims to refine EPN efficacy by investigating their persistence and recycling capabilities in different soil types, host-finding mechanisms, application methods, interactions with other biological control agents, and the potential for isolating more virulent strains. Application technique significantly influences efficacy to control *H. abietis. Steinernema carpocapsae* performs better when covered with soil immediately after application (sub-surface drench) compared to soil surface application (standard drenching), where UV exposure reduces EPN viability (Brixey *et al.*, 2006). Furthermore, *S. carpocapsae* exhibits superior control when applied by standard soil drenching around the stump, compared to "top" application by the top edges of the stump. Conversely, in the case of *H. downesi*, higher efficacy was achieved when IJs were applied to the top edges of the stumps (Kapranas *et al.*, 2017). Field trials on *H. downesi*, indicate that the standard operational dose can be reduced by half (to 1.75 million IJs) and the water volume lowered to 25% of the recommended amount without compromising efficacy (Dillon *et al.*, 2007). Similar findings reducing dosage of both *S. carpocapsae* and *H. downesi* found that half their recommended operational dosage can be as efficient as full-doses (Williams *et al.*, 2013b).

These findings highlight the importance of selecting the appropriate EPN species and refining application methods to optimise *H. abietis* management. By improving IJ survival and selecting the appropriate application methods, EPN-based control can be enhanced for sustainable forestry pest management.

EPN Environmental Safety

The inundative application of augmentative biological control agents requires an assessment of potential impacts on non-target species and overall ecosystem dynamics. Even commercial strains of local EPNs are considered "exotic" when applied in inundative fashion due to their differential virulence and potential unintended effects (Harvey *et al.*, 2016). When nematode species or strains are poorly adapted to forest ecosystems, their long-term persistence is generally low, reducing the risk of permanent establishment (Van Lenteren *et al.*, 2003, Dillon *et al.*, 2012). However, short-term studies—typically spanning up to three years—may overestimate establishment risk or fail to capture longer-term populationdynamics. For example, in northern Scotland, indigenous *S. kraussei* persisted in the soil for over a year post-application, whereas S. *carpocapsae* exhibited lower persistence. This raised concerns regarding potential risks to local entomofauna due to high numbers of native nematodes remaining in the environment (Torr *et al.*, 2007).

Longer studies provide a more nuanced perspective. A five-year mesocosm study assessing the establishment of both exotic and native EPN species, including *H. downesi*, and *S. feltiae*, demonstrated that all species could be recovered three years post-application. However, only the native *S. feltiae* persisted in years four and five, albeit at low prevalence (1–2% of soil samples) (Dillon *et al.*, 2008b). In the same study, horizontal dispersal was minimal, with nematodes remaining near the initial application site. Only *S. feltiae*, showed minor dispersion into adjacent stumps, without extending beyond the treated area. Furthermore, evidence of introgression indicated that local genotypes persisted over exotic strains after 48 months, reinforcing the transient nature of introduced populations (Dillon *et al.*, 2008b).

Expanding on this topic, it was confirmed that EPNs used for *H. abietis* control posed negligible risks on non-target species (Dillon *et al.*, 2012). Further environmental risk assessments on EPN considering five risk categories (Van Lenteren *et al.*, 2003): establishment, dispersal, host range, direct non-target effects, and indirect non-target effects, concluded that, compared to conventional chemical insecticides, EPNs represent a low-risk, viable alternative for *H. abietis* control in managed forests (Harvey *et al.*, 2016).

Entomopathogenic fungi against *H. abietis*

Entomopathogenic fungi (EPFs) are an essential component of terrestrial ecosystems, naturally regulating arthropod populations, including significant pest species such as *Hylobius abietis* (Scheepmaker and Butt, 2010, Sporleder and Lacey, 2013, Araujo and

Hughes, 2016). These fungi are widely distributed in soil environments, existing in complex interactions with other microorganisms, and playing a crucial role in the suppression of insect populations through infection and mortality (Scheepmaker and Butt, 2010, Bueno-Pallero *et al.*, 2020, Majchrowska-Safaryan and Tkaczuk, 2021) Several fungal genera within the order Hypocreales (phylum Ascomycota), particularly *Metarhizium, Beauveria,* and *Clonostachys*, have been extensively studied for their ability to infect a wide range of insect pests and have been successfully formulated as biological control agents (Faria and Wraight, 2007, Zimmermann, 2008, Scheepmaker and Butt, 2010).

Infection mechanisms, host-pathogen interactions and commercial use of EPFs

EPFs exhibit complex life cycles involving both saprophytic and well-developed parasitic phases. Unlike EPNs, which require host ingestion or penetration through natural openings, EPFs initiate infection externally when fungal mycelium or asexual fungal spores (conidia) come into direct contact with the insect's cuticle (Donatti *et al.*, 2008, Oreste *et al.*, 2012, Altinok *et al.*, 2019). The infection process is facilitated by enzymatic degradation of the cuticle, mechanical penetration, and the production of secondary metabolites that suppress the host immune response and facilitate fungal invasion while avoiding invasion by opportunistic saprophytes (Sporleder and Lacey, 2013, Lacey *et al.*, 2015, McNamara *et al.*, 2019). Once inside the hemocoel, fungal hyphae proliferate throughout the insect's body, disrupting physiological processes, ultimately leading to mortality. Following the host's death, the fungus sporulates externally, releasing conidia into the environment, where it can infect new hosts, thus continuing the disease cycle (Scheepmaker and Butt, 2010, Araujo and Hughes, 2016).

Virulence among EPF species and strains varies widely, influenced by fungal genetics, host species susceptibility, environmental conditions, and infection strategy (Barta *et al.*, 2019, Bueno-Pallero *et al.*, 2020). Commercially formulated EPFs, including *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin 1912, *Metarhizium anisopliae* (Metschnikoff) Sorokin 1883, and *Isaria fumosorosea* Wize 1904, have demonstrated broad host ranges and high virulence against insect pests (Torrado-Leon *et al.*, 2006, Zimmermann, 2008, Garrido-Jurado *et al.*, 2015, Yapa *et al.*, 2024). However, their persistence and specificity in forest ecosystems remain key areas of investigation.

Commercial and operational use of EPFs is already well established beyond forestry with mycoinsecticides based mainly on *Beauveria bassiana* and *Metarhizium anisopliae sensu lato* that have been used against a wide range of arthropod pests in greenhouse, horticultural,

arable, and semi-natural environments, demonstrating that EPFs are not merely experimental agents but practical components of biological control programmes (Faria and Wraight, 2007, Lacey *et al*., 2015). A prominent large-scale application of EPFs is in the control of locusts and grasshoppers (Orthoptera: Acrididae), where oil-based formulations of *Metarhizium anisopliae sensu lato*, particularly *M. anisopliae* var. *acridum*, have been developed and deployed in Africa, Australia, and South America (Lomer et al., 2001). These mycoinsecticides typically achieve 70–90% mortality within 2–3 weeks under field conditions, with minimal non-target impacts, making them suitable for integration into preventive and environmentally sensitive pest management programmes (Lomer *et al*., 2001). Such systems are now incorporated into integrated pest management (IPM) strategies alongside other biological agents (e.g. *Nosema locustae* Canning 1953 and parasitoids), representing one of the most advanced operational uses of EPFs at landscape scale (Lomer *et al*., 2001).

More recently, Gao *et al*. (2026) further demonstrated the continued development of *Metarhizium*-based locust control by integrating *M. anisopliae* var. *acridum* with a predatory beetle in a multi-agent biocontrol system, highlighting both the versatility of EPFs and the expanding scope of their deployment in sustainable pest management.

Beyond acridid systems, EPFs are widely used or under advanced development in agricultural crops against diverse pests. For example, *Beauveria bassiana* and *Metarhizium* spp. have been applied in arable systems such as wheat to target soil-dwelling pests like wireworms (Coleoptera: Elateridae), using granular or in-furrow formulations that can improve yield and provide cost-effective control (Sharma *et al*., 2019). Similarly, EPFs have been successfully deployed against lepidopteran pests such as *Spodoptera littoralis* Boisduval 1833, through soil-drench and foliar applications, reducing both survival and reproductive output and demonstrating their compatibility with IPM frameworks (Garrido-Jurado *et al*., 2020). Collectively, these examples highlight that EPFs are not only experimentally validated but are already implemented across a range of crops, environments, and pest systems, from large-scale rangeland locust control to intensive agricultural production.

Environmental Distribution and Bioprospecting for EPFs

The effectiveness of EPFs as biological control agents depends on their environmental distribution and ecological adaptations. Their occurrence in soil varies with habitat type, soil depth, organic matter content, and microbial interactions (Sun and Liu, 2008). Studies

indicate that EPF are more abundant in topsoil layers rich in organic matter and microbial activity, where they exhibit increased sporulation and host-seeking behaviour (Posadas *et al.*, 2012, Coleman *et al.*, 2018). However, their spatial distribution is heterogeneous, necessitating diverse sampling strategies for effective bioprospecting (Quesada-Moraga *et al.*, 2007, Lacey *et al.*, 2015).

Bioprospecting for novel EPF strains typically involves soil collection from natural and agricultural habitats, followed by baiting assays using laboratory model organisms such as *Galleria mellonella* (L., 1758) (Inglis *et al.*, 2008, Sun and Liu, 2008, Oulevey *et al.*, 2009, Uzman *et al.*, 2019) or *Tenebrio molitor* L., 1758 (Glare *et al.*, 2008, Korosi *et al.*, 2019). Some studies have employed a dual baiting approach, combining both insect baits to increase the diversity of isolated fungal pathogens and enhance the likelihood of identifying highly virulent strains (Oreste *et al.*, 2012, Sharma *et al.*, 2018).

Despite the widespread use of these methods, knowledge gaps persist regarding the most suitable habitats for EPF collection, the optimal soil depths for isolation, and the bait species that yield the highest fungal diversity (Quesada-Moraga *et al.*, 2007, Bueno-Pallero *et al.*, 2020). Addressing these gaps is critical for advancing the development of EPFs as biological control agents in forestry applications.

Application of EPFs for Hylobius abietis Control

Laboratory studies have demonstrated the potential of both commercial and wild EPFs for *H. abietis* control. With "dry" conidia formulation proved more effective than the "wet" conidia application method, several fungal strains have exhibited high virulence against all developmental stages of the *H. abietis* (Williams *et al.*, 2013b). The species tested were *Metarhizium robertsii, Metarhizium brunneum,* and *Beauveria bassiana* that shown high efficacy, with the strain *M. robertsii* ARSEF4556 causing 100% mortality of larvae, pupae, and adults within 4, 6, and 12 days, respectively (Ansari and Butt, 2012).

Beyond commercially available EPFs, native fungal isolates also present strong potential as biological control agents. *Beauveria caledonica* Bissett & Widden 1988, emerged as suitable candidate for weevil control due to its ability to infect bark beetle pests in pine plantations in New Zealand, (Reay *et al.*, 2008). Further investigations confirmed that *B. caledonica* is a natural pathogen of *H. abietis* and other bark beetle pests in northern UK and Ireland, from where it was exported to New Zealand (Glare *et al.*, 2008). The ability of *B. caledonica* to infect *H. abietis* in its native range reinforces its potential as a locally adapted and effective biological control agent.

While laboratory experiments indicate high virulence of EPFs against *H. abietis,* field trials provided variable results. Williams *et al.* (2013) conducted a three-year study

evaluating EPF-only, EPN-only, and combined EPF+EPN applications across five different field trials. Results indicated that while EPF-only applications demonstrated limited efficacy, their combination with EPNs led to additive effects, achieving pest suppression levels comparable to or exceeding those of EPN-alone treatments. Further spatial analysis of fungal spread revealed that weevils infected by *B. bassiana* were found at depths of 18 cm and distances of 28 cm from stump boles, while *B. caledonica* demonstrated even greater dispersal, infecting *H. abietis* up to 30 cm below soil and 33 cm from the stumps' bole (Williams *et al.*, 2013b).

McNamara *et al.* (2018a) further validated the potential of EPF-EPN interactions in *H. abietis* control. Their study demonstrated that *B. caledonica* primarily infected weevils above soil at depths greater than one centimetre, whereas *M. brunneum* was more effective in infecting weevils below soil at depths of less than one centimetre. Interestingly, *M. brunneum* was found to persist in the treated sites for over two years, highlighting its potential for long-term suppression of *H. abietis* populations (McNamara *et al.*, 2018a). These findings emphasize the importance of selecting fungal strains based on their spatial distribution and effectiveness at different soil depths to optimize field performance.

The virulence of EPFs is not solely dependent on direct infection, as fungal secondary metabolites also influence host susceptibility. Research by McNamara *et al.* (2019) explored the role of oosporein, a secondary metabolite commonly produced by *B. bassiana* and *B. caledonica*. Surprisingly, oosporein did not exhibit direct insecticidal or anti-feeding properties. Instead, it appeared to act as an immunomodulatory compound, reducing haemocyte counts and altering the humoral immune response in *H. abietis*. This immune suppression facilitated fungal infection, potentially increasing mortality rates in infected individuals. Unexpectedly, exposure to oosporein also increased insect feeding behaviour in both *H. abietis* and the model insect *G. mellonella*, suggesting complex interactions between fungal metabolites and host physiology (McNamara *et al.*, 2019).

The implications of these findings suggest that oosporein, or similar metabolites, could be leveraged to enhance EPF virulence, either through genetic selection for high-producing strains or by co-applying fungal biopesticides with immunosuppressive compounds. Accordingly, it has been proposed that future research should explore the potential of integrating secondary metabolites into biological control strategies to improve efficacy against *H. abietis* (McNamara *et al.*, 2019).

Future Research and Challenges in EPF Biocontrol

Despite promising results, significant knowledge gaps remain regarding the use of EPFs for *H. abietis* control. One key challenge that cannot be addressed due to the limited duration of this project is ensuring long-term persistence in forest environments. While studies have demonstrated that *M. brunneum* can persist for over two years post-application, there is limited data on the long-term survival and recycling of other EPF strains in forestry settings (McNamara *et al.*, 2018a). Expanding field studies to assess persistence beyond two years would provide valuable insights into the sustainability of EPF applications.

Interactions with non-target species are another important consideration. While EPFs are generally regarded as environmentally safe, their impact on native insect populations and ecosystem processes requires further investigation. The use of native fungal strains, such as *B. caledonica*, reduces establishment risks associated with exotic biocontrol agents but does not eliminate the need for ecological impact assessments (Scheepmaker and Butt, 2010, Dara *et al.*, 2019, Chiew *et al.*, 2022).

Optimizing application methods remains a crucial research priority. Previous studies have shown that EPF efficacy is influenced by formulation type, with "dry" conidia outperforming "wet" conidia in laboratory assays (Ansari and Butt, 2012). Field trials should further investigate the effects of conidial formulation, application timing, and environmental factors such as humidity and temperature on fungal performance. Additionally, the observed differential activity of EPFs above and below soil highlights the need for tailored application strategies, where specific fungal strains are selected based on their preferred infection environment (McNamara *et al.*, 2018a).

Bioprospecting efforts aimed at isolating novel EPF strains with enhanced virulence and persistence will be vital for improving biological control strategies. While significant progress has been made in isolating EPFs from soil using baiting techniques with *Galleria mellonella* (L.) 1758 and *Tenebrio molitor* (L.) 1758, further refinement of these methods could enhance the discovery of more effective fungal pathogens (Quesada-Moraga *et al.*, 2007, Bueno-Pallero *et al.*, 2020). The development of molecular tools to assess EPF diversity and monitor persistence post-application could also provide valuable insights for improving field performance.

Integrating EPFs with EPNs for *Hylobius abietis* Control

Increasing evidence suggests that the combination of EPFs and EPNs can enhance *H. abietis* control through additive and potentially synergistic interactions. Research has shown that while EPF-only applications often provide inconsistent results in the field, their

combined use with EPNs improves efficacy and overall pest suppression (Williams *et al.*, 2013b, McNamara *et al.*, 2018a).

Building on these findings, future research should explore the mechanisms underlying EPF-EPN interactions, particularly how fungal infection influences nematode host-seeking behaviour and establishment. Given the promising results observed in previous trials, the integrated use of EPFs and EPNs represents one of the most effective biological control strategies available for *H. abietis*. Further optimization of strain selection, application timing, and delivery methods will be critical for maximizing the potential of this combined approach in sustainable forestry pest management.

Thesis structure and overview

This thesis builds on the extensive body of research into the use of entomopathogenic fungi (EPFs) and entomopathogenic nematodes (EPNs) as biological control agents for *Hylobius abietis*. Recognizing the demonstrated efficacy of these biological control agents in both laboratory and field conditions, this study aims to refine and optimize their application by identifying highly virulent, locally adapted EPF strains and integrating them with commercial EPN formulations. The overarching goal is to improve the effectiveness and sustainability of biological control strategies for *H. abietis* in commercial forestry.

The integration of EPFs and EPNs represents a pivotal step toward optimizing biological control, as previous studies have demonstrated additive and potentially synergistic effects when these agents are applied in combination. By systematically testing co-application strategies in controlled and field settings, this thesis aims to refine biological control protocols, ensuring that forestry managers have access to scientifically validated, environmentally responsible alternatives to chemical insecticides.

Ultimately, this research seeks to advance the application of biological control for *H. abietis*, contributing to the broader goal of reducing reliance on synthetic insecticides in forestry management. By identifying and deploying highly virulent, locally adapted EPFs—alone or in conjunction with EPNs—this work aims to enhance the efficacy, reliability, and sustainability of biopesticide-based strategies against one of the most damaging forest pests in Europe.

CHAPTER 2:

To achieve this, the study undertakes bioprospecting for wild EPFs, employing a dual baiting technique with *Galleria mellonella* and *Tenebrio molitor* larvae to maximize the diversity of fungal species and strains isolated. By sampling at different soil depths across a

range of habitats, this approach aims to capture a broad spectrum of naturally occurring EPFs with potential for *H. abietis* control. The isolated fungal strains will then be identified using molecular techniques, ensuring accurate taxonomic classification, and facilitating comparisons with known virulent EPF species.

CHAPTER 3:

Following isolation and identification, laboratory bioassays will be conducted to assess the pathogenicity of the EPF strains against adult *H. abietis*. These experiments will investigate both lethal and infection effects, including mortality rates, and shifts on weight and feeding behaviour in order to identify the most virulent strains and other potentially non-lethal effects e.g. feeding stimulation/inhibition.

CHAPTER 4:

The best-performing EPF candidates will then be selected for field trials, where their efficacy will be evaluated in different treatment regimes:

- EPF-alone applications, where full-dose fungal conidia are applied in an inundative fashion targeting H. *abietis* populations.
- EPF + EPN combined half-dose treatments, where EPFs will be co-applied with the commercial nematode Steinernema carpocapsae, leveraging potential additive or synergistic interactions between these biocontrol agents.
- EPN-alone treatments, serving as a full-dose benchmark to compare the relative efficacy of fungal and nematode-based control strategies.

To complement larval-stage control, mark-recapture experiments will test the feasibility of using freshly cut billets to attract surviving emerged adult *H. abietis* to be infected by a biopesticide agent placed under the billets, in order to offer additional control targeting adult weevils.

Additionally, the impact of EPF and EPN applications on non-target invertebrates will be assessed to ensure ecological safety. These field trials will provide valuable data on the impact of EPF and EPN treatments under real-world conditions, addressing key knowledge gaps in biological control implementation.

CHAPTER 5:

General discussion and synthesis of the findings in the previous chapters

CHAPTER 2:

The effects of habitat, soil depth, edaphic factors and bait type on the diversity and composition of entomopathogenic fungi in Lancashire Nature Reserves: implications for bioprospecting.

Abstract

Entomopathogenic fungi (EPF) are key regulators of arthropod populations and represent important biological control agents for sustainable pest management. This study evaluated the effects of habitat type, soil depth, edaphic factors and bait insect on EPF diversity, abundance and community composition in Lancashire Nature Reserves, with the aim of optimising bioprospecting strategies. Using a dual baiting approach (*Galleria mellonella* and *Tenebrio molitor*) combined with molecular identification and multivariate analyses, 197 EPF isolates representing 13 genetically distinct strains across three genera (*Metarhizium*, *Beauveria* and *Clonostachys*) were recovered.

Site was the dominant driver of EPF community composition across both non-pooled and soil-pooled datasets, with consistent site-specific indicator strains identified. Soil depth and bait insect did not generate distinct compositional structuring, although the combined use of both bait insects increased detection of low-frequency EPF strains. Pooling topsoil and bottom soil substantially increased isolate abundance, strain richness and co-occurrence at the sampling-point scale, but did not alter dominance patterns, ordination structure or multivariate effect sizes. Environmental fitting revealed that inorganic carbon, organic carbon and soil pH were significant correlates of EPF community composition, underscoring the role of soil chemistry in shaping fungal assemblages.

These results demonstrate that habitat-level environmental conditions primarily structure EPF communities, while sampling design influences recovery efficiency without altering ecological inference. The study provides an evidence-based framework for EPF bioprospecting that prioritises ecologically diverse sites, integrates dual baiting to maximise strain detection, and incorporates edaphic profiling to improve targeting of biologically and commercially valuable EPF strains.

Introduction

Entomopathogenic fungi (EPF) are integral components of natural and agricultural terrestrial ecosystems, with soils serving as an important reservoir for diverse EPF species alongside other microorganisms (Scheepmaker and Butt, 2010, Sporleder and Lacey, 2013, Araujo and Hughes, 2016). These fungi play a crucial role in regulating arthropod populations, including insect pests (Scheepmaker and Butt, 2010, Bueno-Pallero *et al.*, 2020, Majchrowska-Safaryan and Tkaczuk, 2021). Prominent EPF genera, such as *Metarhizium, Clonostachys,* and *Beauveria* from the order Hypocreales (Phylum Ascomycota), exhibit complex lifecycles characterised by a well-developed parasitic phase that infect insect hosts

via contact with mycelium or asexual conidiospores. The infection process is facilitated by secondary metabolites and biological compounds that enable penetration through the insect cuticle, digestive tract, or spiracles. Once the host dies, EPF biologically active compounds are responsible in preventing colonization by opportunistic organisms (Donatti *et al.*, 2008, Bueno-Pallero *et al.*, 2020, McNamara *et al.*, 2019).

Interactions between fungal pathogens and their insect hosts exhibit considerable variability, including saprophytic interaction, reflecting the complexity of fungal morphologies and host-pathogen dynamics. EPF predominantly target Coleopterans and Lepidopterans while *Clonostachys* also infects Hemipterans, often during their larval or pupal stages, though adult stages can also be susceptible (Toledo *et al.*, 2006, Mayerhofer *et al.*, 2015, Araujo and Hughes, 2016). Several EPF such as *Metarhizium anisopliae* (Metschnikoff) Sorokin 1883, *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin 1912, and *Isaria fumosorosea* Wize 1904, have been developed and commercialised as biological control agents (BCAs), offering environmentally benign alternatives to synthetic insecticides. These fungi have demonstrated efficacy against agricultural and forest pests, highlighting their potential for integrated pest management (Faria and Wraight, 2007, Zimmermann, 2008, Posadas *et al.*, 2012). Importantly, the impacts of EPFs on non-target organisms are generally considered to be dose-dependent and transient, particularly under field conditions where environmental constraints limit persistence (Scheepmaker and Butt, 2010).

The distribution of EPF is influenced by habitat type, soil depth, and edaphic factors such as pH, organic carbon, and nitrogen content (Quesada-Moraga *et al*., 2007). These factors significantly affect the prevalence and diversity of fungal communities across forest, grassland, and agricultural soils (Sun and Liu, 2008). However, their vertical distribution within soil profiles remains poorly resolved. Rather than exhibiting consistent stratification with depth, EPF occurrence reflects complex interactions among abiotic conditions, microbial competition, vegetation and host availability (Lacey *et al*., 2015). These interacting drivers can obscure clear vertical patterns, making it difficult to predict how EPF communities are distributed within soil profiles. Additionally, habitat-specific differences in fungal diversity underscore the importance of selecting diverse sampling sites for bioprospecting efforts (Quesada-Moraga *et al.*, 2007, Lacey *et al.*, 2015).

Bioprospecting for EPFs as sustainable alternatives to chemical pesticides, often involves collecting soil from natural habitats and baiting with laboratory model organisms such as *Galleria mellonella* L., 1758 (Inglis *et al.*, 2008, Sun and Liu, 2008, Oulevey *et al.*, 2009, Uzman *et al.*, 2019) or *Tenebrio molitor* L., 1758 (Glare *et al.*, 2008, Korosi *et al.*, 2019). In

some cases, a dual baiting technique is used combining both larvae baits, which are highly susceptible to EPF infection. This approach has been shown to enhance the variability of isolated fungal strains within bait species (Oreste *et al.*, 2012, Sharma *et al.*, 2018). Despite the widespread use of these methods, there is very little information in the published literature advising on which habitats are the most profitable for obtaining EPF, how deep in the soil EPF reside in various habitats and which baits are the most useful for obtaining a diversity of EPF strains from natural habitats. Not only are diversity (species richness and strain richness) and abundance (number of isolates obtained) important, but differences in composition or beta-diversity is important when assessing the collecting strategy for EPF bioprospecting.

This study investigates the effects of habitat, soil depth, edaphic factors, and bait type on the diversity and composition of EPF in Lancashire Nature Reserves. By integrating ecological and molecular approaches, this research aims to identify optimal strategies for EPF bioprospecting, with potential applications in sustainable pest management and agricultural practices. Through targeted sampling and innovative baiting techniques, the findings are expected to enhance our understanding of EPF ecology and the development of effective BCAs.

Materials and Methods

Wild entomopathogenic species of Ascomycota fungi were obtained from local nature reserves across Lancashire. To target EPFs with affinity to Lepidoptera and/or Coleoptera a dual-baiting technique was used based on Sharma *et al.* (2018). To account for potential vertical stratification of EPF communities, soil cores were divided into two depth fractions (0–10 cm and 10–20 cm), reflecting differences in biological activity and root density in upper soil layers (Coleman *et al.*, 2018).

Soil sampling

Soil samples were collected from the Nature Reserves network in Lancashire, Manchester and North Merseyside between 2020 and 2023 (https://www.lancswt.org.uk/nature-reserves). The natural reserves to be sampled were selected based on their online descriptions prioritising reported high diversity of insects and/or insectivorous species within well-established and well-preserved vegetal communities (Appendix table S2.1). The first sampling was conducted across eight nature reserves during July and September 2020. From this survey, twelve EPF isolates exhibiting high virulence were retained and subsequently

used in laboratory (*Chapter 3*) and field (*Chapter 4*) bioassays. The remaining isolates were stored for extended periods and losing its viability, preventing their molecular identification. However, their morphological features, recorded prior to deterioration, allowed classification into *Beauveria/Clonostachys* and *Metarhizium*-like morphotypes, which are included in the present chapter for comparative purposes. Thus, the 2020 dataset is presented as a preliminary survey to inform site selection and to provide ecological context for the strains investigated in subsequent chapters.

A second baiting campaign was conducted in March 2022, targeting the four reserves with the highest EPF recovery in 2020. However, these samples were lost due to incubator failure and were therefore excluded from further analysis.

The third and final sampling in March 2023 was processed immediately, as no parallel experiments delayed its processing. As a result, this sampling yielded the most complete dataset, including molecular identification of isolates, which forms the core of the present thesis chapter.

The four final sites selected included grassland (Middleton Nature Reserve "MD", SD 41740 59239), a semi-natural woodland (Foxhill Bank Local Nature Reserve "FX", SD 74059 27978) and two grassland quarries (Salthill Quarry Local Nature Reserve "SQ", SD 75508 42490 / Cross Hill Quarry Local Nature Reserve "CQ", SD 74471 43356) (Table 2.1). The four discarded sites for 2023 sampling included semi-natural woodland (Boilton, Nab, Redscar and Tunbrook Woods "BN", SD 580 313), lowland dune heath (Freshfield Dune Heath "FF", SD 298 081), raised bog (Cadishead & Little Woolden Moss "CM", SJ698 951), and lowland heath (Highfield Moss "HM", SJ 609 961) (Table 2.2).

Sampling strategy followed a randomised design adapted from Orozco *et al.*, (2014). At each site, a 5m x 5m plot was established, within which nine equidistant points were defined. At each point, soil was collected at two depths (0-10 cm and 10-20 cm), yielding eighteen 500 g soil samples that were collected in 500 ml plastic take-away containers with the lid pierced five times for ventilation, using a gauge auger that was sterilised between each sample. The samples were stored separately for 2-3 weeks at 4^{o}C until they were processed (Figure 2.1).

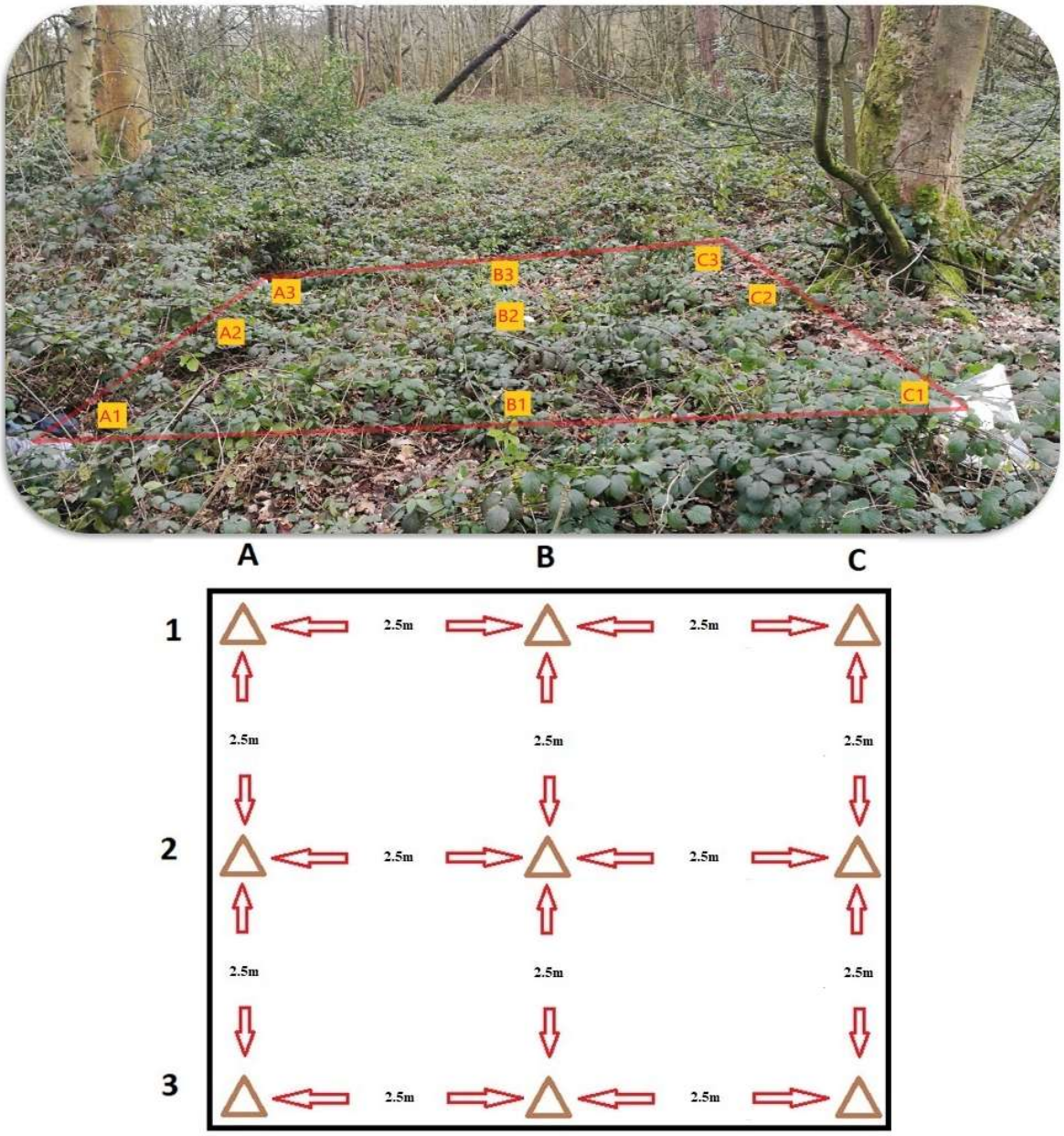


Figure 2.1. Schematic representation of the soil sampling design used for the collection of entomopathogens from natural habitats: At each site, a 5 m × 5 m plot was established and subdivided into nine equidistant sampling points arranged in a grid. At each point, soil was collected at two depths (0–10 cm and 10–20 cm) using a sterilised gauge auger, yielding a total of 18 samples per site (n = 18). Each sample consisted of approximately 500 g of soil placed into 500 ml ventilated plastic containers (lid perforated to allow airflow). Samples were stored individually at 4 °C for 2–3 weeks prior to processing. The sampling design was adapted from Orozco *et al*. (2014).

Extra vegetation and soil surveys were undertaken on the 14th and 25th of September 2023 on each natural reserve's previously sampled areas. Vegetation surveys were conducted in

triplicates from each of the sampling points to obtain their NVC categories (Appendix tables S2.2, S2.3, S2.4 and S2.5). Soil cores were collected with a gauge auger; in Middleton, the core consisted of four fractions of 20 cm each, whereas from the other reserves (FX, SQ and CQ) only three of such fractions were collected as bedrock was reached. In the laboratory, using PRIMACS ™ SNC-100 Carbon/Nitrogen analyser (Skalar Analytical B.V.) following manufacturer's indications, the percentage of total Nitrogen (TN) and the percentage of total Carbon (TC) were obtained from 100 mg of their respective oven dried core fractions. In the same fashion, other 100 mg were used to obtain the percentage of inorganic Carbon (IC) and organic Carbon (OC). The pH value from each soil fraction was measured from a solution of 10g of dried soil according to British Standards (BSI,1990).

Each reserve was assigned an NVC category based on its dominant vegetation and edaphic properties (Table 2.1):

- Salthill Quarry was classified as NVC MG5 (*Cynosurus cristatus* - *Centaurea nigra* grassland). The mean pH was 6.52 ± SE 0.51. Soil contained 3.23 ± SE 0.35% TC, 0.25 ± SE 0.03% TN, -0.006 ± SE 0.000% IC, and 3.24 ± SE 0.35% organic carbon. The mean moisture content was 45.23 ± SE 11.17%, with the highest values recorded at 40–60 cm depth.
- Cross Hill was classified as NVC MG1 (*Arrhenatherum elatius* grassland). The mean pH was 8.18 ± SE 0.05. Soil contained 2.95 ± SE 0.97% TC, 0.08 ± SE 0.01% TN, 2.97 ± SE 0.73% IC, and -0.02 ± SE 1.69% organic carbon. The mean moisture content was 45.89 ± SE 7.84%.
- Foxhill was classified as NVC W8 (*Fraxinus excelsior* - *Acer pseudoplatanus* - *Mercurialis perennis* woodland). The mean pH was 7.65 ± SE 0.39. Soil contained 2.53 ± SE 0.69% TC, 0.06 ± SE 0.02% TN, 0.06 ± SE 0.05% IC, and 2.47 ± SE 0.73% organic carbon. The mean moisture content was 47.72 ± SE 3.33%
- Middleton was classified as NVC M23 (Juncus effusus - Galium palustre rush pasture). The mean pH was 6.90 ± SE 0.14. Soil contained 1.19 ± SE 0.21% TC, 0.04 ± SE 0.03% TN, -0.006 ± SE 0.001% IC, and 1.19 ± SE 0.21% organic carbon. The mean moisture content was 49.72 ± SE 9.56%.

Table 2.1. Vegetation classification (NVC), characteristic plant species, and soil physicochemical properties by depth for four natural reserve sites sampled in September 2023: Sites are classified according to the UK National Vegetation Classification (NVC) system based on dominant vegetation communities. At each site, soil cores were collected using a gauge auger and subdivided into depth fractions (0–20, 20–40, and 40–60 cm). For each depth, total carbon (TC, %), total nitrogen (TN, %), inorganic carbon (IC, %), organic carbon (OC, %), soil pH, and moisture content (%) were measured. Mean values represent averages across depth fractions within each site (n = 3 per site), with associated standard error (± SE). Characteristic plant species associated with each NVC category are listed.

Site	NVC & Species	Depth (cm)	TC (%)	TN (%)	IC (%)	OC (%)	pH	Moisture (%)
Salthill Quarry	MG5	0-20	3.9	0.26	-0.006	3.91	5.93	32.58
	Deschampsia flexuosa	20-40	2.97	0.29	-0.006	2.98	6.09	36.03
	Amblystegium serpens	40-60	2.81	0.19	-0.006	2.82	7.56	67.07
	Centaurea nigra	**Mean**	3.23	0.25	-0.006	3.24	6.52	45.23
		± SE	0.35	0.03	0	0.35	0.51	11.17
Cross Hill	MG1	0-20	3.12	0.1	2.016	1.1	8.26	61.2
	Holcus lanatus	20-40	4.5	0.06	2.413	2.09	8.19	41.09
	Kinderbergia praelonga	40-60	1.23	0.07	4.47	-3.24	8.1	35.38
	Equisetum arvense	**Mean**	2.95	0.08	2.97	-0.02	8.18	45.89
		± SE	0.97	0.01	0.73	1.69	0.05	7.84
Foxhill	W8	0-20	3.89	0.11	0.003	3.89	6.83	52.17
	Rubus fruticosus	20-40	2.14	0.03	0.016	2.12	8.07	41.21
	Climacium dendroides	40-60	1.55	0.04	0.158	1.39	8.04	49.77
	Geranium robertianum	**Mean**	2.53	0.06	0.06	2.47	7.65	47.72
		± SE	0.69	0.02	0.05	0.73	0.39	3.33
Middleton	M23	0-20	1.62	0.11	-0.006	1.63	7.13	68.88
	Juncus effesus	20-40	0.93	0.01	-0.005	0.94	6.93	41.79
	Pulicaria dysenterica	40-60	1.01	0.01	-0.007	1.01	6.63	38.5
	Deschampsia flexuosa	**Mean**	1.19	0.04	-0.006	1.19	6.9	49.72
		± SE	0.21	0.03	0.001	0.21	0.14	9.56

Dual baiting technique

The baiting technique that we used to collect entomopathogenic fungi was adapted from the dual baiting technique proposed by Sharma *et al.*, (2018). Processing of the soil samples started by removing rocks and organic matter before dividing each sample in halves into two 500 ml ventilated containers where to apply separately two insect bait types. Five late instar larvae of *G. mellonella* (Lepidoptera) were placed in one of the containers and five late instar larvae of *T. molitor* (Coleoptera) in the other incubaiting them at 22ºC for four weeks in dark conditions. During the first week, the containers were checked daily for dead insects and from the second week they were checked every second day. The containers were gently shaken after every check throughout the twenty-eight days ensuring that the baits entered in contact with as much soil sample as possible.

The selection of dead insects started by discarding the ones presenting a putrid smell due to bacterial infection and displaying soft and dark bodies. The rest of dead insects were collected as they were likely to have been killed by fungi/nematodes. Collected insects were rinsed prior the removal of saprotrophs by surface flaming of the bodies previously dipped in 70% ethanol according the recommendations of (McNamara *et al.*, 2018a). To favour development of entomopathogens, collected bodies were incubated in moist chambers (9cm diameter Petri dishes with wet filter paper) at 22ºC in the dark. When the bodies displayed mycelium, they were rinsed and incubated at 27ºC for 15-20 days in dodine-free selective media consisting of Oats 20 g/l, Agar 20 g/l, Chloramphenicol 0.5 g/l and cetyl trimethyl ammonium bromide (CTAB) 0.6 g/l following indications of Posadas *et al.*, (2012). This media inhibits saprophyte fungi and bacteria growth while allowing development of entomopathogenic fungi on it. In order to obtain pure cultures, grown mycelium was incubated in potato dextrose agar (PDA) media under the same conditions. When contaminations or mixed colonies were detected at PDA, samples were plated back into a fresh oat selective media plate then to a PDA one repeating the process until obtaining pure sporulated cultures (Figure 2.2).

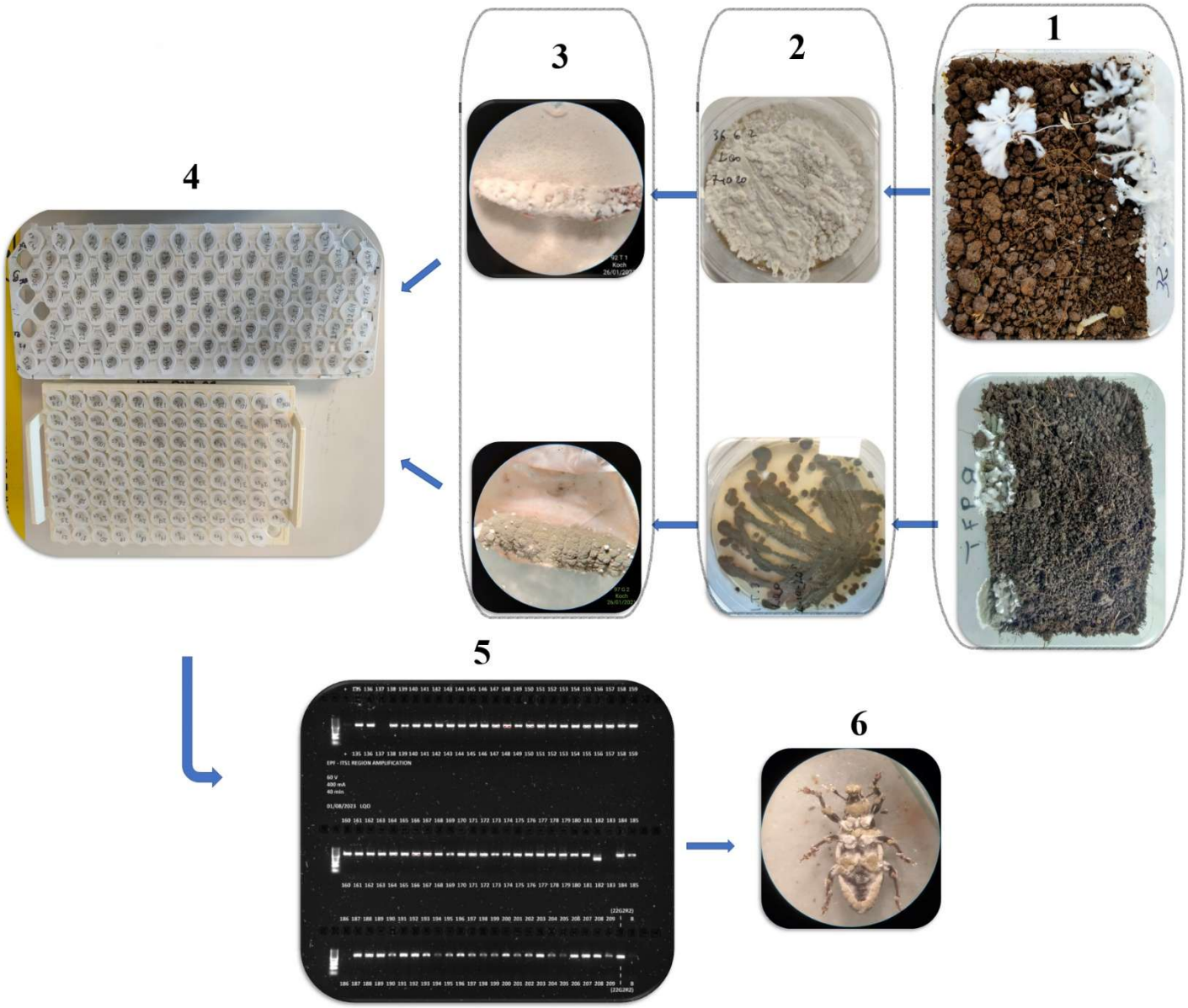


Figure 2.2. Workflow of EPF isolation, screening, and molecular identification from soil samples using a dual insect baiting approach: (1) Soil samples were processed using a dual baiting technique, where each sample was divided and baited separately with five larvae of *Galleria mellonella* and five larvae of *Tenebrio molitor* (n = 5 larvae per bait type), and incubated at 22 °C in the dark for four weeks. (2) Transfer to selective oat-based medium and subsequent subculturing on potato dextrose agar to obtain pure isolates. (3) Pathogenicity of isolates was confirmed by re-infecting host larvae and verifying fungal outgrowth consistent with the original isolate. (4) Genomic DNA was extracted from pure cultures using a commercial plant/fungal DNA extraction kit. (5) The fungal internal transcribed spacer (ITS) region was amplified using ITS1-F and ITS4 primers and sequenced for molecular identification. (6) Confirmed EPF isolates were subsequently tested against

target insect pests in downstream bioassays. The diagram summarises the sequential pipeline from soil baiting to functional validation of EPF isolates.

Pathogenicity test to select EPF from other isolated fungi:

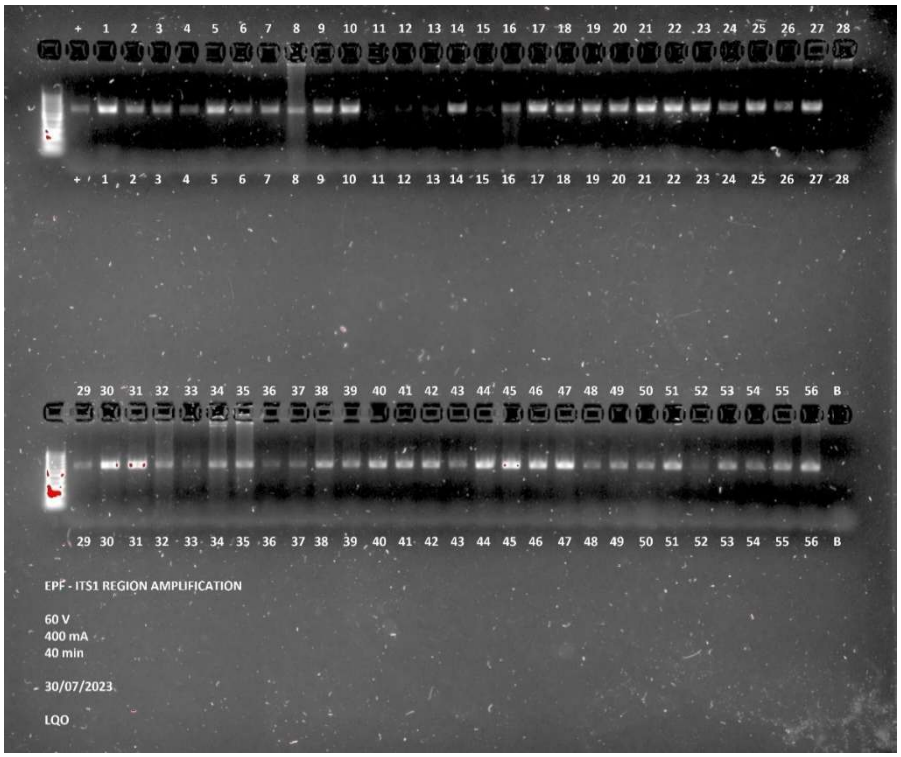
To test the pathogenicity of the pure cultures we followed a protocol based on Sun and Liu, (2008) where each fungus was tested against two of their original bait hosts (*Galleria-Galleria*, *Tenebrio-Tenebrio*) to confirm that the isolate was the cause of death. Spores were rinsed out from PDA plates using 1ml sterile distilled water containing 0.03% (v/v) viscous Tween-80 (Sigma-Aldrich, Dorset, UK), then collected into a 1.5 ml Eppendorf tube from each pure isolate. From each Eppendorf tube,100µl of the extraction were plated in PDA media to grow fresh pure mycelium at 27ºC to be used in the next stages. Proceeding with the pathogenicity test, two insect larvae were dipped, one at time, for 5s in each suspension using sterilised tweezers and both incubated in a moist chamber at 22ºC in the dark for 4 weeks where they were inspected daily until pupation or death (Sun and Liu, 2008). Moist chamber humidity conditions were tailored according to our own experience using 1 ml sterile water for *G. mellonella* and 0.5 ml for *T. molitor* to avoid premature death of the second ones. To identify if larvae were killed by the original fungi, we compared the growth of mycelium and spores to their respective original pure cultures and when the same morphology was obtained those strains moved onto the next phases, otherwise, they were discarded. Finally, 209 isolated fungi were moved to the next phases for their molecular identification.

DNA extraction Molecular Identification of entomopathogenic fungi

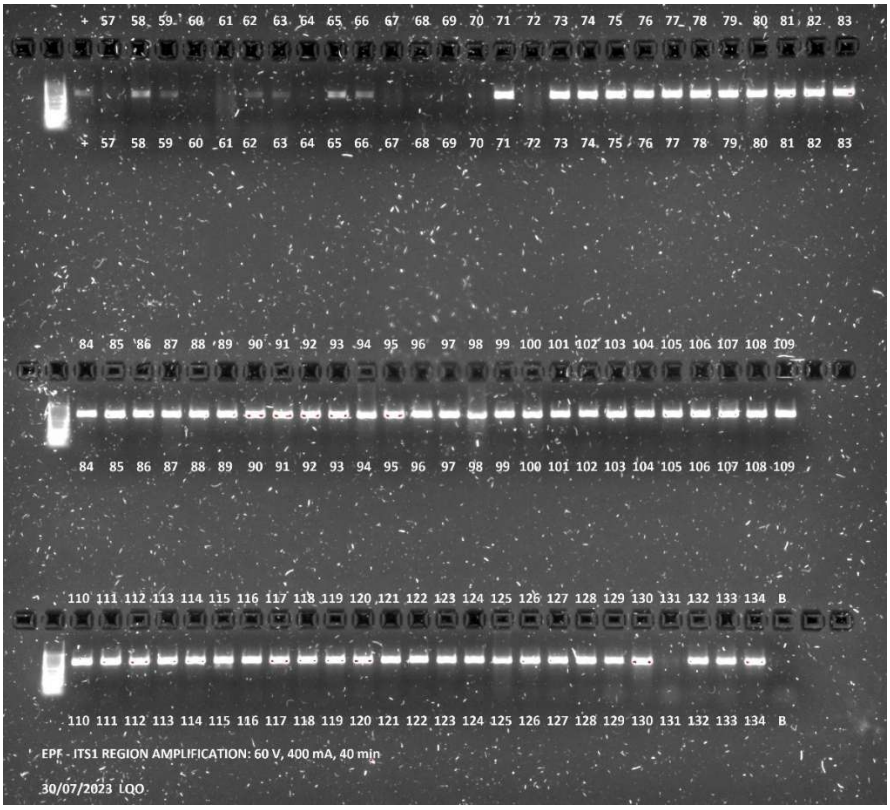
50-100 mg of freshly grown fungi and spores were collected from 209 PDA plates as source of DNA to be extracted using sterilised tweezers, then placing the fungal material in 2ml tissue disruption tubes from DNeasy ® Plant Pro kit (Quiagen). Each sample was prepared following recommendations of Williams *et al.*, (2013b) proceeding to quick freeze the tubes in liquid nitrogen (LN2), followed by tawing at 60ºC in a hot plate, then quick freezing them for a second time obtaining samples ready to be processed according to DNeasy Plant Pro kit protocol. The concentration of DNA obtained was measured using a microvolume UV-Vis spectrometer (NanoDrop™ One, Thermo Scientific), then standardised to 5ng/µl before proceeding with PCR following a modified protocol of Taq PCR Master Mix Kit (Qiagen).

To amplify the fungal internal transcribed spacer region (ITS), the forward primer was ITS1-F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and the reverse primer was ITS4 (5'-

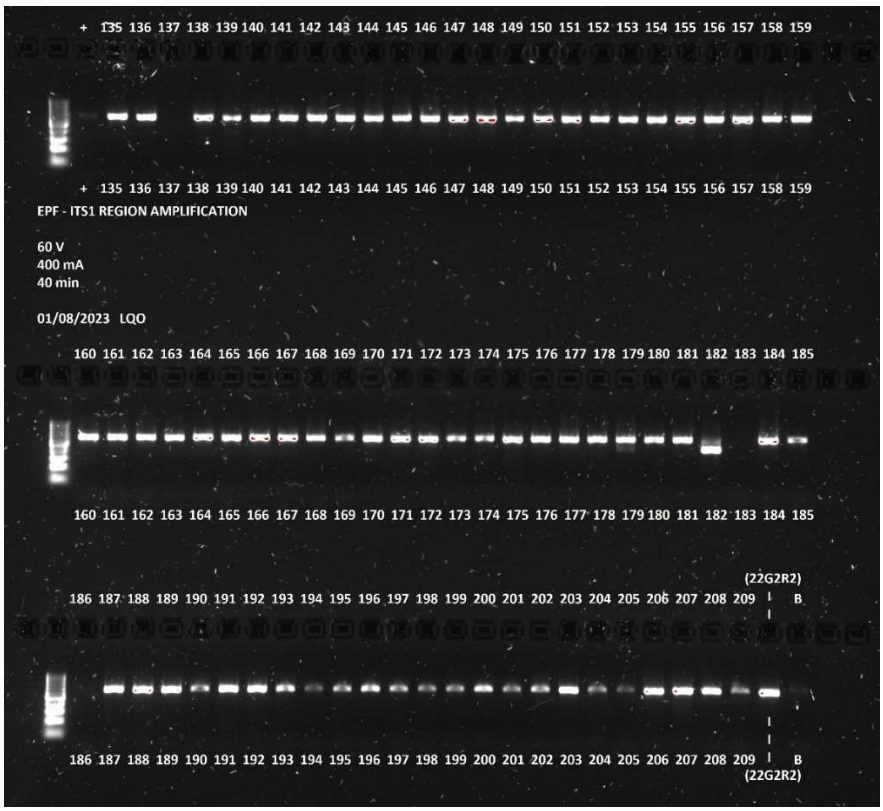
TCCTCCGCTTATTGATATGC-3') (Gardes and Bruns, 1993). For each reaction the setup for a total volume of 25µl was 0.4µM of each primer and 8ng of DNA (12.5µl Master mix, 2µl of each primer [from 5µM], 5µl DNA, 3,5µl water RNase-free). PCR conditions were: Initial denaturation for 5 min at 95 ºC, followed by 35 cycles of 30 sec denaturation at 95ºC, annealing for 30 sec at 50ºC and extension for 2 min at 72ºC. Reactions were completed with a final elongation at 72ºC for 7 min (Yurkov *et al.*, 2015). PCR product was visualised in agarose gel to confirm ITS amplification fragments to be purified using QIAquick ® PCR Purification Kit (Quiagen) and sent for forward and reverse Sanger sequencing to Eurofins Genomics (Figure 2.3.A, B and C).



A



B



C

Figure 2.3. Agarose gel electrophoresis of PCR-amplified ITS region for fungal isolates used in this study: PCR products targeting the internal transcribed spacer (ITS) region were

separated by agarose gel electrophoresis following amplification with ITS1-F and ITS4 primers. Gels were run for 40 min at 60 V (constant current ~400 mA). (A) Isolates 1–56; (B) isolates 57–134; (C) isolates 135–209, including a re-run of isolate 22G2R2 for verification. Lanes labelled "B" correspond to blank (no-template) controls. Successful amplification is indicated by the presence of clear bands at the expected ITS size (~500–700 bp). A total of 209 isolates were screened (n = 209).

From the 209 samples only 197 samples gave quality ITS region sequences that were processed with SeaView 4.7 (Gouy *et al.*, 2010). Reverse sequences were translated into their complement sequences to be aligned with its respective forward sequence using Multiple Sequence Comparison by Log-Expectation (MUSCLE) generating a consensus sequence. Each EPF isolated sequence was processed in BLAST® (National Center for Biotechnology Information) obtaining identities > 88.74% (Appendix Table S2.6).

Statistical analyses and community ecology methods

All statistical analyses were conducted using R version 4.5.2 (R Core Team, Vienna, Austria). Data handling, statistical modelling and visualisation were performed using the packages *tidyverse*, *vegan*, *emmeans*, *indicspecies* and associated dependencies.

Data structure and preprocessing

For the 2023 bioprospecting campaign, two bait insect species (*Galleria mellonella* and *Tenebrio molitor*) were used at each sampling point. The primary sampling unit for unpooled analyses was therefore defined as an individual soil sample (topsoil or bottom soil), with paired observations, one for each bait insect species. Response variables included the number of isolates per sample, number of strains per sample, and number of species per sample.

In addition to depth-stratified analyses, pooled soil datasets were generated by combining topsoil and bottom soil samples collected at each sampling point (e.g. samples 1 + 2, 3 + 4) to produce a single composite soil sample. Pooled analyses were used as a secondary validation to assess EPF diversity and abundance at the scale of individual soil cores.

All analyses were conducted at the strain level for the 2023 dataset. For the 2020 dataset, which lacked molecular identification, analyses were conducted using morphologically assigned genera (*Metarhizium* and *Beauveria/Clonostachys*).

Bait insect comparisons

Because two bait insects were used, all analyses treating bait as a factor were conducted as two-level paired comparisons. Data were structured at the sample level such that each soil sample yielded paired observations, one for each bait insect species. Normality of response variables (number of isolates per sample, number of strains per sample and number of species per sample) was assessed using Shapiro–Wilk tests and indicated non-normal distributions; therefore, bait effects were evaluated using Wilcoxon signed-rank tests. Analyses were performed overall and stratified by site and soil depth to confirm consistency of patterns.

To assess whether individual fungal strains showed differential recovery between bait insects, strain-by-bait contingency tables were generated from isolate-level data and tested using Fisher's exact test due to sparse counts and low expected cell frequencies. Strain detection overlap was summarised by identifying strains recovered by both bait insects versus strains recovered by only one bait.

Alpha diversity analyses

Alpha diversity was quantified at the strain level using richness (number of strains per sampling unit) and Shannon diversity (H′), calculated from strain abundance matrices.

Strain richness, being count-based, was analysed using generalised linear models (GLMs) with a Poisson error distribution and log link function. Shannon diversity was analysed using linear models (LMs) assuming Gaussian errors. For both metrics, site (FX, CQ, SQ, MD), soil depth (topsoil vs bottom), soil pH and moisture content were included as explanatory variables.

Estimated marginal means (EMMs) were computed for categorical predictors using the *emmeans* package to facilitate interpretation and graphical presentation. Pairwise comparisons among sites were conducted using Tukey-adjusted contrasts. Model assumptions were assessed through inspection of residual plots, and overdispersion was evaluated for Poisson models.

Separate alpha diversity analyses were performed for unpooled and pooled soil datasets to compare the effects of vertical stratification on diversity estimates.

Community composition analyses

Compositional differences in entomopathogenic fungal communities among sampling units were analysed using multivariate techniques based on Bray–Curtis dissimilarity of strain abundance data. A matrix of strains by samples was established using isolate counts

per strain, and a corresponding metadata table was assembled including site, soil depth, bait insect and edaphic variables Bray–Curtis dissimilarity was selected as it is appropriate for sparse ecological community data, gives greater weight to abundant taxa and is robust to joint absences. Differences in community composition among categorical grouping variables (site, soil depth and bait insect) were formally tested using Multi-Response Permutation Procedure (MRPP), based on Bray–Curtis distances and 9999 permutations. Effect sizes were quantified using the chance-corrected within-group agreement statistic (A) and the observed within-group dissimilarity (δ). Values of A close to zero indicate no stronger within-group similarity than expected by chance, whereas values approaching one indicate strong within-group homogeneity.

Complementary permutational multivariate analysis of variance (PERMANOVA) was also conducted to quantify the proportion of community variation explained by each factor.

To evaluate whether differences detected by PERMANOVA could be influenced by unequal multivariate dispersion among groups, homogeneity of group dispersions was assessed using permutational analysis of multivariate dispersions (PERMDISP). This procedure calculates the average distance of samples to their group centroid in multivariate space and tests whether dispersion differs among groups using permutations. PERMDISP analyses were conducted using Bray–Curtis distances with 9999 permutations. Significant PERMDISP results indicate differences in within-group variability rather than differences in community centroid position, allowing distinction between true compositional shifts and heterogeneity of dispersion.

Ordination and environmental fitting

Classical multidimensional scaling (Principal Coordinates Analysis, PCoA) was used to visualise patterns of compositional dissimilarity among samples. The proportion of variance explained by each ordination axis was calculated from positive eigenvalues.

Environmental fitting (envfit) was applied to the PCoA ordination to assess correlations between fungal community composition and measured edaphic variables using 9999 permutations. Only unpooled soil data were used for envfit analyses, as pooling removes vertical structure and may obscure relationships between fungal assemblages and soil chemistry gradients.

Indicator species analysis

Indicator Species Analysis (ISA) was performed to identify EPF strains significantly associated with levels of each grouping variable (site, soil depth and bait insect). ISA was conducted using the IndVal.g statistic, which combines measures of relative abundance and

relative frequency while correcting for unequal group sizes. Statistical significance was assessed using 5000 Monte Carlo permutations.

Results

Community composition, abundance and dominance.

2020 morphotype-level survey

The 2020 survey provided a preliminary assessment of EPF occurrence across eight sites, with community composition resolved at the morphotype level (Table 2.2). At FX and CQ, EPF assemblages were dominated by *Metarhizium*-like morphotypes in terms of both prevalence and abundance. In the unpooled dataset, *Metarhizium*-like morphotypes were detected in 13 samples at FX, compared with three samples containing *Beauveria/Clonostachys*-like morphotypes, and in 16 samples at CQ, compared with nine samples containing *Beauveria/Clonostachys*-like morphotypes. Mean isolate abundance per positive sample was also higher for *Metarhizium*-like morphotypes at both sites (FX: 1.56 vs 1.00; CQ: 2.14 vs 2.08; Table 2.2).

Table 2.2. Distribution of EPF morphotypes recovered from unpooled and soil-pooled samples during the 2020 bioprospecting survey: Unpooled data represent individual soil samples collected at two depths (topsoil: 0–10 cm and bottom soil: 10–20 cm; n = 18 samples per site), whereas pooled data combine both depths within each sampling point (n = 9 pooled samples per site). Values indicate the number of samples in which each morphotype was detected (samples positive) and the mean number of isolates recovered per positive sample. Abbreviations: *Beauveria/Clonostachys* = isolates morphologically consistent with *Beauveria* spp. or *Clonostachys rosea* f. *catenulata*; *Metarhizium* = isolates morphologically consistent with *Metarhizium* spp. Site codes: FX = Foxhill Bank; CQ = Cross Hill Quarry; SQ = Salthill Quarry; MD = Middleton; HM = Highfield Moss.

Site	Morphotype	Samples positive (unpooled)	Mean isolates (unpooled)	Samples positive (pooled)	Mean isolates (pooled)
FX	*Beauveria/Clonostachys*	3	1.00	2	1.50

FX	*Metarhizium*	13	1.56	8	2.33
CQ	*Beauveria/Clonostachys*	9	2.08	7	2.50
CQ	*Metarhizium*	16	2.14	9	3.36
SQ	*Beauveria/Clonostachys*	8	1.56	5	2.33
SQ	*Metarhizium*	4	1.25	3	1.67
MD	*Beauveria/Clonostachys*	3	1.67	3	1.67
MD	*Metarhizium*	2	2.00	2	2.00
HM	*Beauveria/Clonostachys*	3	1.00	3	1.00
HM	*Metarhizium*	2	1.00	2	1.00

In contrast, *Beauveria/Clonostachys*-like morphotypes were more prevalent than *Metarhizium*-like morphotypes at SQ, MD and HM. This pattern was most pronounced at SQ, where *Beauveria/Clonostachys*-like morphotypes were detected in eight unpooled samples compared with four for *Metarhizium*-like morphotypes (Table 2.2). These results indicate site-level variation in morphotype composition rather than uniform dominance of a single morphotype group across all sites.

Clear differences in overall EPF recovery were also observed among sites. CQ and FX exhibited the highest EPF recovery, SQ showed intermediate recovery, and MD and HM yielded comparatively low levels. No EPF were detected at FF, CM or BN (Table 2.3). Based on these preliminary results, FX, CQ, SQ and MD were selected for structured resampling in 2023.

Pooling topsoil and bottom-soil samples increased the number of isolates represented within individual sampling points but did not substantially alter the broad ranking of productive sites (Tables 2.2 and 2.3).

Table 2.3. Mean number of EPF isolates recovered per soil sample across sites sampled in 2020 and 2023: Unpooled values represent arithmetic means of EPF isolates recovered from individual soil samples collected at two depths (topsoil: 0–10 cm; bottom soil: 10–20 cm; n = 18 samples per site). Pooled values represent arithmetic means obtained after combining topsoil and bottom soil samples from each sampling point (n = 9 pooled samples per site). Habitat descriptors are based on dominant vegetation communities and site management classifications obtained from the Lancashire Wildlife Trust Nature Reserves (https://www.lancswt.org.uk/nature-reserves). A dash (–) indicates sites not sampled in 2023.

Site code	Site name	Habitat descriptor	Mean isolates per sample 2020	Mean isolates per sample 2023	Mean isolates per pooled sample 2020	Mean isolates per pooled sample 2023
FX	Foxhill Bank LNR	Semi-natural woodland	0.86	2.19	3.44	8.78
CQ	Cross Hill Quarry LNR	Grassland quarry	2	1.53	8	6.11
SQ	Salthill Quarry LNR	Grassland quarry	0.53	1.28	2.11	5.11
MD	Middleton NR	Grassland	0.25	0.47	1	1.89
HM	Highfield Moss	Lowland heath	0.14	-	0.56	-
FF	Freshfield Dune Heath	Lowland dune heath	0	-	0	-
CM	Cadishead & Little Woolden Moss	Raised bog	0	-	0	-
BN	Boilton–Nab–Redscar–Tunbrook Woods	Semi-natural woodland	0	-	0	-

2023 strain-level community structure

These morphotype-level patterns were subsequently confirmed in the 2023 survey, where molecular identification revealed a *Metarhizium*-dominated community structure with consistent site-level differences in strain composition. Across the four sites sampled, 72 depth-stratified soil samples were processed, of which 54 yielded EPF isolates. In total, 197 isolates representing 13 genetically distinct strains were recovered. These strains corresponded to five species (*Metarhizium anisopliae*, *Metarhizium sp*., *Beauveria bassiana*, *Beauveria brongniartii,* and *Clonostachys rosea*), belonging to three genera: *Metarhizium* (8 strains), *Beauveria* (4 strains) and *Clonostachys* (1 strain) (Table 2.4).

Table 2.4. Site-level frequency and abundance of entomopathogenic fungal (EPF) strains recovered during the 2023 survey (unpooled dataset). Values are presented as *x (y)*, where *x* represents the number of physical soil samples in which each strain was detected, irrespective of bait insect, and *y* represents the total number of isolates recovered. The Total column gives the number of soil samples in which each strain was detected and the total number of isolates recovered across all four sites. A total of 72 physical soil samples were examined (n = 18 per site). Site codes: FX = Foxhill Bank Local Nature Reserve; CQ = Cross Hill Quarry Local Nature Reserve; SQ = Salthill Quarry Local Nature Reserve; MD = Middleton Nature Reserve. Abbreviations: Ma = *Metarhizium anisopliae*; Msp = *Metarhizium* sp.; Bb = *Beauveria bassiana*; B. brongniartii = *Beauveria brongniartii*; Cr f. catenulata = *Clonostachys rosea* f. *catenulata*.

Strain	FX	CQ	SQ	MD	Total
Ma 687	14 (48)	7 (11)	13 (27)	5 (14)	39 (100)
Msp RMK-2011b	12 (19)	12 (25)	6 (11)	1 (1)	31 (56)
B. brongniartii	0	0	4 (4)	0	4 (4)
Bb DAOM210087	0	0	0	2 (2)	2 (2)
Ma PL52-1	4 (5)	7 (7)	1 (1)	0	12 (13)
Cr f. catenulata	3 (3)	3 (4)	1 (1)	0	7 (8)
Ma T5R2	2 (2)	3 (3)	1 (1)	0	6 (6)
Ma var. anisopliae strain LRC 142	2 (2)	1 (1)	0	0	3 (3)
Bb DAOM195005	0	0	1 (1)	0	1 (1)
Ma ARSEF5851	0	1 (1)	0	0	1 (1)
Ma var. anisopliae isolate F4	0	1 (1)	0	0	1 (1)
Bb KK2	0	1 (1)	0	0	1 (1)

Ma var. anisopliae isolate GMX10.2-3	0	1 (1)	0	0	1 (1)

Table 2.4 summarises the frequency of recovery and isolate abundance for each strain at each site, allowing comparison of strain prevalence across habitats. *Metarhizium* dominated recoveries, accounting for 181 of 197 isolates (91.9%). Two strains were highly prevalent: *M. anisopliae* strain 687 (100 isolates recovered from 39 samples) and *Metarhizium* sp. RMK-2011b (56 isolates from 31 samples). The remaining *Metarhizium* strains were recovered at much lower frequencies, including PL52-1 (13 isolates; 12 samples), T5R2 (6 isolates; 6 samples), LRC 142 (3 isolates; 3 samples), and three singletons (ARSEF5851, F4 and GMX10.2-3; one isolate each).

Beauveria was represented by three *B. bassiana* strains (DAOM195005, DAOM210087 and KK2; one to two isolates each) and one *B. brongniartii* strain (four isolates). *Clonostachys rosea* f. *catenulata* was recovered eight times from seven samples (Table 2.4).

Bait insect recovery patterns and co-occurence

To evaluate potential differences in recovery efficiency between bait insects, strain recovery frequencies and isolate counts were partitioned by bait species (Appendix Table S2.7). Across all sites and soil depths, *Galleria mellonella* and *Tenebrio molitor* recovered comparable numbers of strains and isolates, and the two dominant strains (*M. anisopliae* 687 and *Metarhizium sp.* RMK-2011b) were recovered frequently by both bait insects at broadly similar frequencies, with only minor differences in isolate counts between bait species. Statistical comparisons confirmed these patterns: paired Wilcoxon signed-rank tests detected no significant differences between bait insects in the number of isolates recovered per sample ($V = 553.5$, $p = 0.8896$), number of strains per sample ($V = 458.5$, $p = 0.7085$), or number of species per sample ($V = 176.0$, $p = 0.5026$). Similarly, Fisher's exact tests performed at the strain level indicated no evidence of differential recovery bias between bait insects ($p = 0.9734$).

Despite the absence of statistically significant bait effects, some low-frequency strains were detected by only one bait insect. For example, *Galleria mellonella* uniquely recovered *B. bassiana* KK2, *B. bassiana* DAOM195005, and *M. anisopliae* GMX10.2-3, whereas *Tenebrio molitor* uniquely recovered *M. anisopliae* ARSEF5851 and *M. anisopliae* F4. Consequently, five strains (38% of the total strain diversity) would not have been detected if only a single bait insect species had been used (Figure 2.4).

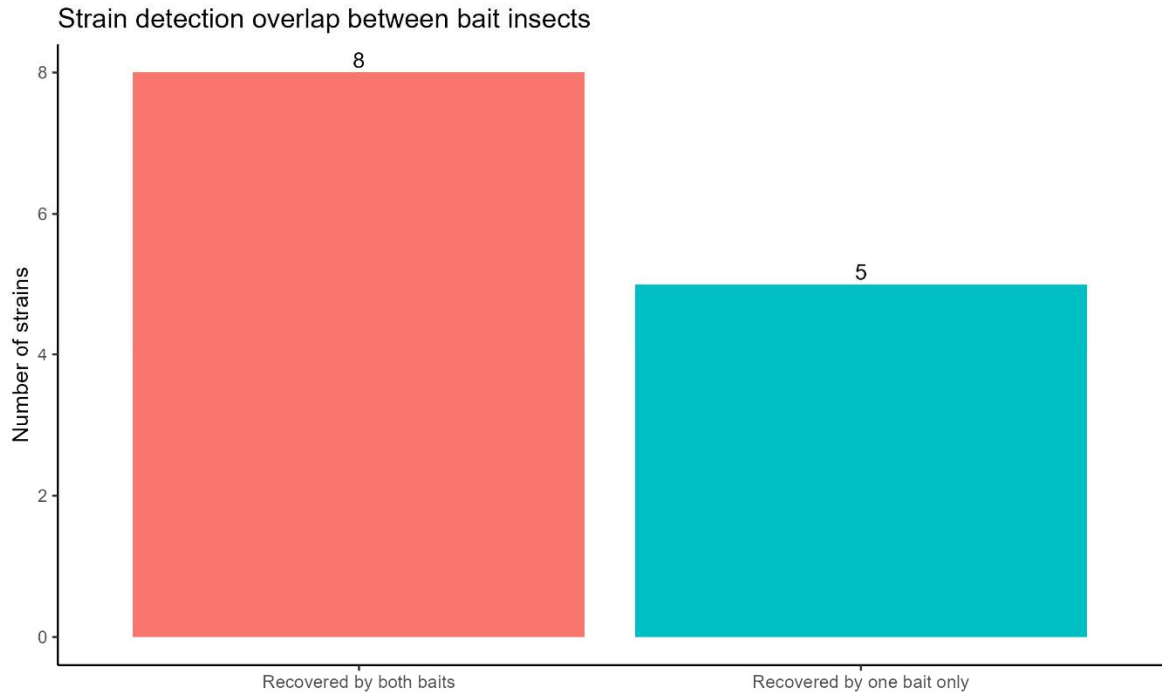


Figure 2.4. Comparison of EPF strain recovery between bait insects during the 2023 survey: Bar chart showing the number of EPF strains recovered by both bait insects (*Galleria mellonella* and *Tenebrio molitor*) versus strains recovered exclusively by one bait species. Across 72 physical soil samples (n = 18 per site), 13 EPF strains were identified; eight were recovered using both bait insect species and five were detected using only one bait species. Each physical soil sample was divided between the two bait insect treatments.

Multiple strains frequently co-occurred within individual soil samples. A total of 72 non-pooled soil samples were analysed across the four sites. EPF were recovered from 54 samples (75%), while the remaining 18 samples yielded no EPF isolates. Considering all samples collected, 30.6% of samples contained more than one EPF strain and 11.1% contained strains from more than one species, indicating that co-occurrence of taxa within individual soil samples was relatively common.

No entomopathogenic nematodes were recovered from any soil sample during the 2023 campaign.

Despite this increase in co-occurrence, pooled analyses did not alter overall dominance patterns, with *M. anisopliae* strain 687 and *Metarhizium* sp. RMK-2011b remaining the most prevalent and abundant strains across both bait types (Figure 2.5).

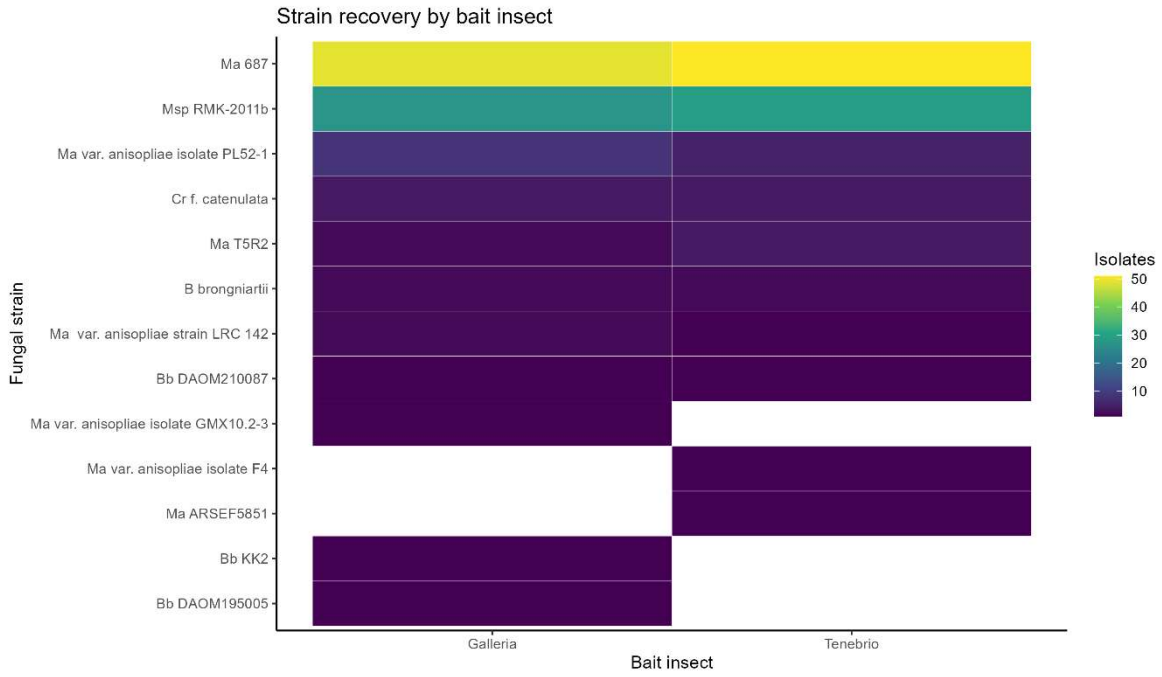


Figure 2.5. Heatmap of isolate abundance for EPF strains recovered using *Galleria mellonella* and *Tenebrio molitor* bait insects during the 2023 survey: Heatmap showing the total number of isolates recovered for each EPF strain–bait insect combination across 72 physical soil samples (n = 18 per site). Each soil sample was divided between the two bait insect treatments. Each tile therefore represents the total isolate abundance for a strain–bait combination across the complete survey rather than an individual replicate. Colour intensity is proportional to isolate abundance. Strains are ordered according to their total number of isolates across both bait insects.

Alpha diversity of EPF communities.

Tukey-adjusted pairwise comparisons detected no significant differences in unpooled strain richness among individual sites (Figure 2.6A). For unpooled Shannon diversity, MD exhibited significantly lower diversity than FX ($p = 0.0475$) and CQ ($p = 0.0093$), but did not differ significantly from SQ ($p = 0.2553$) (Figure 2.6B). Soil depth and bait insect did not show significant effects on strain richness, while the effect of bait insect on Shannon diversity was marginal but non-significant ($p = 0.097$).

Following pooling of topsoil and bottom-soil samples, Tukey-adjusted pairwise comparisons detected no significant differences in strain richness among sites (Figure 2.6C). For pooled Shannon diversity, MD was significantly lower than FX ($p = 0.0018$) and CQ ($p = 0.0024$), but did not differ significantly from SQ ($p = 0.1074$) (Figure 2.6D). Thus, site-level differences were more evident for Shannon diversity than for strain richness.

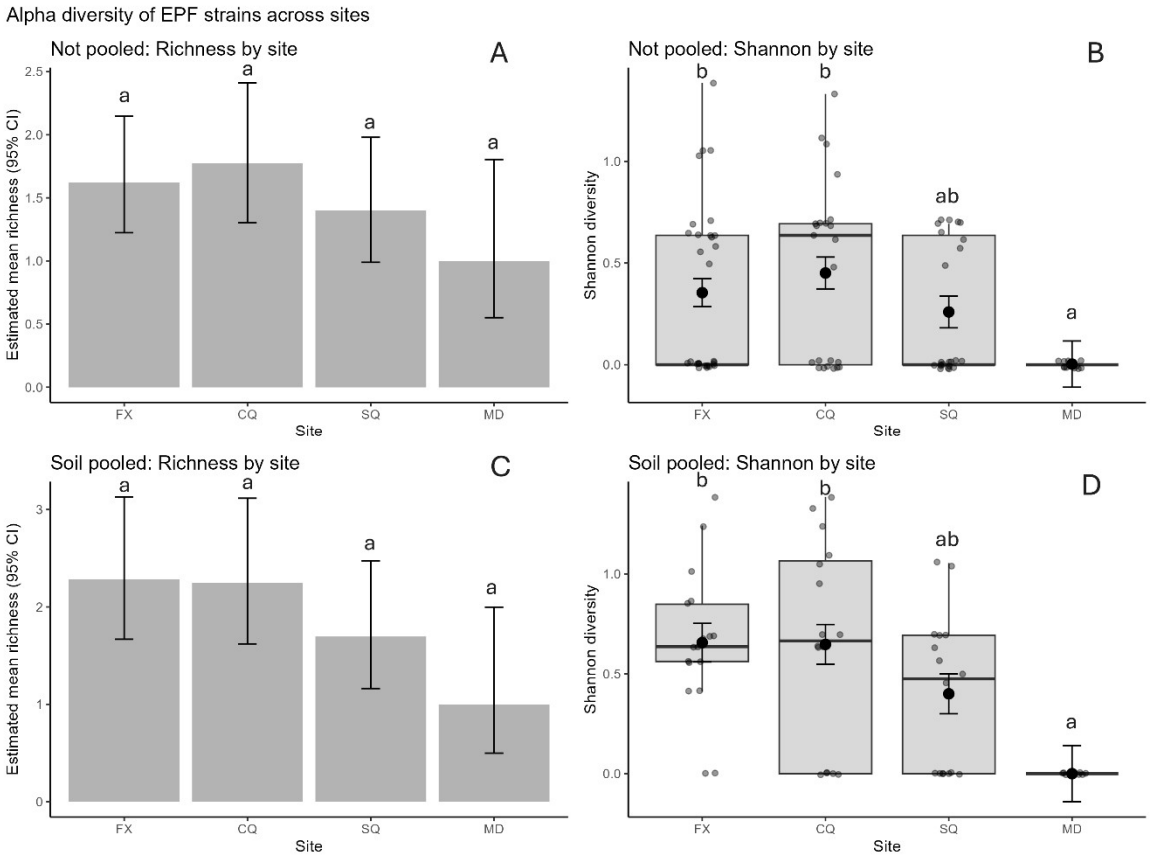


Figure 2.6 Alpha diversity of EPF communities across four sites sampled in 2023: Panels show (A) unpooled strain richness, (B) unpooled Shannon diversity index, (C) pooled strain richness, and (D) pooled Shannon diversity index. Unpooled analyses were based on 18 physical soil samples per site (n = 18; 72 samples in total). For pooled analyses, topsoil and bottom-soil samples from each sampling point were combined, resulting in 9 pooled samples per site (n = 9; 36 pooled samples in total). Points represent individual unpooled or pooled samples, and bars represent estimated marginal means ± standard error (SE) derived from the respective statistical models. Different letters indicate statistically significant differences among sites based on Tukey-adjusted pairwise comparisons ($\alpha = 0.05$); sites sharing the same letter did not differ significantly.

Soil pH and moisture content were included as continuous covariates in alpha diversity models; however, neither variable showed a significant association with strain richness or Shannon diversity in either the unpooled or pooled datasets (all $p > 0.05$), and their inclusion did not alter site-level patterns. These variables were therefore not retained in final reported models. By contrast, these variables were significantly associated with multivariate

community structure in ordination analyses (see below), indicating that edaphic factors influenced EPF composition rather than local richness.

Indicator species analysis.

Indicator Species Analysis (ISA) conducted on the unpooled dataset identified five EPF strains significantly associated with specific sites (IndVal.g; 5,000 permutations; Table 2.5). *Metarhizium anisopliae* strain 687 showed the strongest indicator value and was associated with sites FX, SQ and MD (IndVal = 0.81, p = 0.001). *Metarhizium* sp. RMK-2011b was associated with FX, CQ and SQ (IndVal = 0.69, p = 0.011). *Beauveria brongniartii* was significantly associated with SQ (IndVal = 0.42, p = 0.014), whereas *Beauveria bassiana* strain DAOM210087 was uniquely associated with MD (IndVal = 0.43, p = 0.017). *Metarhizium anisopliae* isolate PL52-1 showed affinity for FX and CQ (IndVal = 0.46, p = 0.046).

No EPF strains were significantly associated with soil depth or bait insect in the unpooled dataset ($p > 0.05$ for all taxa). ISA conducted on soil-pooled data revealed no significant bait-associated indicator strains (Appendix Table S2.10), confirming that bait insect did not generate consistent strain-level filtering.

Table 2.5. Indicator Species Analysis (IndVal.g; 5,000 permutations) identifying EPF strains associated with specific sites in the unpooled dataset: Analysis was based on 87 EPF-positive analytical sampling units, defined by site × sample × soil depth × bait insect. These analytical units derive from the 72 physical soil samples and should not be interpreted as 87 independent physical soil samples. For each strain, columns FX, CQ, SQ, and MD indicate presence (1) or absence (0) of association with each site based on the IndVal grouping structure. The IndVal statistic (IndVal.g) quantifies the strength of association between each strain and site(s), combining measures of specificity and fidelity. Permutation-based p-values indicate the statistical significance of these associations. Statistically significant indicator strains are marked with an asterisk (*), where $p < 0.05$.

Strain	FX	CQ	SQ	MD	IndVal	p-value
Ma 687	1	0	1	1	0.810	0.001*
Msp RMK-2011b	1	1	1	0	0.693	0.010*
B. brongniartii	0	0	1	0	0.417	0.014*
Bb DAOM210087	0	0	0	1	0.426	0.017*

Strain	FX	CQ	SQ	MD	IndVal	p-value
Ma PL52-1	1	1	0	0	0.455	0.046*
Cr f. catenulata	1	1	0	0	0.313	0.451
Ma T5R2	1	1	1	0	0.281	0.809
Ma LRC142	1	1	0	0	0.238	0.645
Bb DAOM195005	0	0	1	0	0.209	0.648
Ma ARSEF5851	0	1	0	0	0.209	0.651
Ma F4	0	1	0	0	0.209	0.651
Bb KK2	0	1	0	0	0.209	0.656
Ma GMX10.2-3	0	1	0	0	0.209	0.664

Community composition and multivariate structure.

Unpooled strain composition (Site × Sample number x Depth × Bait; n = 87)

Principal Coordinates Analysis (PCoA) of Bray–Curtis dissimilarities showed clear site-level structuring of EPF strain composition along the first two ordination axes (Figure 2.7). The first axis explained 40.7% of variation and the second axis explained 14.3% (cumulative 55.0%; Appendix Table S2.9). Samples from CQ and FX formed partially overlapping but distinct clusters, whereas MD samples clustered tightly and were separated from the other sites, consistent with reduced community complexity at MD. Separation by bait insect was limited relative to the site effect, and any bait-associated differences were not expressed as consistent directional shifts in ordination space.

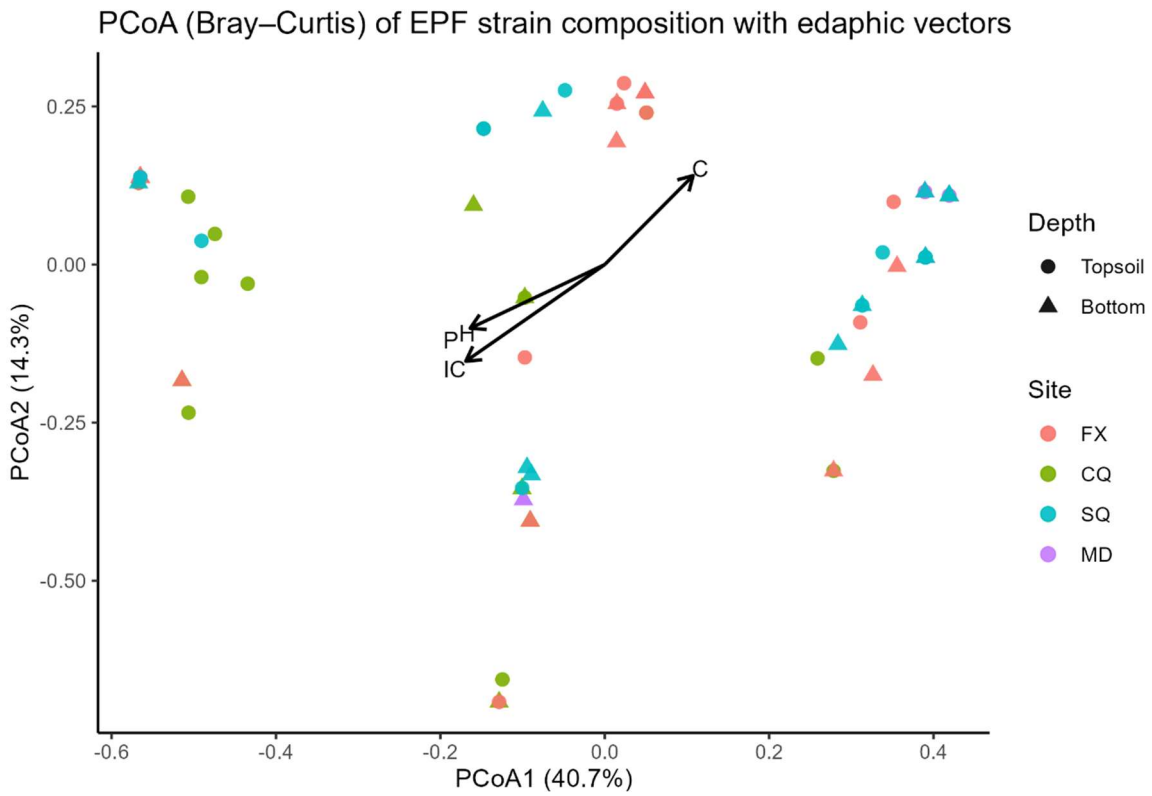


Figure 2.7. Principal Coordinates Analysis (PCoA) of EPF strain composition across soil samples collected in 2023 (unpooled dataset): Ordination is based on Bray–Curtis dissimilarities calculated from EPF strain abundance across 87 EPF-positive analytical sampling units, defined by site × sample × soil depth × bait insect and derived from 72 physical soil samples. Each point represents one analytical sampling unit; colours indicate site and point shapes denote soil depth (topsoil: 0–10 cm; bottom soil: 10–20 cm). Axis labels show the percentage of total variation explained by each principal coordinate axis. Arrows show significant edaphic variables fitted using envfit ($p \leq 0.05$; 9,999 permutations); arrow length is proportional to the strength of correlation (r), and arrow direction indicates the gradient of increasing values for each variable.

Environmental fitting (envfit) of edaphic variables onto the Bray–Curtis PCoA ordination of the unpooled dataset identified inorganic carbon (IC) ($r^2 = 0.2109$, $p = 0.0002$), soil pH ($r^2 = 0.1500$, $p = 0.0012$) and organic carbon (C) ($r^2 = 0.1274$, $p = 0.0038$) as significant correlates of EPF community composition (Table 2.6; Figure 2.7). Vector directions aligned with the main site gradient represented on PCoA1 and PCoA2, indicating that variation in carbon fractions and pH covaried with among-site differences in EPF assemblage composition. In contrast, total nitrogen (TN) ($r^2 = 0.0216$, $p = 0.3916$), total carbon (TC) ($r^2 = 0.0102$, $p = 0.6484$) and soil moisture ($r^2 = 0.0069$, $p = 0.7399$) were not significantly

associated with ordination structure. Envfit analyses were conducted exclusively on unpooled soil data because pooling topsoil and bottom soil removes vertical structure and may obscure relationships between EPF assemblages and depth-specific soil properties.

Table 2.6. Environmental fitting (envfit) results for edaphic variables associated with the PCoA ordination of unpooled EPF communities (2023): Environmental vectors were fitted onto Bray–Curtis dissimilarity-based PCoA ordination space using 9,999 permutations. Coefficients for PCoA1 and PCoA2 represent directional cosines (vector coordinates) used for plotting in ordination space. The coefficient of determination (r^2) indicates the strength of correlation between each environmental variable and the community composition. Statistically significant relationships are indicated by an asterisk (*), where $p < 0.05$.

Variable	PCoA1	PCoA2	r^2	p-value
IC	-0.3407	-0.3080	0.2109	0.0002*
pH	-0.3300	-0.2028	0.1500	0.0012*
C	0.2167	0.2836	0.1274	0.0038*
TN	0.0995	0.1081	0.0216	0.3916
TC	-0.0559	0.0843	0.0102	0.6484
Moisture	-0.0639	0.0532	0.0069	0.7399

PERMANOVA on Bray–Curtis dissimilarities indicated that the model including Site, Soil depth and bait explained a significant proportion of variation in EPF strain composition in the unpooled dataset (Df = 5; $R^2 = 0.1547$; F = 2.9657; p = 0.0004; 9,999 permutations; Table 2.7A)

Table 2.7. PERMANOVA (adonis2; 9,999 permutations) testing the effects of site, soil depth, and bait insect on EPF strain composition based on Bray–Curtis dissimilarities: Analyses were conducted separately for (A) the unpooled dataset (n = 87 EPF-positive analytical sampling units, defined by site × sample × soil depth × bait insect) and (B) the soil-pooled dataset (n = 57 EPF-positive analytical sampling units, defined by site × pooled sample × bait insect). Reported statistics include degrees of freedom (Df), proportion of variation explained (R^2), pseudo-F statistic (F), and permutation-based p-values. Statistically significant models are indicated by an asterisk (*), where $p < 0.05$.

(A) Unpooled dataset				
Model	**Df**	**R^2**	**F**	**p-value**
Site + Depth + Bait	5	0.1547	2.9657	0.0004*
(B) Soil pooled dataset				
Model	**Df**	**R^2**	**F**	**p-value**
Site + Bait	4	0.1614	2.5027	0.0014*

MRPP effect sizes indicated that only site showed meaningful within-group agreement (A = 0.0739, δ = 0.638, p = 0.0001), whereas soil depth (A = −0.00437, p = 0.766) and bait (A = −0.00299, p = 0.629) did not show evidence of compositional structuring (Table 2.8).

Table 2.8. Multi-Response Permutation Procedure (MRPP; Bray–Curtis; 9,999 permutations) testing differences in EPF community composition among grouping factors: Analyses were conducted separately for (A) the unpooled dataset (n = 87 EPF-positive analytical sampling units, defined by site × sample × soil depth × bait insect) and (B) the soil-pooled dataset (n = 57 EPF-positive analytical sampling units, defined by site × pooled sample × bait insect). The chance-corrected within-group agreement A reflects the degree of within-group similarity (A = 1 indicates identical samples; $A \approx 0$ indicates random grouping; $A < 0$ indicates greater heterogeneity within groups than expected by chance). δ represents the observed mean within-group dissimilarity. Statistically significant differences are indicated by an asterisk (*), where $p < 0.05$.

(A) Unpooled dataset	**(n = 87)**		
Factor	***A***	**δ**	**p-value**
Site	0.0739	0.638	0.0001*
Depth	−0.00437	0.692	0.766
Bait	−0.00299	0.691	0.629
(B) Soil pooled dataset	**(n =57)**		
Factor	***A***	**δ**	**p-value**
Site	0.0677	0.62	0.0006*
Bait	−0.0124	0.674	0.997

PERMDISP did not detect significant differences in multivariate dispersion among sites (p = 0.754) or between depth categories (p = 0.393), but did indicate a significant difference

in dispersion between bait groups (p = 0.0196; Table 2.9). Thus, site-related multivariate differences reflect genuine compositional structuring, whereas bait-related differences are better interpreted as differences in recovery variability rather than shifts in community centroid position.

Table 2.9. PERMDISP (betadisper + permutest; 9,999 permutations) testing homogeneity of multivariate dispersions for EPF communities based on Bray–Curtis dissimilarities: Analyses were conducted separately for (A) the unpooled dataset (n = 87 EPF-positive analytical sampling units, defined by site × sample × soil depth × bait insect) and (B) the soil-pooled dataset (n = 57 EPF-positive analytical sampling units, defined by site × pooled sample × bait insect). Reported values are permutation-based p-values assessing differences in within-group dispersion (i.e. beta diversity variance). Statistically significant results indicate heterogeneity of dispersion among groups, which may influence PERMANOVA interpretation. (*) indicates $p < 0.05$.

(A) Unpooled dataset (n=87)	
Factor	**p-value**
Site	0.754
Depth	0.393
Bait	0.0196*
(B) Soil pooled dataset (n=57)	
Factor	**p-value**
Site	0.165
Bait	0.211

Soil-pooled strain composition with bait retained (Site × Pooled sample × Bait; n = 57)

PCoA of the soil-pooled dataset showed comparable site-level clustering (Figure 2.8). The first axis explained 33.2% of variation and the second axis explained 14.7% (cumulative 47.9%; Appendix Table S2.9). Separation between bait insects remained minimal, supporting the interpretation that bait type contributed little to overall compositional differentiation once soil depths were pooled within sampling points.

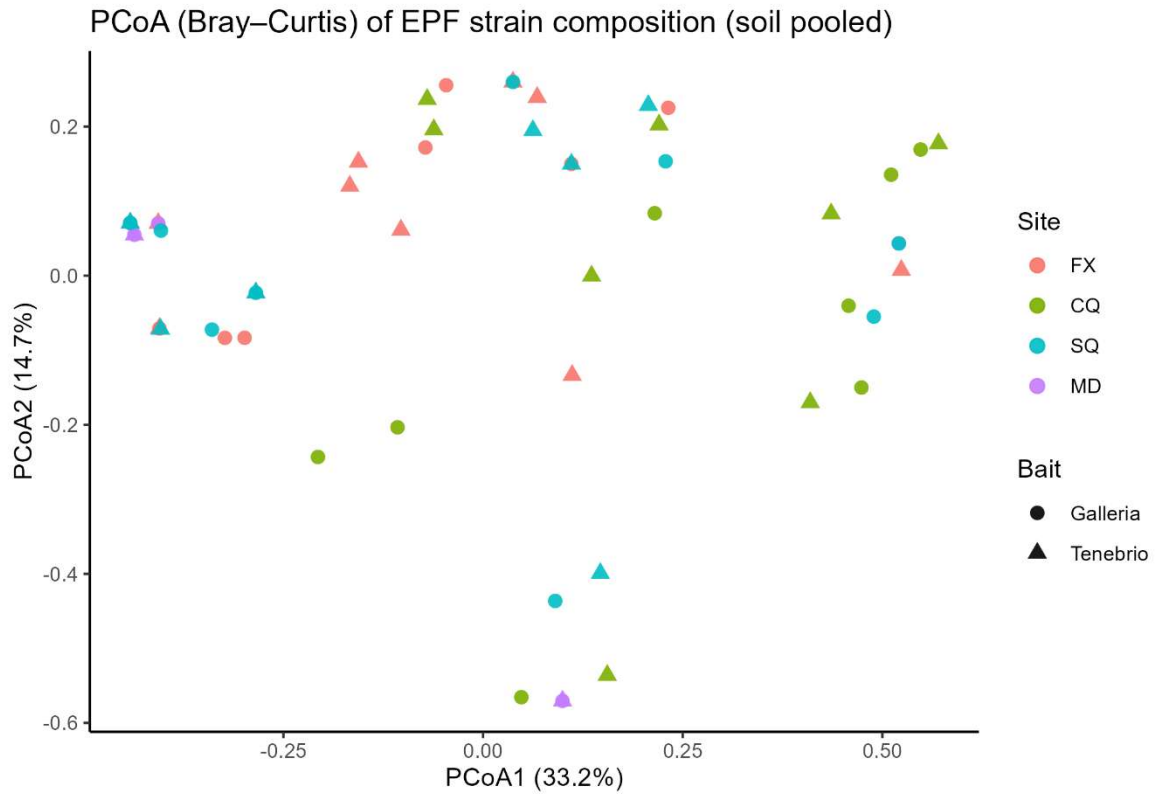


Figure 2.8. Principal Coordinates Analysis (PCoA) of EPF strain composition in soil-pooled samples collected in 2023: Ordination is based on Bray–Curtis dissimilarities calculated from EPF strain abundance data across pooled sampling units defined by site × sampling point × bait insect (n = 57). Topsoil (0–10 cm) and bottom soil (10–20 cm) samples were combined within each sampling point prior to analysis. Each point represents a pooled sampling unit; colours indicate site, and point shapes denote bait insect (*Galleria mellonella* vs *Tenebrio molitor*). Axis labels show the percentage of total variation explained by each principal coordinate axis.

PERMANOVA indicated that the model including site and bait explained a significant proportion of variation in EPF strain composition in the pooled dataset (Df = 4; $R^2 = 0.1614$; F = 2.5027; p = 0.0014; 9,999 permutations; Table 2.7B), together these factors accounted for approximately 15–16% of the observed variation in EPF community composition (Table 2.10). However, MRPP indicated that the compositional signal was attributable to Site only (A = 0.0677, δ = 0.620, p = 0.0006), whereas bait showed no evidence of compositional structuring (A = −0.0124, p = 0.997; Table 2.8). PERMDISP did not detect significant differences in dispersion for either Site (p = 0.165) or bait insect (p = 0.211; Table 2.9).

Synthesis of pooled versus unpooled analyses.

Pooling topsoil and bottom soil within paired sampling points substantially increased quantitative recovery metrics without altering qualitative community structure (Table 2.10). Mean isolate recovery per sampling unit increased from 2.27 in the unpooled dataset to 5.96 in the soil-pooled dataset, while mean strain richness increased from 1.53 to 2.63 and mean species richness from 1.11 to 1.41. Co-occurrence frequency increased markedly following pooling, with 75.0% of pooled samples containing more than one strain compared to 30.6% in the unpooled dataset, and 40.6% containing more than one species compared to 11.1% in the unpooled design (Appendix Table S2.8).

Despite these increases in abundance and co-occurrence, ordination structure and multivariate effect sizes remained highly consistent between designs. The proportion of variation explained by the first two PCoA axes was similar in both datasets (PCoA1: 40.7% vs 33.2%; PCoA2: 14.3% vs 14.7%), and overall PERMANOVA effect sizes were nearly identical (R^2 = 0.1547 for unpooled; R^2 = 0.1614 for pooled). MRPP effect sizes for Site were also comparable (A = 0.0739 vs 0.0677), indicating that site-level compositional structuring was robust to sampling design.

Environmental fitting identified inorganic carbon, organic carbon and soil pH as significant predictors of community structure in the unpooled dataset. Dominance patterns were conserved across both designs, with *Metarhizium anisopliae* strain 687 and *Metarhizium* sp. RMK-2011b remaining the most prevalent strains, and low-diversity sites remaining consistently species-poor.

Table 2.10. Comparison of EPF community metrics between unpooled and soil-pooled datasets (2023). Analyses were conducted separately for the unpooled dataset (n = 87 EPF-positive analytical sampling units, defined by site × sample × soil depth × bait insect) and the soil-pooled dataset (n = 57 EPF-positive analytical sampling units, defined by site × pooled sample × bait insect). Reported values summarise mean isolate recovery, taxonomic richness, co-occurrence frequency, ordination variance (PCoA), and multivariate effect sizes (PERMANOVA and MRPP). Envfit variables indicate edaphic factors significantly associated with community composition ($p < 0.05$)

Metric	Unpooled (n = 87)	Soil pooled (n = 57)
Mean isolates per sample	2.27	5.96
Mean strains per sample	1.53	2.63
Mean genera per sample	1.11	1.41

Metric	Unpooled (n = 87)	Soil pooled (n = 57)
% samples with >1 strain	30.6%	75.0%
% samples with >1 genus	11.1%	40.6%
PCoA1 variance explained	40.7%	33.2%
PCoA2 variance explained	14.3%	14.7%
PERMANOVA R^2 (model)	0.1547	0.1614
MRPP A (Site)	0.0739	0.0677
Significant envfit variables	IC, C, pH	–

Discussion

The results of this study provide a detailed ecological context for the distribution, diversity, and recovery of entomopathogenic fungi in natural soils across Lancashire Nature Reserves, with direct implications for optimising EPF bioprospecting strategies. By integrating dual baiting, depth-stratified sampling, edaphic measurements, and multivariate community analyses, this work identifies site as the strongest and most consistent factor associated with EPF community composition, whereas bait insect type and soil depth exerted comparatively minor influences. Significant associations between community composition and soil pH and carbon fractions further indicate that edaphic conditions contribute to the observed site-level structuring.

Interannual comparison: preliminary survey (2020) versus structured resampling (2023)

The 2020 survey functioned as a preliminary screening of EPF occurrence across eight nature reserves. Because isolates from this campaign lost viability before molecular identification could be completed, community composition could only be resolved at the morphotype level. Despite this limitation, the survey provided valuable baseline information on the spatial distribution of EPF across the eight sampled sites, revealing strong site-level heterogeneity in EPF recovery, with CQ and FX consistently yielding the highest numbers of isolates, while several sites (FF, CM and BN) produced no detectable EPF.

The structured resampling conducted in 2023 confirmed and expanded upon these preliminary patterns. Molecular identification resolved the community composition to the strain level, revealing a community dominated by *Metarhizium* taxa, particularly *Metarhizium anisopliae* strain 687 and *Metarhizium* sp. RMK-2011b. Importantly, the sites selected for resampling based on the 2020 survey (FX, CQ, SQ and MD) again yielded EPF

isolates in 2023, confirming that the preliminary morphotype-level screening successfully identified productive habitats for EPF recovery.

Although the two surveys differed in taxonomic resolution and analytical depth, several broad community patterns were consistent between them. In both years, *Metarhizium* dominated EPF recoveries across most sites, whereas *Beauveria* and *Clonostachys* occurred at lower frequencies. The agreement between morphotype-level patterns in 2020 and strain-level identification in 2023 therefore strengthens confidence that the observed community structure reflects genuine ecological patterns rather than methodological artefacts.

This pattern resembles some surveys from managed temperate systems, including vineyard soils in Germany and Australia, where *Metarhizium* also occurred frequently (Uzman *et al.*, 2019; Korosi *et al.*, 2019). However, the predominance of *Metarhizium* in the present study contrasts with other soil surveys. In one of the few detailed studies conducted in the United Kingdom, Chandler *et al.* (1997), for example, reported *Beauveria bassiana* as the dominant EPF recovered from soils in Warwickshire, while Sharma *et al.* (2018) found *Clonostachys rosea* to be the most prevalent taxon in Portuguese vineyard soils.

The contrasting dominance patterns among studies likely reflect differences in habitat type, land management, sampling intensity, and methodological design. The survey conducted by Chandler *et al.* (1997) focused primarily on cultivated fields and woodland habitats using a single bait insect (*Galleria mellonella*), whereas the present study employed dual baiting with both *Galleria mellonella* and *Tenebrio molitor* across multiple natural habitats. Similarly, Sharma *et al.* (2018) investigated agricultural vineyard systems and found strong habitat-specific associations among taxa, including the predominance of *Clonostachys* in semi-natural hedgerow soils.

The present work expands current knowledge of EPF distribution in the United Kingdom in several important ways. First, it provides a detailed strain-level characterisation of EPF communities from natural habitats, whereas previous UK surveys reported only species-level (Chandler *et al.* 1997). Second, the integration of dual baiting approaches allowed a broader assessment of functional EPF diversity than studies relying on a single bait insect (Chandler *et al.* 1997; Korosi et al., 2019), supporting recommendations by Sharma *et al.*, (2018) for the application of dual baiting techniques in EPF bioprospecting. Third, the combination of depth-stratified sampling and edaphic analysis provides new insight into the ecological drivers of EPF distribution, demonstrating that site-level environmental context is a stronger determinant of community structure than soil depth or bait insect type.

The large differences in EPF recovery among sites may reflect several environmental and ecological factors. From an ecological perspective, EPF distribution is strongly influenced

by vegetation structure, soil chemistry, and host insect availability (Bidochka *et al.*, 2001; Quesada-Moraga *et al.*, 2007). Sites such as CQ and FX represent relatively heterogeneous habitats with well-established vegetation and potentially abundant insect host communities, conditions that may favour the persistence and transmission of entomopathogenic fungi in soil. By contrast, habitats such as raised bogs (CM) or dune heath (FF) may represent more restrictive edaphic conditions, including low pH, strong moisture variation, nutrient limitation or reduced insect host availability, which may limit EPF establishment and persistence.

Methodological differences between campaigns may also have contributed. The 2020 isolates were not processed immediately and many lost viability before molecular identification, potentially underestimating diversity. By contrast, the 2023 campaign benefited from refined processing protocols, immediate culturing, and improved quality control, likely increasing recovery efficiency. Seasonal and interannual climatic variation may have also influenced EPF detectability, as soil moisture and temperature can affect spore survival and infection rates (Inglis *et al.*, 2001; Altinok *et al.*, 2019).

Across both surveys, FX and CQ consistently showed comparatively high EPF recovery, whereas MD showed lower overall recovery and lower Shannon diversity in the 2023 survey. Although Tukey-adjusted comparisons did not identify significant differences in strain richness among individual sites, the broader consistency in recovery and community-composition patterns supports persistent site-level heterogeneity.

Together, the comparison between 2020 and 2023 highlights two key principles for bioprospecting: (1) exploratory broad-scale surveys are valuable for identifying productive habitats, and (2) targeted resampling of ecologically favourable sites substantially increases recovery success without altering fundamental community patterns. Therefore, this phased approach may represent an efficient strategy for future EPF discovery programmes.

Bait insect performance and implications for bioprospecting design

Bait insect type did not significantly influence EPF recovery at the sample level. Across all sites and depths, paired Wilcoxon signed-rank tests indicated no significant differences between *Galleria mellonella* and *Tenebrio molitor* in the number of isolates, strains or species recovered per soil sample. At strain level, Fisher's exact test showed no evidence of differential recovery bias between bait insects, indicating that neither bait preferentially selected for particular EPF taxa in a consistent manner.

Importantly, PERMDISP analyses revealed a significant difference between bait types in the unpooled dataset, indicating that bait insect type influenced the variability among

samples rather than the overall community composition. Community composition or centroid differences were not found as indicated by not significant bait effects on PERMANOVA analysis. In other words, although the two bait insects recovered broadly similar EPF assemblages, the degree of variation among samples differed between bait treatments. This pattern suggests that bait insect types may affect detection variability or recovery efficiency, but do not act as strong ecological filters determining which EPF taxa are present.

These findings align with previous studies reporting broadly comparable EPF recovery success across commonly used bait insects when applied to heterogeneous field soils (Inglis *et al.*, 2008; Medo and Cagáň, 2011). Although several low-frequency strains were detected in association with only one bait insect species, their sparse occurrence suggests stochastic detection rather than bait-specific selectivity. In this context, deploying both bait insects functions primarily as parallel sampling, increasing the likelihood that infrequently encountered strains are captured simply through greater biological replication rather than through ecological filtering. Similar interpretations have been reported where methodological diversity enhances EPF recovery, particularly when communities are sparse or dominated by a few abundant strains (Sharma *et al.*, 2018; Korosi *et al.*, 2019). Consequently, bait insect types should be interpreted primarily as sampling tools that influence recovery probability rather than as indicators of host specificity.

Taken together, these results support the continued use of dual baiting in EPF bioprospecting, where multiple bait insects increase overall detection efficiency and improve the likelihood of recovering rare strains without substantially altering the inferred ecological structure of the community.

Site-level structuring of EPF communities

Site emerged as the strongest and most consistent factor associated with EPF community composition. PERMANOVA and MRPP analyses consistently identified significant site effects in both unpooled and soil-pooled datasets, with positive MRPP *A* values indicating non-random within-site similarity. Crucially, PERMDISP analyses showed no evidence of heterogeneous dispersion among sites, confirming that the observed compositional differences reflect true ecological structuring rather than artefacts of variance.

Ordination analyses further supported this conclusion. Principal Coordinates Analysis revealed clear site-level clustering along the primary ordination axes, with the first two axes explaining a substantial proportion of variation in both datasets. Samples from CQ and FX formed partially overlapping but distinct assemblages, whereas MD samples clustered

tightly and were separated from the other sites, consistent with reduced diversity and simplified community structure at MD.

These findings are consistent with previous work demonstrating strong habitat- and site-specific structuring of EPF communities in soils (Quesada-Moraga *et al.*, 2007; Sun and Liu, 2008; Lacey *et al.*, 2015). Differences in vegetation, soil chemistry, and land-use history can generate distinct ecological niches that favour particular EPF taxa, resulting in patchy and spatially structured distributions even across relatively small geographic scales.

Soil depth effects and vertical stratification

Soil depth did not exert a strong or consistent influence on EPF community composition in this study. Although slightly higher isolate recovery was observed in topsoil layers, MRPP and PERMANOVA analyses provided no evidence of depth-driven compositional structuring, and depth was not associated with significant ordination separation.

Rather than contradicting a well-established vertical pattern, these results are consistent with the broader understanding that EPF distribution in soils is governed by complex and interacting ecological factors (Lacey *et al*., 2015). Entomopathogenic fungi occur within highly heterogeneous soil environments, where their persistence and activity are influenced by microbial competition, nutrient availability, host presence, and environmental conditions such as moisture and temperature (Lacey *et al*., 2015). These interacting drivers can obscure simple vertical gradients resulting in context-dependent patterns of distribution. Site-level heterogeneity appears to override any potential depth-related structuring (Uzman *et al.*, 2019). The lack of depth-associated indicator strains further supports the conclusion that, in these habitats, EPF assemblages are not vertically segregated in a biologically meaningful way within the sampled depth range.

From an applied perspective, these results suggest that restricting sampling to the upper soil layer may be sufficient for EPF bioprospecting in similar environments, reducing sampling effort without substantially compromising recovery of distinct communities. However, this should be interpreted as a pragmatic sampling strategy rather than evidence of a universal ecological pattern.

Edaphic drivers of community structure

Environmental fitting analyses identified inorganic carbon, organic carbon, and soil pH as significant correlates of EPF community structure in the unpooled dataset. Vector orientations aligned with the primary ordination gradient separating sites, indicating that variation in carbon fractions and pH covaried with site-level differences in EPF assemblages.

The importance of soil pH and carbon availability in shaping fungal communities is well established (Donatti *et al.*, 2008; Sun and Liu, 2008; Altinok *et al.*, 2019). According to these studies, soil pH can influence spore germination, enzymatic activity, and competitive interactions, whereas carbon availability affects fungal persistence and saprotrophic competence. In our study, the lack of significant associations with total nitrogen and moisture suggests that, within the range of conditions sampled, these variables were less limiting or were overridden by other soil properties.

The restriction of envfit analyses to the unpooled dataset was methodologically appropriate, as pooling removes vertical stratification and can obscure depth-specific chemical gradients. This reinforces the value of depth-resolved data when linking EPF communities to soil chemistry.

Indicator strains and ecological consistency

Indicator Species Analysis identified several strains significantly associated with particular sites or combinations of sites, including *Metarhizium anisopliae* strain 687 and *Metarhizium* sp. RMK-2011b, which were associated with multiple productive sites (Yapa *et al*., 2024). *Beauveria brongniartii* showed a significant association with SQ, where it was recovered from four soil samples, while *B. bassiana* DAOM210087 was associated with MD. In contrast, no strains were significantly associated with soil depth or bait insect. These patterns are consistent with the broader multivariate analyses and support site-level structuring of strain distributions.

Indicator values should nevertheless be interpreted in conjunction with strain prevalence and abundance, particularly for relatively infrequent taxa.

Pooled versus unpooled sampling designs

Pooling topsoil and bottom soil samples substantially increased quantitative recovery metrics, including mean isolate abundance, strain richness, and co-occurrence frequency. However, pooling did not alter dominant taxa, site-level structuring, ordination patterns, or multivariate effect sizes. PERMANOVA R^2 values and MRPP *A* statistics were highly consistent between pooled and unpooled datasets, demonstrating that sampling design influenced recovery efficiency but not ecological interpretation.

This finding has practical implications. Soil pooling can be used to maximise EPF recovery and diversity detection when logistical constraints exist, whereas depth-resolved sampling remains valuable for linking communities to soil properties.

Conclusions

This study demonstrates that site is the strongest and most consistent factor associated with EPF diversity and community composition in natural soils from Lancashire nature reserves. Multivariate analyses confirmed that differences among sites reflect genuine compositional structuring of EPF assemblages rather than artefacts of unequal dispersion among samples. These results highlight the importance of habitat-specific environmental conditions in shaping the spatial distribution of soil-borne entomopathogenic fungi.

In contrast, dual baiting with *Galleria mellonella* and *Tenebrio molitor* did not produce consistent differences in EPF recovery at the sample or strain level; instead, their combined use increased sampling intensity and complementary detection opportunity, improving the probability of recovering rare strains and supporting dual baiting as a robust bioprospecting strategy.

Soil depth had limited influence on EPF composition within the sampled 0–20 cm profile, suggesting that prioritising the upper 10 cm may provide an efficient sampling strategy in comparable habitats. However, depth-resolved sampling remains appropriate where vertical ecological patterns or relationships with soil properties are specific objectives.

Edaphic factors, particularly soil pH and carbon fractions, were significantly associated with community composition, reinforcing the importance of soil chemistry in determining EPF persistence and distribution, highlighting the importance of integrating soil chemistry into bioprospecting frameworks. Pooling soil depths increased quantitative recovery but preserved compositional patterns and ecological conclusions, providing flexibility in sampling design depending on research objectives.

Overall, this work provides one of the most detailed strain-level characterisations of EPF communities from natural habitats in the United Kingdom. By integrating habitat screening, dual baiting, depth-stratified sampling and molecular identification, this study establishes a robust ecological framework for EPF bioprospecting. These findings suggest that targeting ecologically favourable habitats while maximising baiting intensity represents an effective strategy for discovering diverse and locally adapted EPF strains with potential applications in biological control.

CHAPTER 3:

Mortality and feeding effects of wild entomopathogenic fungi on the large pine weevil (*Hylobius abietis* [L.]).

Abstract

- The large pine weevil (LPW), *Hylobius abietis*, is native to the northern hemisphere in Europe and Asia where it has become a widespread man-made pest affecting the restocking of coniferous plantations.
- Inundative biological control agents, such as entomopathogenic fungi and nematodes, are amongst the safest and most promising alternative to the well-established use of chemicals against *H. abietis*, which have a wide range of action and environmental side effects.
- Research efforts mainly focus on finding fast-killing fungi against *H. abietis* but little work has been done on infection effects of highly virulent fungal strains such as the effects on feeding behaviour and related weight change.
- We have obtained highly virulent wild fungi strains from nature reserves in Lancashire and analysed their lethal and infection effects on adult *H. abietis* kept under conditions emulating forest environments.
- A direct correlation was found between the increase in pathogenic fungi virulence of the strains and diminution of bark consumption of the weevils presumably due to infection effects. From a biocontrol perspective, targeting adult weevils with the most efficacious fungi is likely to reduce medium-term damage to trees due to these infection effects on LPW and more research is encouraged to find new wild strains of entomopathogenic fungi against this and other insect pests.

Introduction

The large pine weevil (LPW), *Hylobius abietis* (L.), is a native and widespread weevil in the northern hemisphere in Europe and Asia that is highly attracted to volatiles, such as alpha-pinene, that indicate availability of fresh decaying conifer stumps for egg laying (Leather *et al.*, 1999, Moore 2001, Inward *et al.*, 2012). Under natural conditions, the availability of stumps of fallen trees to reproduce is low and is a factor limiting the increase of populations, but this is not the case in expansive clearfell environments (Willoughby *et al.*, 2017). The establishment of large coniferous plantations, including large areas of clear-felling, sets the conditions for the demographic explosion of *H. abietis* populations (Leather *et al.*, 1999, Inward *et al.*, 2012).

The adult LPW feeds on the bark of young conifer saplings post-emergence. A single adult's bark-feeding behaviour can kill several saplings up to five years old and hence the

re-stocking of the forest has become a major issue when they are present in high numbers as they can kill all the new saplings if protective measures are not undertaken (Leather *et al.*, 1999, Kapranas *et al.*, 2016). Additionally, warmer conditions due to climate change, shorten their life cycle, shift their suitable distribution areas, and increases their dispersion (Inward *et al.*, 2012). In Europe, LPWs threaten 3.4 million ha of forest causing estimated annual losses of €140 million (Långström and Day 2007), with those in UK private and public forestry in 1998 estimated at €4.6 million (Leather *et al.*, 1999, Kapranas *et al.*, 2016, McNamara *et al.*, 2018a)

On many sites in the UK and Ireland, LPW integrated control management practices can include the application of synthetic chemicals under derogation although acetamiprid is currently the main insecticide pre-applied in nurseries to protect seedlings for forest planting which is normally followed by additional top-up spraying on site (Kapranas *et al.*, 2016). Not all pesticides used have the same impact; for example, acetamiprid is not classified as "highly hazardous." However, many pesticides do not effectively reduce local *H. abietis* populations, as they tend to have a repellent rather than lethal effect on *H. abietis* (Leather *et al.*, 1999, Torr *et al*, 2005). Additionally, concerns remain regarding the environmental impact of certain pesticides, which are classified as “highly hazardous chemicals” according to the Forest Stewardship Council (FSC) guidelines (Kapranas *et al.*, 2016, McNamara *et al.*, 2018a). For instance, studies on cypermethrin have highlighted its high toxicity to freshwater non-target organisms including ground beetles (Carabidae) and pollinators (Desneux *et al.*, 2007, Saha and Kaviraj, 2008, Merivee *et al.*, 2015).

The use of biological control agents (BCA) is a less environmentally impactful option amongst the alternative treatments to supress *H. abietis* infestations (Calvo *et al.*, 2012, van Lenteren *et al.*, 2017). Kapranas *et al.*, (2017) reported suppression of adult *H. abietis* emergence below the threshold of 20 *H. abietis* per stump on field trials when treated with the entomopathogenic nematode *Steinernema carpocapsae*. Previous works also found that *S. carpocapase* applied in half operational doses of 1.75 x10^6 nematodes in 500 ml presented additive effects when applied together with half doses of the entomopathogenic fungi (EPF) *Beauveria caledonica* (5 x10^8 spores per stump) obtaining similar suppression than full doses of the nematodes (Williams *et al.*, 2013, Mc Namara *et al.*, 2018a).

Entomopathogenic BCAs have traditionally been isolated from soil with the soil baiting technique. This uses vulnerable and nutrient rich insect larvae to attract soil biota to feed on them as predators, parasites, commensals, and saprotrophs (Sun and Liu, 2008). In most of the studies *Galleria melonella* (L.) larvae have been used as bait and these collect mainly nematodes and fungi but *Tenebrio molitor* (L.) has also been used as bait on a smaller scale.

Both baits are sometimes combined in a dual method "G*alleria-Tenebrio*-bait*"* that has demonstrated efficacy in obtaining EPFs as some of their species have more affinity to either of the baits, increasing the spectrum of isolates that could also include species isolated for first time in environments without previous records of its presence (Sharma *et al.*, 2018).

The efficacy of entomopathogenic fungi as BCAs against *H. abietis* under laboratory conditions make them excellent candidates to be further studied as bioinsecticides in field integrated pest management programmes as they cause minimum effect on beneficial non-target organisms (Dara *et al.,* 2019). The Ascomycota fungi of the order Hypocreales contains the largest diversity of entomopathogens with lifecycles involving a well-developed parasitic phase infecting the host´s body, mainly infecting coleopterans and lepidopterans in their larval stages but also adults (Araujo and Hughes, 2016). Following host dead, the fungi grow and reproduce, in the case of the genera *Beauveria* and *Metarhizium*, inside the dead insect as yeast-like hyphal bodies multiplying asexually by budding, producing anamorphic asexual spores (Araujo and Hughes, 2016). *Metarhizium* and *Beauveria* has been commercially produced for use against various insect pests and have been tested against *H. abietis* by applying conidia directly (dry conidia) or in water suspension (wet conidia) submerging either LPWs or twigs to be eaten by *H. abietis* in concentrations ranging from 1 $x10^5$ to 1 x 10^{10} conidia per cm^2 or ml respectively (Ansari and Butt, 2012, Williams *et al.*, 2013b). Some commercially available EPF species such as *Metarhizium robertsii* (Metchnikoff) Sorokin 1883, *Metarhizium brunneum* Petch 1935 and *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin 1912 are efficacious at killing all developmental stages of *H. abietis* in laboratory assays (Ansari and Butt, 2012). In the latter study, *Metarhizium robertsii* ARSEF4556 was the most virulent strain causing 100% mortality of pupae, larvae and adults after 4, 6 and 12 days, respectively. In contrast to these laboratory results, field trials on two-year-old Sitka spruce transplants by Willoughby et *al* (2020) found poor levels of protection provided by *Metarhizium anisopliae* var. *anisopliae* strain F52 (Met 52) against intermediate to high populations of *H. abietis*.

Infection effects of EPFs on arthropods have been poorly documented but there are some studies showing physiological effects of *Beauveria* and *Metarhizum* strains on various insect pests and these have been shown to affect their developmental stages in different ways (Torrado-Leon *et al.*, 2006, Liu *et al.*, 2020). Insects under EPF infective treatments can manifest changes on their next generations such as was the case for *B. bassiana* applied to "whiteflies" *Bemisia tabaci* (Gennadius) where the fitness of subsequent generations of whiteflies was reduced (Torrado-Leon *et al.*, 2006). (Maehara *et al.*, 2007) reported a different response to *B. bassiana* after a 4-day treatment, observing a decrease in feeding by

adult *Monochamus alternatus* Hope (Coleoptera: Cerambycidae) after 30 days, with infected beetles consuming 14.8 cm^2/individual and controls 103.4 cm^2/individual. Studies of infection activity of EPFs on *H. abietis* found behavioural effects of the secondary metabolite oosporein of *B. bassiana* where adult *H. abietis* increased their feeding behaviour when exposed to oosporein (McNamara *et al.*, 2019). In contrast to this, another study showed that adult *H. abietis*' daily bark consumption was lower when adult weevils were treated with *B. bassiana* strain AMEP20 and consumed area was smaller as higher concentrations of conidia were applied (Barta *et al.*, 2019).

Some studies, however, have not found any of the aforementioned effects caused by the specific EPF strains used, for example for *Cyclocephala lurida* Bland (Coleoprtera: Scarabaeidae) where neither *M. brunneum* or *B.bassiana* had significant infection effects ((Wu *et al.*, 2016).

The use of EPF as biocontrol agents for *H. abietis* is focused on adults and finding the most virulent strains. However, we suggest that it is also of value to investigate the post-infection feeding stimulation / inhibition effects of EPF on *H. abietis*. In this study, from a range of wild EPF strains, we aim to find those effective at killing adult *H. abietis* investigating their post-infection feeding effects and to investigate any link between feeding effects and the time required by each EPF strain to kill 50% of *H. abietis* (Lethal Time 50 or LT_{50}). We hypothesise that the most efficacious EPFs, as indicated by shorter LT_{50} values, will induce significant post-infection effects in *H. abietis,* leading to a noticeable increase in weight. This increase is expected to result from the weevils consuming more bark to counteract the effects of infection by the more virulent EPF strains.

Materials and Methods

Source and rearing conditions of *H. abietis*.

Hylobius abietis were collected from pine/spruce forestry sites in Scotland between 2021 and 2022 using 20cm long freshly cut billets of Scots pine and Sitka spruce. In September 2021, Forest Research, Roslin, Midlothian, Scotland, EH25 9SY (NT 24918 63980) provided us with their collected adult weevils for the first experiment that took place in April 2022. For the second part of the experiment in November 2022, we collected weevils manually using spruce billets between June-September 2022 from Clatteringshaws, Scotland (NX 54621 79109).

In the laboratory, up to 50 adult *H. abietis* were kept at 15ºC in sterile plastic containers (18cm x 12cm x 6cm) pierced on the lid and on each side for aeration. Each box was provided

with fresh pine and spruce twigs and a 5cm Petri dish with sterile filter paper soaked in 15ml of distilled water. Every two weeks weevils were transferred to a clean box with fresh twigs and water until the experiments started.

Source of entomopathogenic agents

Locally sourced entomopathogenic agents were obtained from the Nature Reserves network in Lancashire, Manchester and North Merseyside in July and September 2020 (https://www.lancswt.org.uk/nature-reserves). The selection of reserves was based on high diversity of insects and/or insectivorous species in well-established and preserved communities according to their online descriptions. Selected habitats included grassland (Middleton Nature Reserve "MD"), semi-natural woodlands (Foxhill Bank Local Nature Reserve "FX" / Boilton, Nab, Redscar and Tunbrook Woods "BN"), lowland dune heath (Freshfield Dune Heath "FF"), grassland quarries (Salthill Quarry Local Nature Reserve "SQ" / Cross Hill Quarry Local Nature Reserve "CQ"), raised bog (Cadishead & Little Woolden Moss "CM"), and lowland heath (Highfield Moss "HM").

From each reserve, eighteen soil cores of 500g were collected at nine equidistant points and two depths (0-10cm = "Top fraction" and 10-20cm = "Bottom fraction") from a 5m x 5m square using a gauge auger that was sterilised between each sample. These were stored separately for 2-3 weeks at 4ºC in 500 ml plastic take away containers with the lid pierced five times for ventilation.

To obtain the entomopathogenic agents we used a dual baiting technique adapted from Sharma *et al.*, (2018). Rocks and organic matter were removed prior to halving each sample in two 500 ml containers. Next, five late instar larvae of *G. melonella* (Lepidoptera) were added to one container and five late instar larvae of *T. molitor* (Coleoptera) to the other. Soils were incubated at 22ºC in the dark for four weeks and were gently shaken to check the insects were alive. Samples were checked daily during the first week and from the second week every two days. Dead insects with a putrid smell and soft and dark bodies were discarded due to bacterial infection. Other dead insects thought to be killed by fungi, were collected and rinsed with distilled water prior to removal of saprotrophs on the surface. Dead rinsed specimens were dipped in 70% ethanol and flamed following the recommendations of (McNamara *et al.*, 2018a). The incubation of entomopathogenic fungi from dead bodies was carried out in moist chambers (9cm diameter Petri dishes with wet filter paper) at 22ºC in the dark. Insects displaying mycelium were rinsed and incubated at 27ºC for 15-20 days in dodine-free selective media based on Oats 20 g/l, Agar 20 g/l, Chloramphenicol 0.5 g/l and cetyl trimethyl ammonium bromide (CTAB) 0.6 g/l as recommended by Posadas et *al*

(2012). This medium allows growth of EPFs while inhibiting saprophyte fungi and bacteria. The fungi isolated were incubated in potato dextrose agar (PDA) medium under the same conditions. When mixed colonies or contamination was present, samples were plated again firstly into a selective medium followed by PDA until single fungal cultures alike to the mycelium obtained in the baits were isolated.

Pathogenicity test and Selection of the best EPF candidates:

A fungal pathogenicity test was carried out following a modified protocol based on Sun and Liu (2008). Fungal cultures were selected based on their pathogenicity against the original hosts (*Galleria-Galleria*, *Tenebrio-Tenebrio*). From PDA fungal cultures, spores were extracted using 1ml sterile distilled water mixed with 0.03% (v/v) viscous Tween-80 (Sigma-Aldrich, Dorset, UK), and collected into 1.5 ml Eppendorf tubes. From the collected spores, 100ul were plated in PDA to obtain fresh fungal cultures for the following stages. In the remaining suspension, insect larva were dipped one by one for 5 seconds using sterilised forceps, then, transferred to individual moist chambers incubated at 22ºC in the dark for 4 weeks with daily inspects until pupation or death (Sun and Liu, 2008). To avoid premature death of the larvae, humidity was maintained using 1 ml sterile water for *G. mellonella* and 0.5 ml for *T. molitor*. Growth of mycelium and spores served to identify if larvae were killed by the original fungi and, if so, they were then either moved to the next phase or were discarded. In this work, we use the term "EPF morphology" when there is a distinctive mycelial growth from the spiracles, mouth or anus, and this covers the insect and produces spores. To shortlist the most promising candidates from the isolates we selected those with shorter lethal time and clear EPF morphology. Based on these criteria, 12 samples showed the desired EPF structures and killed the original host within five days. Those samples were shortlisted, identified with molecular methods and used against *H. abietis*.

Molecular methods to Identify EPFs

DNA extraction from mycelium and spores of the 12 EPF fungal cultures was performed using DNeasy ® Plant Pro kit (Quiagen). Mycelium preparation followed the indications of Williams *et al* (2013) extracting 50-100 mg of fungi in 1.5 ml Eppendorf tubes to quick freeze in liquid nitrogen (LN_2), then, thawed at 60ºC in a hot plate and frozen again in LN_2 to next be crushed with a micro-pestle (Bel-Art SP Scienceware). After mycelium were prepared, we followed the DNeasy Plant Pro kit protocol. DNA concentration was measured using a microvolume UV-Vis spectrometer (NanoDrop™ One, Thermo Scientific) and samples were standardised to 5ng/µl for PCR.

PCR followed a modified protocol of Taq PCR Master Mix Kit (Qiagen). The fungal internal transcribed spacer (ITS) region was amplified using the forward primer ITS1-F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and reverse primer ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (Gardes and Bruns, 1993). For each reaction 0.4µM of each primer was used and 8ng of DNA obtaining a total volume of 25µl. (12.5µl Master mix, 2µl of each primer [from 5µM], 5µl DNA, 3,5µl water RNase-free). Thermocycler conditions were set following (Yurkov *et al.*, 2015) with initial denaturation for 5 min at 95 ºC, followed by 35 cycles of 30 sec denaturation at 95ºC, annealing for 30 sec at 50ºC and extension for 2 min at 72ºC. The final extension step was carried out at 72ºC for 7 min. The amplified sequences were visualised using an agarose gel electrophoresis and confirmed fragments were purified using QIAquick ® PCR Purification Kit (Quiagen). Purified fragments were sent for forward and reverse Sanger sequencing to Eurofins Genomics using the primers ITS1-F and ITS4, respectively.

Forward and reverse sequences were processed with SeaView 4.7 (Gouy *et al.*, 2010). Complement sequences were generated from the reverse readings. Next, each forward and its respective complement sequence were aligned using Multiple Sequence Comparison by Log-Expectation (MUSCLE) to obtain a consensus sequence for each EPF isolate. Finally, the 12 sequences were processed in BLAST® (National Center for Biotechnology Information) obtaining identities > 97.43% (Table 3.1).

The effect of EPF on feeding behaviour and mortality of adult *H. abietis*.

To evaluate lethal, infection and behavioural effects of the selected EPFs on *H. abietis* we designed an experiment based on Tudoran *et al.*, 2021. Spores were extracted from the PDA plates of the 12 shortlisted candidates as mentioned above and counted using a haemocytometer (Hawksley, Sussex, UK) placed in a light microscope (400 x magnification). Calculation of the number of spores was done following (Lacey, 1997) obtaining 10^8 spores/ml in 0.03% (v/v) viscous Tween-80 solution used as a full concentration of the 12 EPFs to be tested each on 10 adult *H. abietis* on April 2022 (120 treatments + 10 controls). The 2 most effective isolates were selected based on their LT_{50} to be further tested at medium and low concentrations in November 2022 (10^7 and 10^6 spores/ml) in the same fashion (40 treatments + 10 controls). Before starting the experiment, weevils were starved for 48h recording their individual weight (grams) on the day of the experiment (t = 0). Weevils were submerged 5s in its respective treatment and placed individually in a 9 cm Petri dish containing an 8cm long fresh Scots pine twig and a 2cm damp paper filter ball to provide access to clean distilled water. Insect mortality was recorded

daily for 21 days to obtain the respective lethal times, whereas weight change and area consumed was recorded for 14 days. On days 4, 7 and 14, weevils were weighed, the wood pieces collected, labelled, and replaced with fresh twigs of the same size. The amount of bark consumed was recorded using transparent acetate to manually copy the surface eaten from each twig with a permanent black marker. Acetates were scanned together with a ruler as scale to be analysed using the image analysis software ImageJ (U. S. National Institutes of Health, Bethesda, Maryland, USA) to obtain feeding areas in mm^2.

Statistical Analysis

Mortality analysis was carried out in OASIS using the Log-rank test to identify statistical differences in lifespan (Yang *et al.*, 2011). Variations in area consumption were analysed based on the area consumed per day and the accumulated area at T=4, T=7 and T=14. Weight changes were assessed as the difference between final and initial weight, as well as weight change per surviving days. Some data on area variation were found to be non-normally distributed, with a P-value < 0.05 according to the Kolmogorov-Smirnov test (K-S). For those datasets, a non-parametric Kruskal-Wallis (K-W) test was applied, as the data were either non-normal or did not meet the assumptions required for a standard analysis of variance (ANOVA). Post-hoc pairwise comparisons were performed using Dunn's test (Lee and Lee, 2018).

For normally distributed data, ANOVA was applied. Levene's test for equality of variance indicated homoscedasticity (P > 0.05) for most variables, confirming reliability of the ANOVA model, and allowing for post-hoc analysis using Tukey's Honestly Significant Difference (HSD) test. For samples lacking homogeneity of variance, robust tests Welch and Brown-Forsythe were employed, followed by Dunnett T3 post-hoc test. LT_{50} values were correlated with the studied variables. All statistical analysis of weight change and consumed area were performed using SPSS.

Results

Molecular identification of EPFs (GenBank OR805435-OR805447)

Before testing against *Hylobius abietis*, the fungal isolates recovered from the 2020 soil-baiting survey (Chapter 2) were screened for pathogenicity against the same bait host species from which they had originally been isolated on the 25th of January 2021 using a single insect per treatment. Fungal isolates that displayed a clear EPF morphology were further tested in two sequential assays. Said assays were conducted, first with 10 insects per treatment and control on the 23rd of February of 2021, and subsequently on the 24th of March of 2021 with

20 insects per treatment and control to confirm consistency of virulence. Survival analysis indicated substantial differences among isolates in the speed of host killing, with several isolates causing rapid mortality relative to the controls (Appendix Table S3.1). Across the confirmatory assay, the fastest-acting isolates generally killed their original hosts within approximately 4.7–6.8 days on average, whereas the control groups survived markedly longer (mean survival 10.95 days).

Twelve entomopathogenic fungi were selected that, during the screening process, killed 50% of the original host insects the fastest (shortest LT_{50}) and exhibited suitable EPF morphology. These fungi were isolated from soils collected at four nature reserves (FX, CQ, SQ and MD). Using the dual baiting technique, only 23T was obtained using *T. mollitor*, while the remaining EPF isolates were obtained using *G. mellonella.* The EPFs were recovered from both top and bottom soil fractions (Table 3.1).

Table 3.1. Molecular identification of entomopathogenic fungal (EPF) isolates selected for bioassays against *Hylobius abietis*. Isolates were obtained from soil samples collected in 2020 (*Chapter 2*) and identified by sequencing of the ITS region followed by BLAST® comparison against GenBank. The closest match (BLAST consensus), percentage sequence identity, and corresponding GenBank accession numbers (OR805435–OR805447) are shown. Soil fraction indicates the depth from which isolates were recovered (topsoil: 0–10 cm; bottom soil: 10–20 cm).

Origin / Soil fraction	SAMPLE	BLAST Consensus	Percent Identity	GenBank Accession
FX/bottom	6G	*Metarhizium* sp. RMK-2011b	98.79%	OR805435
FX/top	15G	*Metarhizium* sp. RMK-2011b	98.10%	OR805436
FX/top	17G	*Metarhizium* sp. RMK-2011b	99.13%	OR805437
CQ/bottom	22G	*Metarhizium* sp. RMK-2011b	98.80%	OR805438
CQ/top	23T	*Metarhizium* sp. RMK-2011b	97.93%	OR805439
CQ/bottom	30G	*Metarhizium* sp. RMK-2011b	98.78%	OR805440
CQ/top	33G	*Metarhizium* sp. RMK-2011b	98.79%	OR805441
CQ/top	35G	*Beauveria bassiana* isolate DAOM210087	98.99%	OR805442
SQ/top	93G	*Metarhizium* sp. RMK-2011b	99.48%	OR805444
SQ/bottom	102G	*Metarhizium anisopliae* (Metschnikoff) Sorokin, 1883	97.43%	OR805445

MD/top	139G	*Metarhizium* sp. RMK-2011b	98.96%	OR805446
MD/top	143G	*Metarhizium* sp. RMK-2011b	99.30%	OR805447

BLAST analysis of consensus sequences identified four distinct strains, all with identity values greater than 97.43%. Ten EPF candidates were identified as *Metarhizium sp.* RMK-2011b. Strain 35G was identified as *Beauveria bassiana* with 98.99% identity, and strain 102G with the lowest identity percentage (97.43%) was identified as *Metarhizium anisopliae* (Metschnikoff) Sorokin, 1883 (Table 3.1).

Survival Curve and LT_{50}

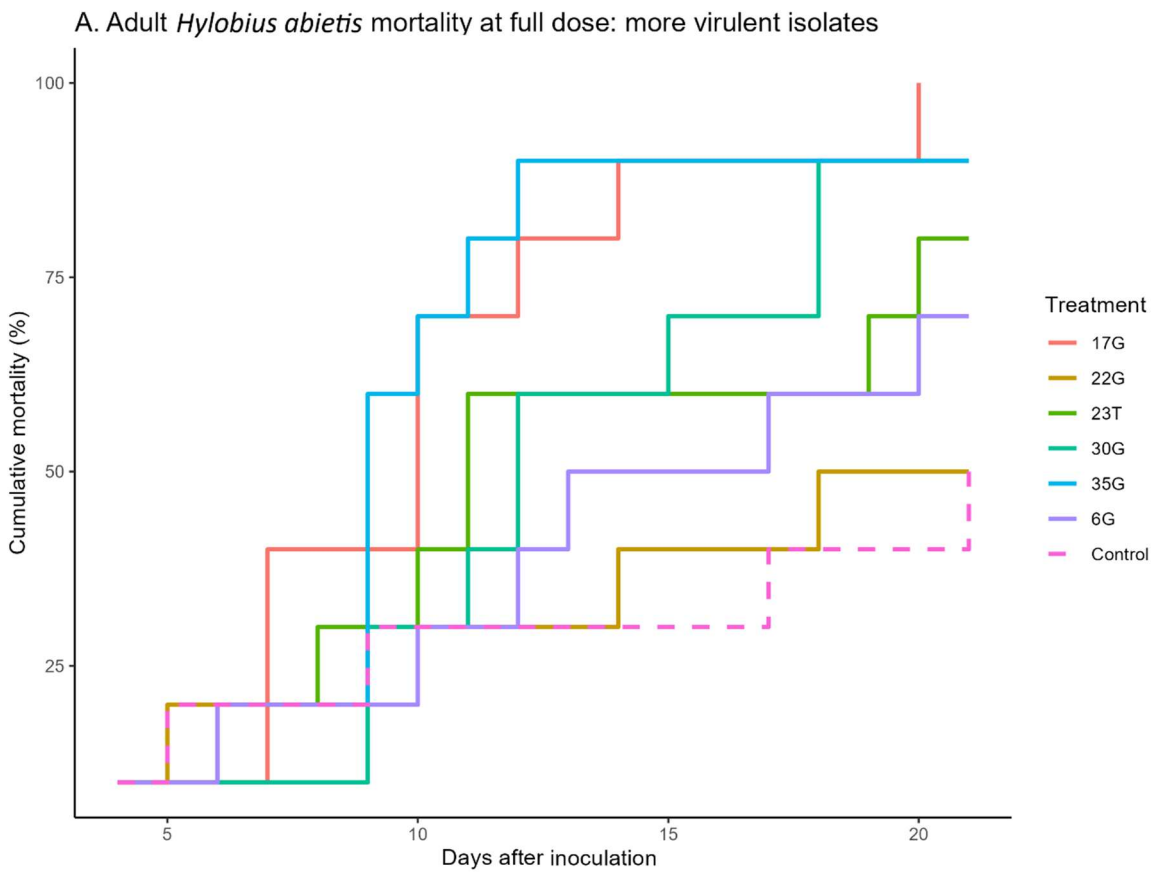
In April 2022, testing full doses of the twelve EPF strains provided LT_{50} values, enabling us to select the most effective EPF candidates to further determine their effects on adult *H. abietis* when applied at lower concentrations. The two most effective isolates were *Metarhizium sp.* 17G and *Beauveria bassiana* isolate 35G with LT_{50} values of 10 and 9 days, respectively, both over two times faster than the control, which had an LT_{50} of 21 days (Table 3.2). A Log-rank test comparing the survival of control *H. abietis* to those treated with the twelve EPF strains revealed significant differences for the fast-killing strain 17G ($p=0.0108$) and the slow-killing strain 139G ($p=0.0494$), with a marginal difference observed for the fast-killing 35G strain ($p=0.052$) (Appendix Table S3.2). In November 2022, when comparing weevil survival due to the fast-killing strains 35G and 17G, at both medium and low concentrations, significant differences ($p<0.05$) were observed in all cases when compared to control *H. abietis.* Additionally, the survival of *H. abietis* treated with the strain 35G at medium dose was significantly different compared to both low concentrations (17 Low and 35 Low) (Appendix Tables S3.2 and S3.3).

Table 3.2. Survival analysis parameters for *Hylobius abietis* adults exposed to twelve entomopathogenic fungal (EPF) isolates at a high conidial concentration (10^8 conidia ml^{-1}): Adult weevils (n = 10 per treatment) were exposed to individual EPF isolates and monitored daily for 21 days under laboratory conditions (April 2022) with access to food and water. The table reports restricted mean survival time (days ± standard error, S.E.), 95% confidence intervals (C.I.), and estimated time (days) to reach specific mortality thresholds (25%, 50%, 75%, 90%, and 100%). Median lethal time (LT_{50}) corresponds to the time to 50% mortality. The "95% Median C.I." represents the confidence interval of the median survival estimate (fiducial limits). The control group consisted of untreated insects maintained under identical conditions. Median lethal time (LT_{50}) corresponds to the time to

50% mortality. The "95% Median C.I." represents the confidence interval of the median survival estimate (fiducial limits). The control group consisted of untreated insects maintained under identical conditions.

		Restricted Mean			Age in days at % mortality					
Name	**No. of subjects**	**Days**	**S.E.**	**95% C.I.**	**25%**	**50%**	**75%**	**90%**	**100%**	**95% Median C.I.**
6G	10	14.50	1.94	10.71 ~ 18.29	10.00	13.00				10.0 ~ 21.0
15G	10	16.40	1.48	13.49 ~ 19.31	12.00	18.00				12.0 ~ 21.0
17G	10	10.10	1.35	7.45 ~ 12.75	7.00	10.00	12.00	14.00	20.00	7.0 ~ 11.0
22G	10	15.50	2.11	11.37 ~ 19.63	9.00	18.00				9.0 ~ 21.0
23T	10	13.10	1.96	9.25 ~ 16.95	8.00	11.00	20.00			8.0 ~ 19.0
30G	10	13.10	1.43	10.29 ~ 15.91	9.00	12.00	18.00	18.00		9.0 ~ 17.0
33G	10	15.60	1.29	13.07 ~ 18.13	11.00	17.00	19.00	21.00		11.0 ~ 18.0
35G	10	10.60	1.16	8.33 ~ 12.87	9.00	9.00	11.00	12.00		9.0 ~ 10.0
93G	10	16.10	1.61	12.95 ~ 19.25	10.00					10.0 ~ 21.0
102G	10	18.20	1.28	15.69 ~ 20.71	17.00	19.00				17.0 ~ 21.0
139G	10	20.60	0.38	19.86 ~ 21.34						- ~ -
143G	10	19.50	1.42	16.71 ~ 22.29						- ~ -
Control	10	16.10	2.16	11.87 ~ 20.33	9.00	21.00				9.0 ~ 21.0

The most lethal strain applied at full dose was 17G which killed 100% of *H. abietis* after 20 days. No other EPF treatment killed all 10 individuals. Three additional strains reached 90% of mortality with 35G being the fastest at 12 days, followed by 17G with 14 days, and 30G and 33G with 18 and 21 days, respectively. Additionally, strain 23T reached 75% of mortality by day 20 (Table 3.2, Fig 3.1). Although these mortality rates were higher than those observed in the control group, which exhibited 50% mortality by day 21, cautious interpretation of these findings is required due to the high control mortality and the small sample size, which may affect the statistical robustness of the results.


A. Adult *Hylobius abietis* mortality at full dose: more virulent isolates
Cumulative mortality (%)
100
75
50
25
5
10
15
20
Days after inoculation
Treatment
17G
22G
23T
30G
35G
6G
Control

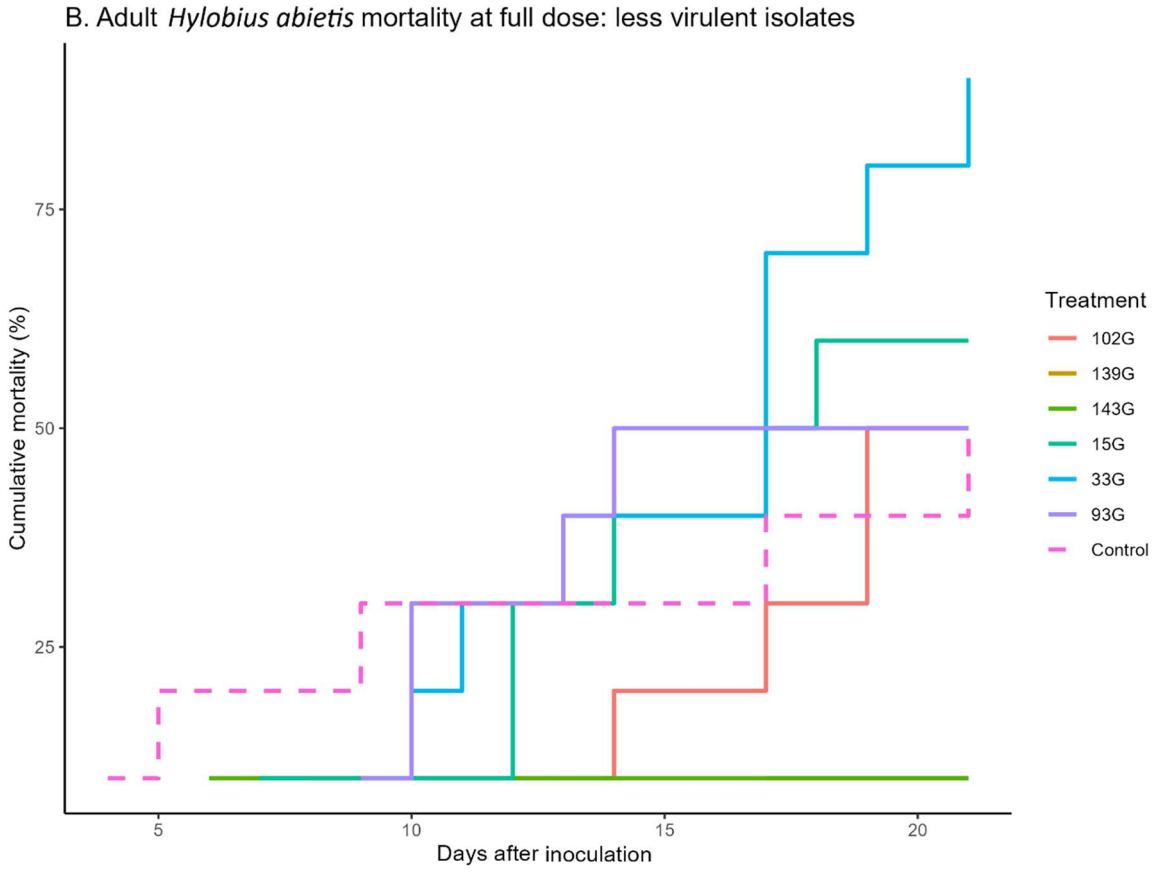

B. Adult *Hylobius abietis* mortality at full dose: less virulent isolates
Cumulative mortality (%)
75
50
25
5
10
15
20
Days after inoculation
Treatment
102G
139G
143G
15G
33G
93G
Control

Figure 3.1. Survival curves for adult *Hylobius abietis* exposed to twelve EPF isolates at a high conidial concentration (10^8 conidia ml^{-1}): Adult weevils (n = 10 per treatment) were monitored daily for 21 days following exposure under laboratory conditions with access to food and water. Survival is expressed as the proportion of individuals remaining alive over time. For visual clarity, isolates are separated into two panels based on relative virulence: (A) the six fastest-acting (more virulent) isolates and (B) the six slowest-acting (less virulent) isolates. The untreated "Control" is included in both panels for comparison. Differences in survival distributions between treatments were assessed using Log-rank tests (see Table 3.2 and Appendix Table 3.3). Steeper declines in survival curves indicate faster host mortality and greater pathogenicity.

The effects of 17G and 35G at medium and lower doses, where control mortality was notably absent (0%), yielded different LT_{50} values compared to their respective full-dose treatments. For 17G, the LT_{50} increased from 10 days at full dose to 12 days at medium dose and further decreased in virulence with a LT_{50} of 16 days at low dose (Fig 3.2, Appendix Table S3.2). No medium or lower dose treatments of 17G killed more than the 50% of *H. abietis* after 21 days. For the strain 35G, the LT_{50} at medium dose was 9 days, similar to the full-dose treatment. However, the low dose of 35G exhibited a slower kill rate with a LT_{50} of 20 days and did not surpass 50% mortality within the 21-day period. (Appendix Table S3.2).

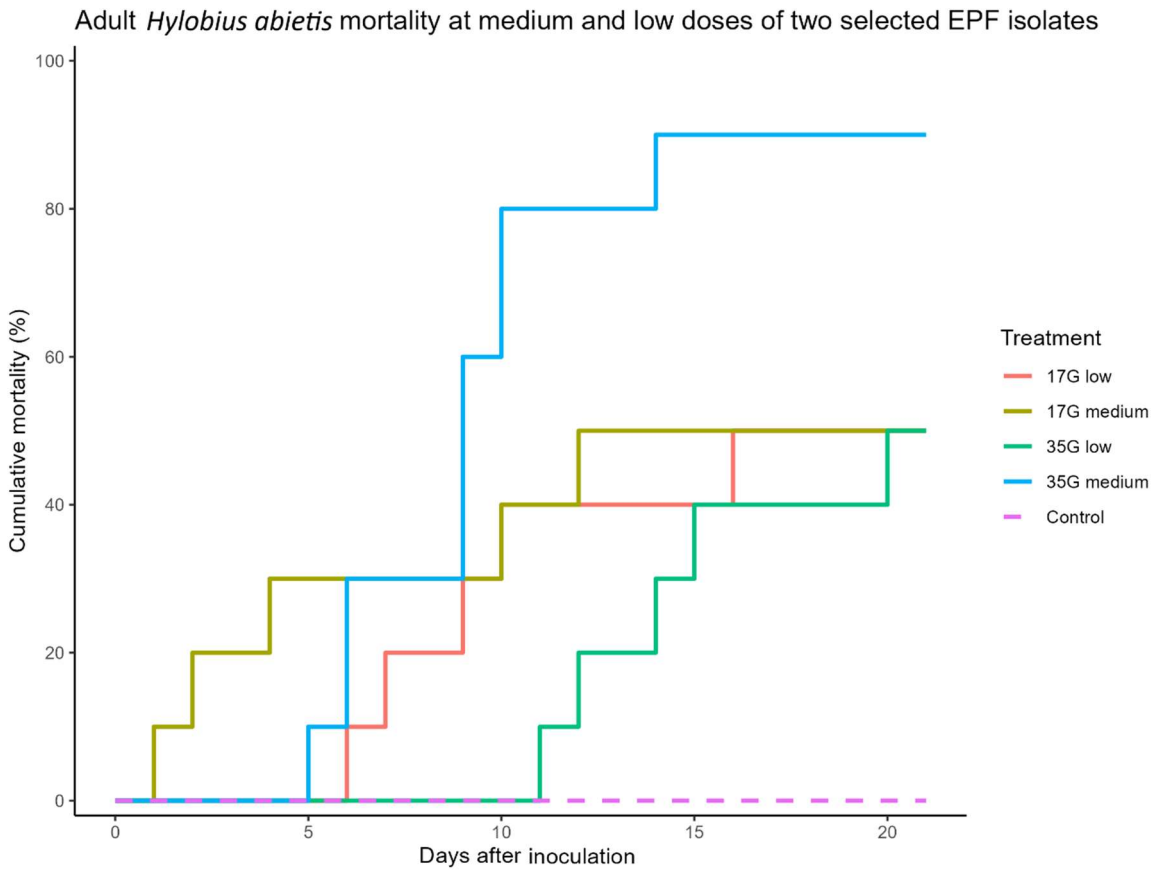


Figure 3.2. Survival curves for adult *Hylobius abietis* exposed to two fast-acting EPF isolates (17G and 35G) at medium and low conidial concentrations: Adult weevils (n = 10 per treatment) were exposed to EPF isolates at 10^7 conidia ml^{-1} (medium) and 10^6 conidia ml^{-1} (low), following selection of these isolates based on high virulence in the full-dose assay (Figure 3.1). Survival was monitored daily for 21 days under laboratory conditions with access to food and water and is expressed as the proportion of individuals remaining alive over time. The untreated "Control" was maintained under identical conditions without fungal exposure. Differences in survival distributions between treatments were assessed using Log-rank tests (see Appendix Tables 3.2 and 3.3). Steeper declines in survival curves indicate faster mortality and greater pathogenicity.

Bark area consumed by adult *H. abietis* exposed to 12 EPF strains at full concentration and 2 most effective EPFs at medium, and low concentrations for 14 days.

When analysing changes in bark area consumed by *H. abietis* after exposure to the 12 full-dose EPF treatments, most datasets were found to be non-normally distributed and were therefore analysed using the Kruskal-Wallis test, except for the area consumed per day. The

K-W identified significant differences in bark consumption at T= 4, 7 and 14 between control and treated weevils exposed to the full doses of the twelve EPF strains (Appendix Table S3.4, Fig. 3.3). At T=4, pairwise comparisons revealed significant differences in ingested bark between the control *H. abietis* and those treated with four *Metarhizium sp.* strains (23T, 30G, 139G and 143G). By T=7, there were found differences in the accumulated feeding area between the control and *H. abietis* exposed to six EPF strains. Notably, at T=14, the final accumulated bark feeding area consumed by control *H. abietis* differed significantly from that of LPWs treated with the two most effective EPFs, 17G and 35G (Appendix Table S3.6), highlighting the substantial impact of these strains in reducing bark consumption.

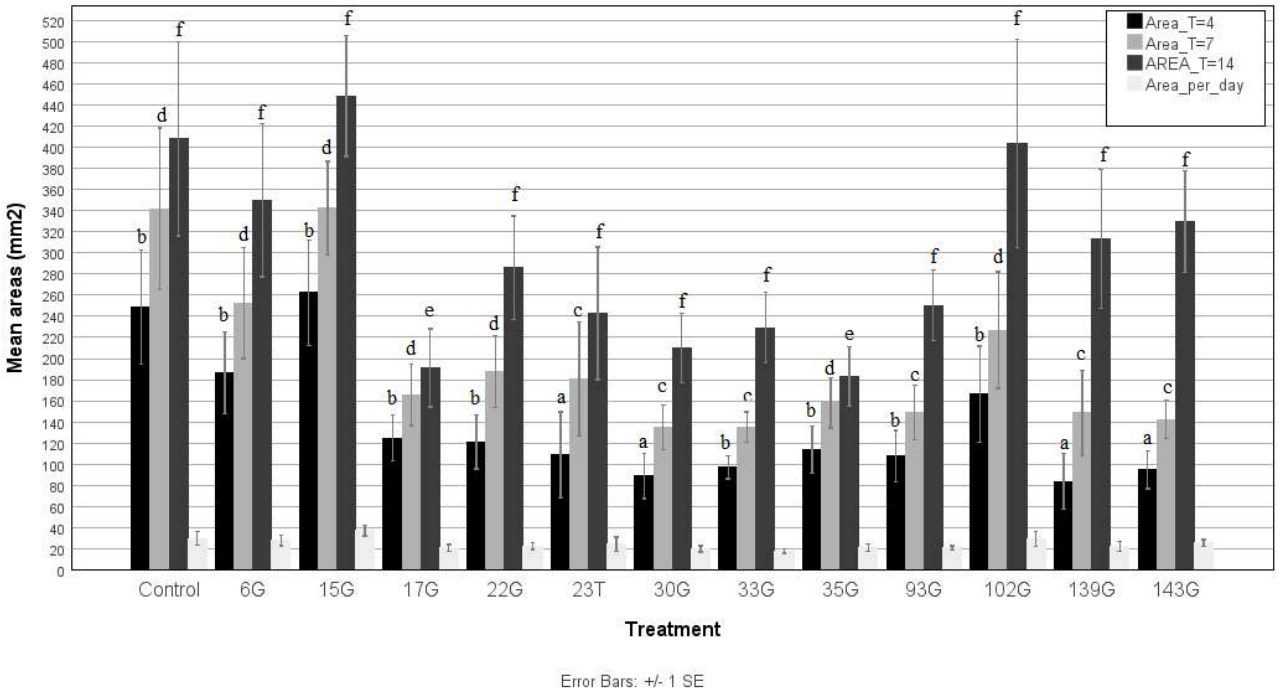


Figure 3.3. Bark consumption by adult *Hylobius abietis* following exposure to twelve EPF isolates at full dose (10^8 conidia ml^{-1}): Mean cumulative bark area consumed (mm^2) per individual weevil (n = 10 per treatment) was measured after 4, 7, and 14 days under laboratory conditions with access to food and water. The untreated control "Control" was maintained under identical conditions without fungal exposure. Bars represent different feeding metrics, distinguished by grey-scale shading as indicated in the legend: cumulative bark area at day 4 (T = 4), day 7 (T = 7), and day 14 (T = 14), as well as mean bark area consumed per day (Area_per_day). Due to non-normal distribution of most datasets (Kolmogorov–Smirnov test; Appendix Table 3.4), differences among treatments were analysed using the Kruskal–Wallis test, followed by Dunn's post hoc pairwise comparisons. Bars show: (a, c, e) treatments that differ significantly from the control and (b, d, f) treatments that do not differ significantly from the control at each time point. Significant

reductions in bark consumption relative to the control were observed for multiple isolates over time, with the strongest effects at day 14, where the fast-acting isolates 17G and 35G showed significantly lower feeding compared to untreated weevils (Appendix Table 3.6). Error bars represent ± standard error of the mean (SE).

In the experiment on bark area consumption by *H. abietis* treated with the fast-killing strains (17G and 35G) at medium and low doses, datasets were normally distributed and analysed using ANOVA, with the exception of data from T=4. Significant differences were found between treatment groups and the control at T=14, as well as in the analysis of area consumed per day (Appendix Table S3.4). *Post-hoc* analysis at T=14 indicated that area consumed by control weevils was significantly different to areas debarked by *H. abietis* exposed to both medium doses. Additionally, a significant difference in bark consumption was observed between the low and medium doses of the 35G treatment. Finally, in the analysis of area consumed per day, the debarked area by control *H. abietis* was significantly different from that of weevils exposed to medium doses of both 17G and 35G (Appendix Table S3.5, Fig. 3.4).

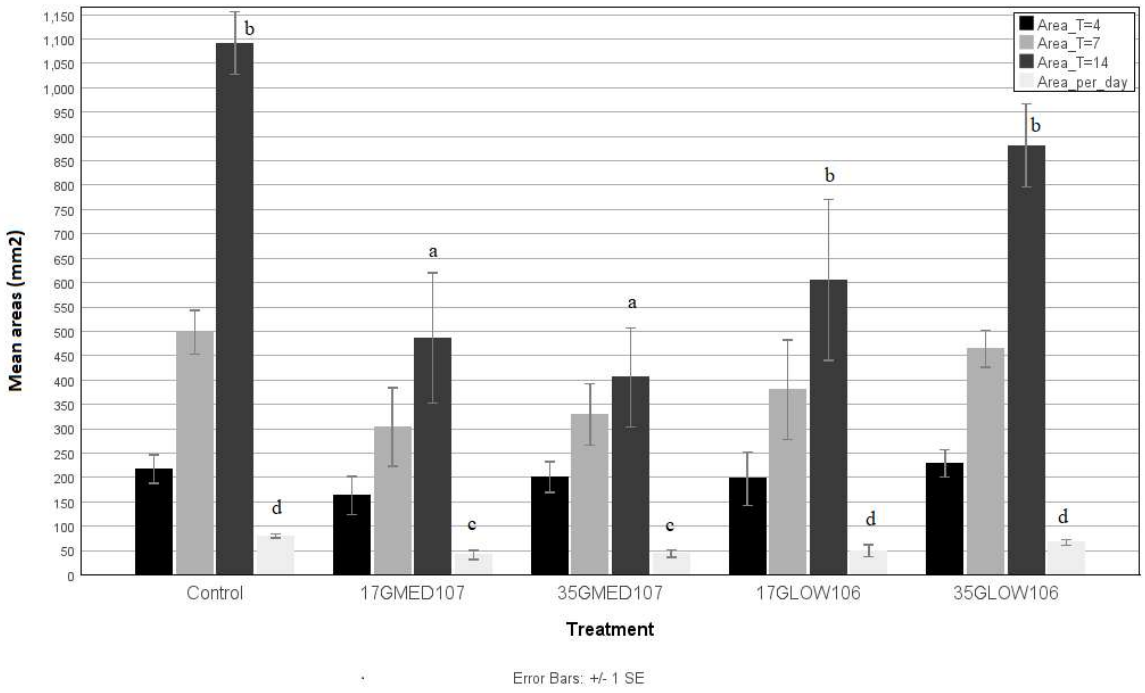


Figure 3.4. Bark consumption by adult *Hylobius abietis* following exposure to two fast-acting EPF isolates (17G and 35G) at medium and low concentrations: Mean cumulative bark area consumed (mm²) per individual weevil (n = 10 per treatment) was measured after 4, 7, and 14 days under laboratory conditions with access to food and water. Treatments included medium (10^7 conidia ml^{-1}; MED107) and low (10^6 conidia ml^{-1};

LOW106) concentrations of each isolate, alongside an untreated "Control". Bars represent feeding metrics distinguished by grey-scale shading as indicated in the legend: cumulative bark area at day 4 (T = 4), day 7 (T = 7), and day 14 (T = 14), as well as mean bark area consumed per day (Area_per_day). Data met normality assumptions (Kolmogorov–Smirnov test; Appendix Table 3.4) and were analysed using one-way ANOVA followed by Dunnett T3 post hoc comparisons. Bars show: (a, c) treatments that differ significantly from the control and (b, d) treatments that do not differ significantly from the control, for cumulative bark area at day 14 and mean bark area consumed per day, respectively. Significant reductions in bark consumption were observed at day 14 and in daily feeding rates for medium-dose treatments, particularly for isolate 35G, indicating a dose-dependent effect on feeding suppression. Error bars represent ± standard error of the mean (SE).

Weight change of adult *H. abietis* exposed to 12 EPF strains at full concentration and 2 best EPFs at medium and low concentrations for 14 days.

No significant differences were observed between control and full-dose treatments in the analysis of weight change T0-T14 (from T=0 to the day of death or T=14 if *H. abietis* survived) (Appendix Table 3.3). However, a single significant difference in weight change analysed per day was detected between *H. abietis* treated with 15G and those treated with 17G (Fig. 3.5).

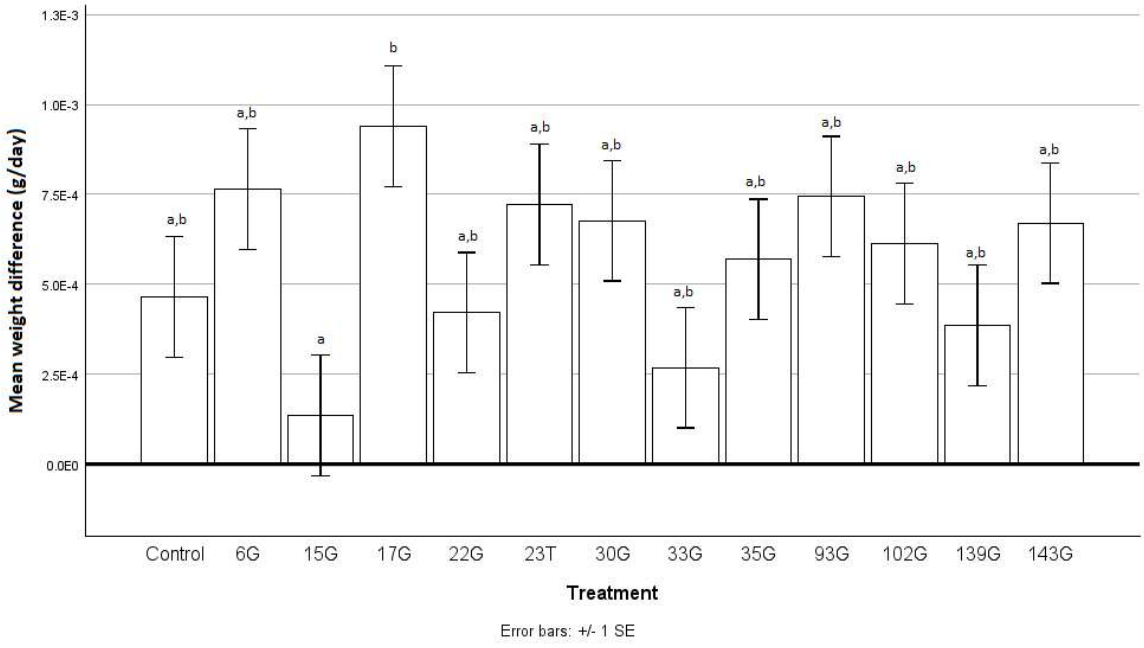


Figure 3.5. Mean daily weight change of adult *Hylobius abietis* following exposure to twelve EPF isolates at full dose (10^8 conidia ml^{-1}): Mean weight change per day (g day^{-1}) was calculated for individual weevils (n = 10 per treatment) over a 14-day period (from T =

0 to death or day 14 for surviving individuals) under laboratory conditions with access to food and water. Untreated "Control" was included for comparison. Data met normality assumptions (Kolmogorov–Smirnov test; Appendix Table 3.4) and were analysed using one-way ANOVA followed by Tukey's HSD post hoc test. Letters (a) and (b) represent homogeneous subsets of treatments with no significant differences within each subset. Overall, no significant differences in total weight change were observed between treatments and the control; however, a significant difference in daily weight change was detected between isolates 15G and 17G. Error bars represent ± standard error of the mean (SE).

Similarly, no significant differences were found between control and treatments in the experiment testing the two fast-killing strains (17G and 35G) at low and medium doses, both for weight change T0-T14 and weight change per day (Appendix Table S3.4). However, the low dose of 35G showed a significant difference compared to both medium doses in both experiments (Appendix Table S3.5, Figs. 3.6 and 3.7).

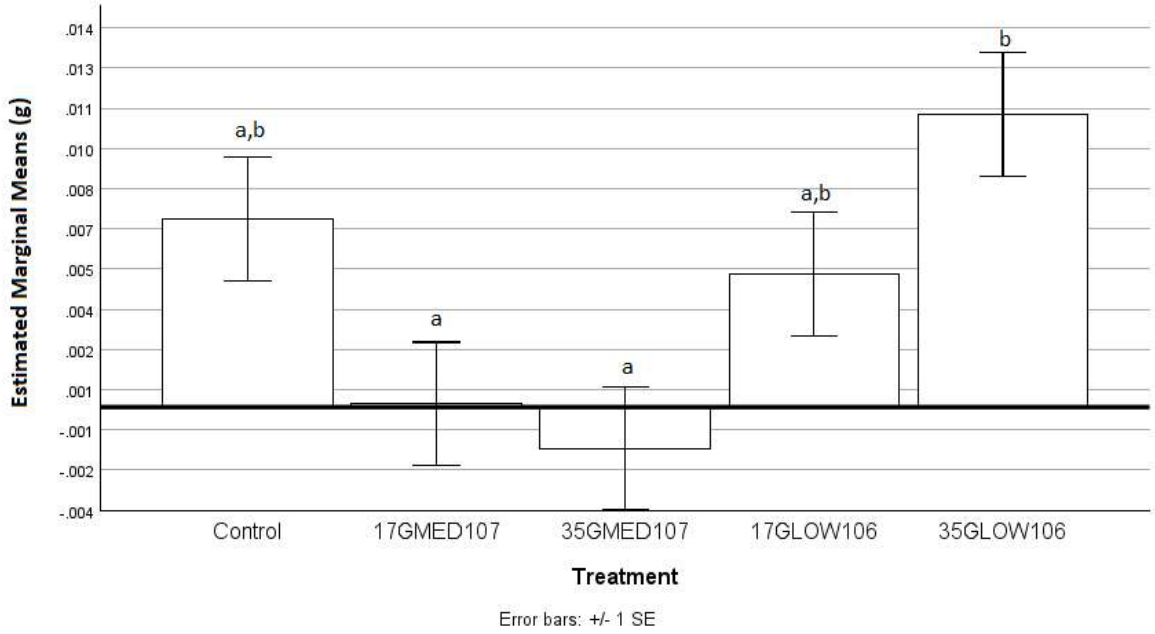


Figure 3.6. Mean total weight change of adult *Hylobius abietis* following exposure to two fast-acting EPF isolates (17G and 35G) at medium and low concentrations: Mean weight change (g) was calculated for individual weevils (n = 10 per treatment) over a 14-day period (from T = 0 to death or day 14 for surviving individuals) under laboratory conditions with access to food and water. Treatments included medium (10^7 conidia ml^{-1}; MED107) and low (10^6 conidia ml^{-1}; LOW106) concentrations of each isolate, alongside untreated "Control". Data met normality assumptions (Kolmogorov–Smirnov test; Appendix Table 3.4) and were analysed using one-way ANOVA followed by Tukey's HSD post hoc

test. Letters (a) and (b) represent homogeneous subsets of treatments with no significant differences within each subset. No significant differences in total weight change were observed between treatments and the control. However, the low concentration of isolate 35G differed significantly from both medium-dose treatments. Error bars represent ± standard error of the mean (SE).

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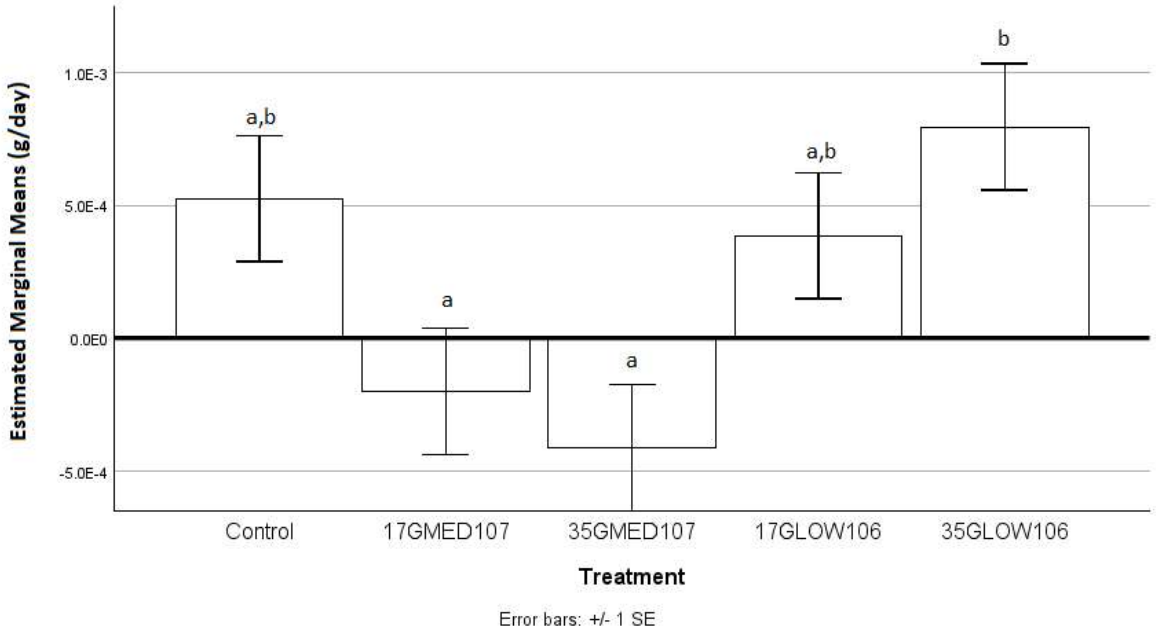
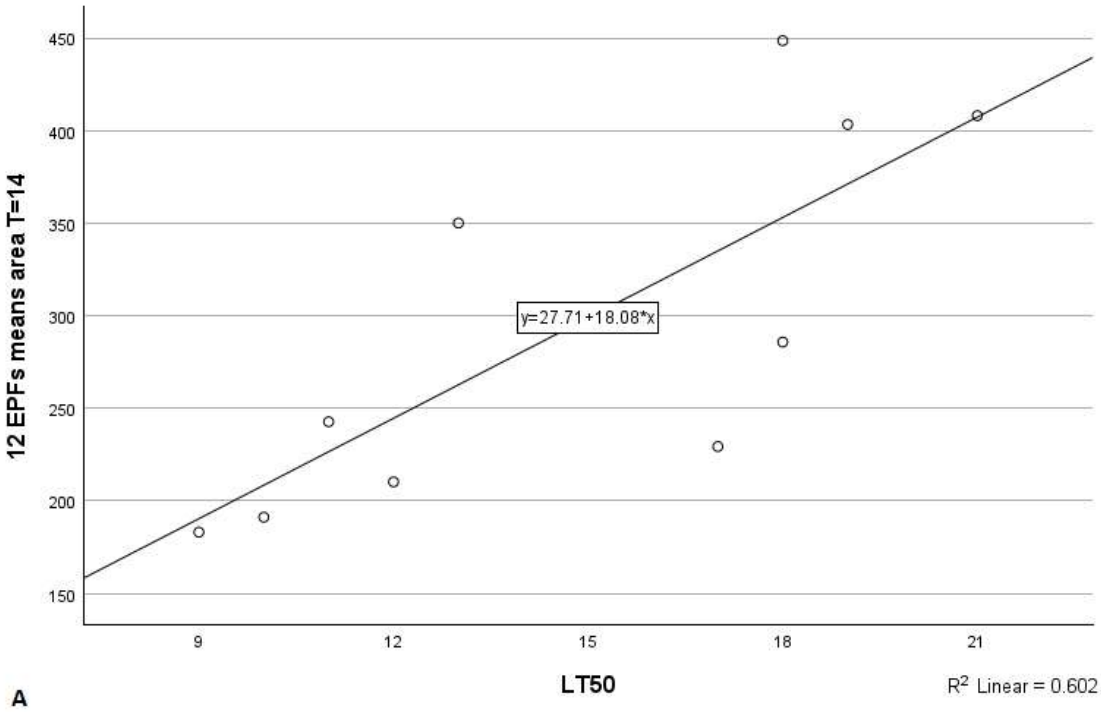


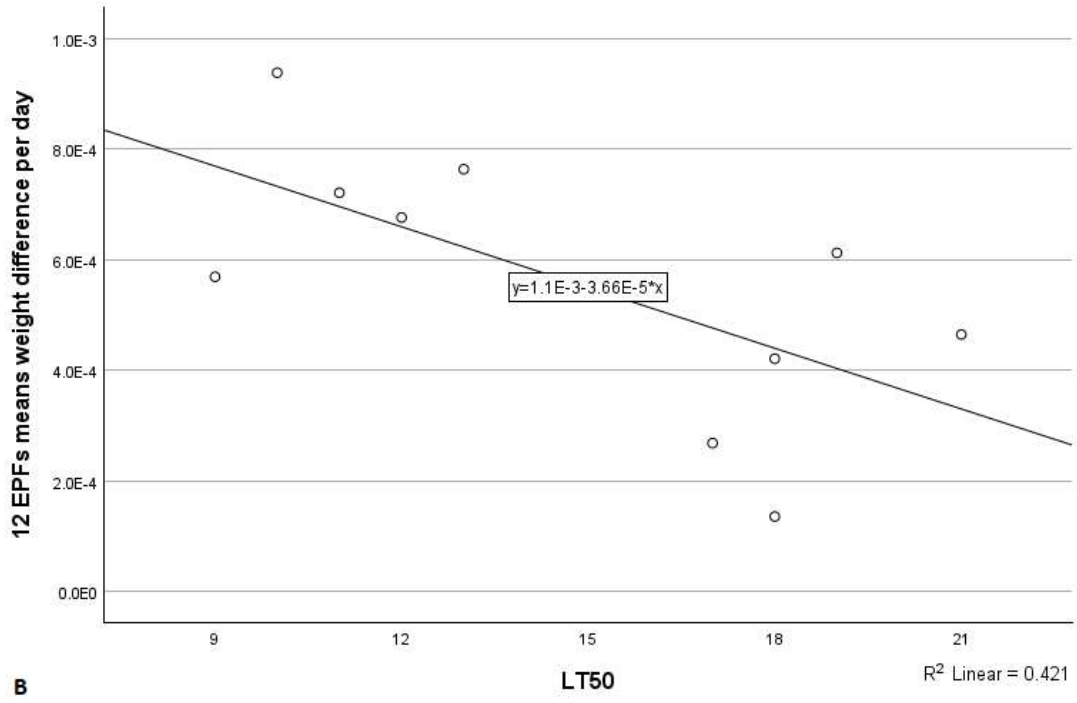
Figure 3.7. Mean daily weight change of adult *Hylobius abietis* following exposure to two fast-acting entomopathogenic fungal (EPF) isolates (17G and 35G) at medium and low concentrations: Mean weight change per day (g day^{-1}) was calculated for individual weevils (n = 10 per treatment) over a 14-day period (from T = 0 to death or day 14 for surviving individuals) under laboratory conditions with access to food and water. Treatments included medium (10^7 conidia ml^{-1}; MED107) and low (10^6 conidia ml^{-1}; LOW106) concentrations of each isolate, alongside untreated "Control". Data met normality assumptions (Kolmogorov–Smirnov test; Appendix Table 3.4) and were analysed using one-way ANOVA followed by Tukey's HSD post hoc test. Letters (a) and (b) represent homogeneous subsets of treatments with no significant differences within each subset. No significant differences in daily weight change were observed between treatments and the control. However, the low concentration of isolate 35G differed significantly from both medium-dose treatments. Error bars represent ± standard error of the mean (SE).

Correlation with LT_{50}.

A significant (P = 0.008) and strong positive correlation (Pearsons = 0.776) was found between the LT_{50} of the twelve full-dose EPF treatments and the accumulated feeding area means at T=14 (Fig. 3.8A). Additionally, a significant (P = 0.042) moderate negative correlation (Pearsons = -0.649) was observed between LT_{50} and the mean weight change per day in weevils treated with the 12 full-dose EPF treatments (Appendix Table S3.7, Fig. 3.8B).



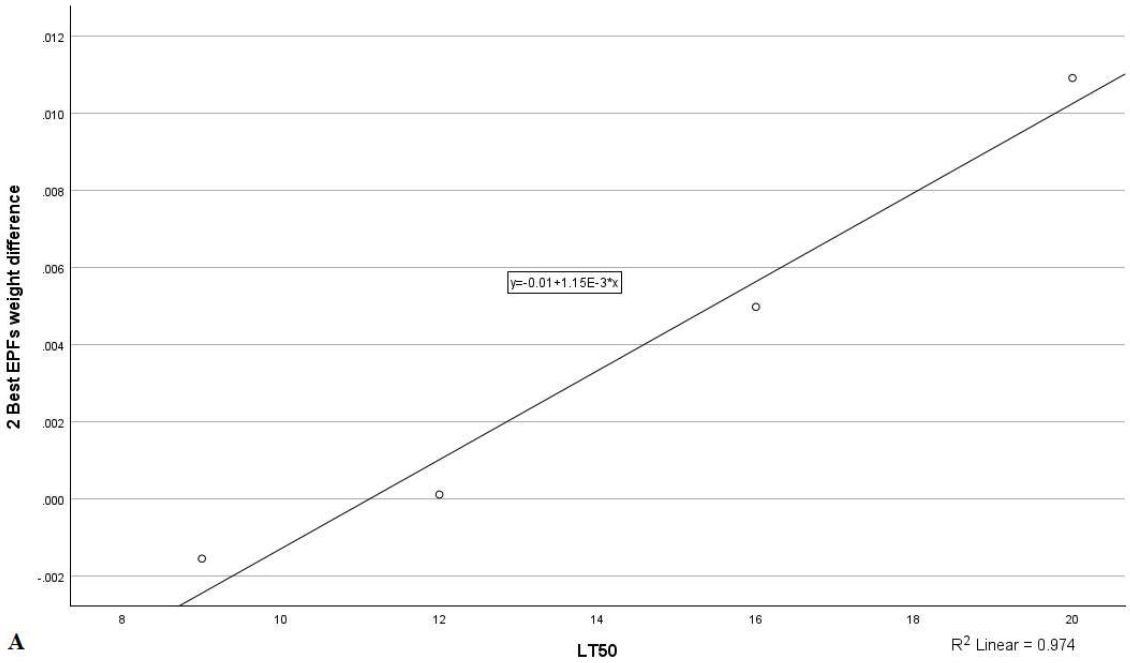
A



B

Figure 3.8. Relationships between virulence (LT_{50}) and feeding and physiological responses of adult *Hylobius abietis* exposed to twelve EPF isolates at full dose (10^8 conidia ml^{-1}). Scatterplots show correlations between LT_{50} (median lethal time, days) and (A) mean cumulative bark area consumed (mm^2) at day 14, and (B) mean daily weight change (g day^{-1}), calculated from individual weevils (n = 10 per treatment). Each point represents the mean response for a given EPF isolate. Pearson's correlation coefficients (r) and associated p-values were calculated to assess the strength and significance of relationships (Appendix Table 3.7). A significant positive correlation between LT_{50} and bark consumption (A) indicates that slower-acting isolates (higher LT_{50}) are associated with higher feeding. A significant negative correlation between LT_{50} and daily weight change (B) indicates that faster-acting isolates tend to be associated with greater reductions in weight over time. Lines represent linear regression fits.

For fast-killing strains, a significant (P = 0.028) strong positive correlation (Pearsons = 0.972) was found between the mean feeding areas of weevils exposed to medium and low doses at T=14 and their LT_{50} (Fig. 3.9C). In the same way, significant strong positive correlations were observed between LT_{50} and both the mean of weight change (P = 0.013, Pearsons = 0.987) (Fig. 3.9A) and weight change per day (P = 0.006, Pearsons = and 0.994) (Fig. 3.9B) (Appendix Table S3.7).



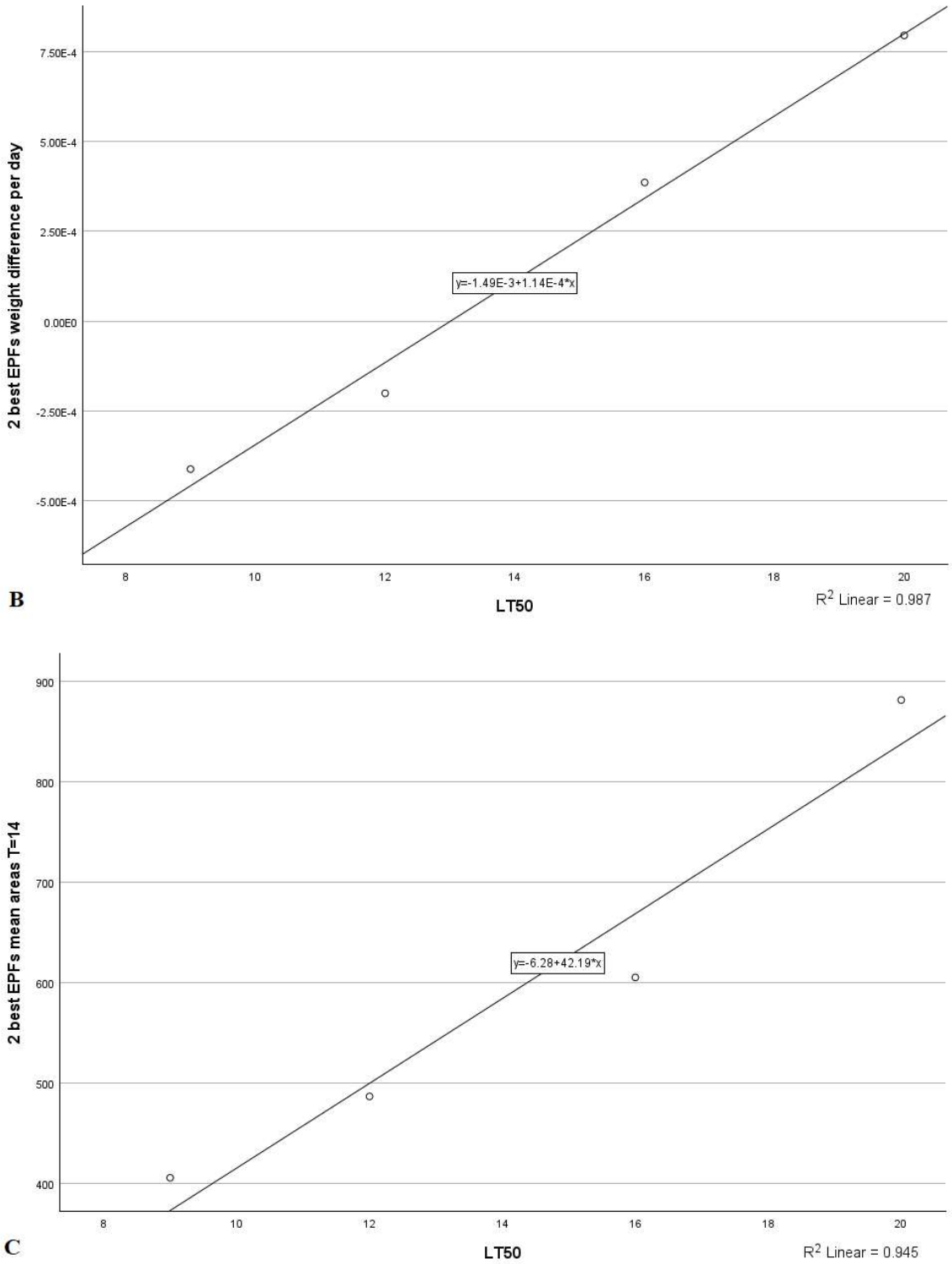


Figure 3.9. Relationships between virulence (LT_{50}) and host responses in adult Hylobius abietis exposed to two fast-acting EPF isolates (17G and 35G) at medium and low concentrations: Scatterplots show correlations between LT_{50} (days) and mean responses of weevils (n = 10 per treatment) for (A) total weight change (g), (B) daily weight change (g day^{-1}), and (C) cumulative bark area consumed (mm^2) at day 14. Treatments include medium (10^7 conidia ml^{-1}) and low (10^6 conidia ml^{-1}) concentrations of each isolate. Each point represents the mean response for a given treatment. Pearson's correlation coefficients (r) and

associated p-values were calculated to assess the strength and significance of relationships (Appendix Table 3.7). Strong positive correlations indicate that increased LT_{50} (reduced virulence) is associated with increased feeding and weight-related responses. Lines represent linear regression fits.

Discussion

Mortality caused on adult *H. abietis* by the most virulent strains in our study showed promising results, reaching 90% mortality on days 12 and 14, akin to other studies e.g. (Ansari and Butt, 2012). Our mortality results are consistent with those from studies using the most virulent strains cited in the literature where direct "dry" conidia applications were used (Ansari and Butt, 2012). However, the results should be interpreted with caution as control mortality on the full doses experiment was over 20%. Moreover, we used the "wet" conidia method, that is generally considered less effective, suggesting that higher mortality rates might be obtained by testing the efficacy of our strains with different inoculation approaches. Notably, *B. bassiana* (35G) was highly effective at both full and medium doses ($10^{\wedge 8}$ and $10^{\wedge 7}$ spores/ml), making it a promising strain to be further tested towards commercial field applications such as control of adult *H. abietis* emergence from tree stumps at clear-felled areas. This aligns with previous studies that tested locally sourced strains applied as wet conidia at operational doses (Williams *et al.*, 2013b).

Feeding behaviour of adult *H. abietis* differed between the two experimental periods, with lower bark consumption observed in the first experiment conducted in April 2022, compared to the second experiment in November 2022. These different feeding rates could be linked to behavioural preparations for overwintering during the later months of the year (Willoughby *et al.*, 2017). For instance, control *H. abietis* in April consumed 29.79 mm^2/day, whereas in November, daily consumption of control weevils rose to 80.47 mm^2/day, possibly in anticipation of overwintering. These findings suggest that the use of suitable EPFs in integrated pest management should be tailored to specific circumstances. The lower feeding levels during the first experiment and the levels of mortality on control *H. abietis* could also being explained by moribund or poor condition of the individuals used for the experiment highlighting the importance of ensuring the highest standards while collecting and/or rearing *H. abietis* for similar investigations.

Our study shows a complex relationship between EPF strain virulence, concentration and feeding inhibition, especially at lower concentrations of fast-killing EPFs. Full-dose EPF treatments produced infection effects on adult *H. abietis,* with bark consumption generally

decreasing across treated groups. Some strains showed statistically significant reductions in bark consumption compared to controls during the first week, culminating in significant differences in total bark consumption after two weeks for the two most virulent strains. These findings are consistent with Barta et *al* (2019), where various wild EPFs reduced bark consumption in treated weevils. In contrast, strain 15G exhibited slightly higher bark consumption than the control; however, this difference was small and not statistically significant. Given the limited sample size and absence of replication, this pattern should be interpreted cautiously and does not provide robust evidence for increased feeding associated with EPF infection. Rather than indicating a true biological effect, such variation likely reflects natural variability in feeding behaviour among individuals. It could mean that the same entomopathogenic species can trigger different behavioural responses, potentially due to differences in infection mechanisms or concentrations of secondary metabolites of each strain, such as oosporein, which has been shown to increase appetite in adult *H. abietis* (McNamara *et al.*, 2019).

The degree of feeding inhibition in each treatment corresponded with LT_{50} values. Strains with lower LT_{50} (greater virulence), generally decreased *H. abietis* bark consumption more effectively, while strains with higher LT_{50} allowed for more substantial feeding. In the case of the fast-killing strains, medium concentrations remained highly virulent reducing significantly bark consumptions, whereas lower concentrations exhibiting less virulence had less impact on feeding inhibition. Similar results were reported by Barta et *al* (2019) using *B. bassiana* and *B. pseudobassiana* strains, where *H. abietis* infected with EPFs exhibiting major virulence ate less bark, whilst those infected by EPF treatments less virulent had major feeding rate.

Our results indicate an unexpected negative correlation between LT_{50} and mean weight change per weevil per day that could imply difference of metabolism of *H. abietis* infected by various strains or an effect of the period over which the measurements were made. However, this does not translate into a greater total area of bark consumed by day 14 among the remaining live weevils. Conversely, the more efficacious EPFs (lower LT_{50}) correlate with lower levels of feeding after 14 days. By the end of the experiment, weevils exposed to more efficacious fungi are alive but moribund, while those exposed to less effective fungi remain active and continue feeding normally. This pattern suggests that sublethal doses or "weaker" EPFs may allow *H. abietis* to maintain relatively normal bark feeding behaviour in adult *H. abietis*, permitting a response to boost their immunity. Previous research shown that both *Metarhizium* and *Beauveria* have different abilities to modulate *H. abietis* immune

responses (McNamara *et al.*, 2018b). The response to EPF infection will determine their capabilities as suitable biocontrol agents.

From a biocontrol perspective, the more efficacious fungi are at killing, the less detrimental infection effects on young trees caused by *H. abietis* feeding will occur in the medium to long term. These findings open opportunities for further investigate these trends by expanding the experiments with more weevils examining metabolic responses.

Conclusions

Our study provides evidence that certain wild EPF strains can significantly reduce both survival and feeding damage in adult *H. abietis* under laboratory conditions. Among the most effective strains, *Beauveria bassiana* isolate 35G and *Metarhizium sp.* 17G produced differing responses depending on concentration. Specifically, higher doses led to lower bark consumption, suggesting that these strains are highly effective when applied at full or medium doses.

As LT_{50} increases, feeding inhibition decreases, leading to continuous bark consumption among *H. abietis* exposed to sublethal EPF concentrations. This continuous feeding may favour immune response acting as a compensatory mechanism for fungal infection, potentially helping to counteract the physiological stress. Thus, employing highly efficacious EPF strains with lower LT_{50} values may limit non-lethal feeding effects and enhance the suppression of *H. abietis* populations in pest management contexts.

The differential impact of wild EPF strains on both mortality and feeding behaviour highlights the importance of ongoing research to identify and test effective local strains. Utilising the methods and procedures outlined in this study may aid in identifying strains with high efficacy and appropriate lethality thresholds, thereby avoiding unintended feeding stimulation.

Based on the promising laboratory results, *B. bassiana* isolate 35G and *Metarhizium* sp. 17G are strong candidates for field trials. We recommend testing these strains at various concentrations and exploring potential additive / synergistic effects when combined with commercial entomopathogenic nematodes such as *Steinernema carpocapsae* (Weiser, 1955). Further field-based studies will help establish their practical utility in integrated pest management strategies targeting *H. abietis* infestations in forestry settings.

Regarding our hypothesis, although smaller LT_{50} values had a negative correlation with weight change per day, the increase of daily mean weight was not translated into greater bark

intake as shorter killing times were correlated with less feeding damage. Therefore, we cannot predict if LT_{50} will increase or decrease bark area consumption after 14 days since infection effects might render adult *H. abietis* moribund. Observed weight changes suggest complex physiological responses involving LPW metabolism caused by the most efficacious EPFs, which do not translate into greater feeding damage, as evidenced by the unexpected negative relationship between LT_{50} and weight change per day.

In summary, targeting adult *H. abietis* with the most efficacious EPF can reduce non-lethal feeding effects, effectively reducing damage in saplings. The differential action of wild EPF strains on *H. abietis* mortality and feeding behaviour in the present study is both interesting and encouraging. Therefore, we suggest continuing the search for even more infective and efficient wild and local EPF strains using the methods and procedures proposed in this paper looking to avoid unintended feeding stimulation. Finally, the promising laboratory results of this research suggest *B. bassiana* isolate 35G and *Metarhizium* sp. 17G as strong candidates for testing at field conditions evaluating their activity against *H. abietis* at different concentrations and possible additive interactions with commercial entomopathogenic nematodes such as *S. carpocapsae*.

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CHAPTER 4:

Field Trials of Wild Entomopathogenic Fungi and Commercial *Steinernema carpocapsae* on the Large Pine Weevil (*Hylobius abietis* [L.]) Including an Assessment of Non-Target Effects

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Abstract

The large pine weevil (*Hylobius abietis*) is a major pest in European and Asian coniferous forests, particularly in managed plantations where clear-felling practices create ideal conditions for its population growth. Traditional management practices involving synthetic insecticides have limited efficacy in terms of reducing pest populations and pose environmental risks. This study evaluated the effectiveness of a wild entomopathogenic fungus (EPF) and the commercial entomopathogenic nematode *Steinernema carpocapsae* (EPN) as biological control agents (BCAs) against *H. abietis* in clear-felled spruce plantations in Wales and Scotland. Field trials used a randomised block design with three treatments (EPN full dose, EPF full dose and a combination of EPF+EPN at half doses each) compared to a control. Emergence trapping and destructive sampling were employed to assess treatment efficacy. All treatments significantly reduced weevil emergence, with the mixed treatment showing the greatest impact. Destructive sampling revealed strong associations between treatment type and infection outcomes in *H. abietis*, with a small but significant relationship between weevil developmental stages and infection types. Importantly, the treatments had no significant impact on the total abundance or taxon richness of non-target invertebrates. These findings suggest that wild EPFs alone and combined with EPNs are effective and environmentally safe alternatives to synthetic insecticides for managing *H. abietis* populations in managed forests.

Introduction

Hylobius abietis (L.) (large pine weevil) has a widespread natural distribution in Europe and Asia. This weevil is highly attracted to freshly cut conifer stumps that release volatiles, such as alpha-pinene, that indicate the availability of wood material for egg laying (Moore, 2001, Inward *et al.*, 2012). The availability of stumps of fallen trees within which to reproduce is low in natural woodlands and, under natural conditions, is a limiting factor for the increase in *H. abietis* populations. However, the opposite scenario is found in expansive coniferous exploitations (Willoughby *et al.*, 2017). The harvesting of large coniferous plantations by clear-felling provides hundreds, or even thousands, of stumps that have made *H. abietis* populations increase, affecting the regeneration of such forests (Leather *et al.*, 1999, Inward *et al.*, 2012).

H. abietis lifecycle ranges from 12 to 36 months depending on the temperature (the climate change-related increase in temperature shortens the cycle and favours dispersion), starting as eggs laid below ground by the stumps (Dillon *et al.*, 2006, Dillon *et al.*, 2012). Hatched larvae feed in the cambial layer until pupation and emergence of callow adults that

mainly feed on the bark of young conifer saplings (often leading to their death by girdling), exposed lower stems and the upper part of coniferous and broadleaved trees (Willoughby *et al.*, 2017). A solitary adult *H. abietis* lethally affects several saplings up to five years old, when young trees can withstand debarking damage. Consequently, adults in high number are a pest for forest re-stocking, as they can kill all the new saplings when no protective measures are undertaken (Leather *et al.*, 1999, Kapranas *et al.*, 2017). In Europe, 3.4 million ha of forest are threatened, producing estimated losses of €140 million per annum; meanwhile, public and private forests within the U.K. suffered estimated losses of €4.6 million in 1998 (Leather *et al.*, 1999, Kapranas *et al.*, 2016, McNamara *et al.*, 2018a).

The integrated control of *H. abietis* in U.K. and Ireland can incorporate the pre-application in nurseries of acetamiprid (amongst other synthetic chemicals used under derogation) to protect the seedlings prior to forest planting, followed by an additional insecticide top-up spraying on site (Kapranas *et al.*, 2016, Willoughby *et al.*, 2017, Moore *et al.*, 2021). There is concern due to the potential environmental impacts of these substances, as they are considered "highly hazardous chemicals" according to the Forest Stewardship Council (FSC) guidelines (Kapranas *et al.*, 2016, McNamara *et al.*, 2018a). For example, cypermethrin presents high toxicity to freshwater and other non-target organisms (Desneux *et al.*, 2007, Saha and Kaviraj, 2008, Merivee *et al.*, 2015). Moreover, the use of synthetic insecticides has a repulsive rather than a lethal effect on *H. abietis* and does not help to reduce the local populations of the pest (Rose *et al.*, 2005, Torr *et al.,* 2005, Leather *et al*., 1999). Alternatives to the use of synthetic chemicals against sapling damage by *H. abietis* include changes in silviculture practices such as mounding (Luoranen and Viiri, 2012), delayed restocking and altered silvicultural practices (Von Sydow, 1997, Örlander and Nilsson, 1999, Örlander and Nordlander, 2003), based on risk management systems integrated with GIS (Wainhouse *et al.*, 2007, Kapranas *et al.*, 2016), and the application on stumps of the decaying fungus *Phlebiopsis gigantea* (Fr.), which makes the stumps unsuitable for oviposition (Skrzecz and Moore, 1997). Another alternative is the use of mechanical barriers such as collar guards and the application of a protective coating (Petersson *et al.*, 2004, Petersson *et al.*, 2006, Willoughby *et al.*, 2017, Hardy *et al.*, 2020a); physical barriers, under determined circumstances, in the U.K. and Ireland can partially replace synthetic chemicals as part of an integrated approach to protect saplings (Moore *et al.*, 2021). A promising approach is the use of biological control agents (BCAs), which represent a less environmentally impactful alternative to treatments when applied as biopesticides in an inundative fashion to target and supress *H. abietis* infestations (Calvo *et al.*, 2012, van Lenteren *et al.*, 2017). These agents are generally considered environmentally

safe for plants, vertebrates and non-target insects, even though they have a broad host range. In addition, BCAs offer practical advantages, including the capacity for mass-production and typically limited persistence in the environment. This is particularly evident for entomopathogenic nematodes (EPNs)(Boemare *et al.*, 1996, Dillon *et al.*, 2012) and entomopathogenic fungi (EPFs) (Scheepmaker and Butt, 2010, Dara *et al.*, 2019, Chiew *et al.*, 2022).

EPN free-living infective juveniles (IJs) are the host-seeking stage that, first, find and enter the insect host via spiracles, the mouth, the anus and/or the cuticle and then, colonise and kill the insect by releasing symbiotic bacteria (*Xenorhabdus* sp. by Steinernematidae, or *Photorhabdus* sp. by Heterorhabditidae) that turn the host tissue into a nutrient medium that favours the continuity of EPN lifecycle until the release of numerous new IJs (Kaya and Gaugler, 1993, Forst *et al.*, 1997). *Steinernema carpocapsae* is recognised as an "ambush forager" that sits and waits for its insect host, sometimes nictating on the substrate. The IJ stage is non-feeding and resistant and the only life stage of an EPN that can survive outside an insect host. The EPFs used as biopesticides against *H. abietis* include species from the genera *Metarhizium* and *Beauveria*, which infect by contact with their mycelium and asexual conidiospores in every stage of target insects with piercing–sucking mouthparts (Ansari and Butt, 2012, Oreste *et al.*, 2012, Williams *et al.*, 2013b). Both EPNs and EPFs have been reportedly used as biopesticides to target developing larvae and pupae of *H. abietis* in field trials, applied in an aqueous suspension drenched around stumps. Earlier studies focused on the use of EPNs alone (Brixey *et al.*, 2006, Dillon *et al.*, 2007, Torr *et al.*, 2007), whereas more recent reseach has explored combined applications of EPN and EPF to enhance control efficacy (Williams *et al.*, 2013b, McNamara *et al.*, 2018a). For example, Kapranas *et al.*, (2017), using the EPN *Steinernema carpocapsae* (Weiser, 1955) at the recommended operational full dose of 3.5×10^6 IJs/500 mL per stump on field trials obtained suppression of emergence below the threshold of 20 adults of *H. abietis* per stump. In other works, similar suppression was obtained when *S. carpocapsae* was applied in half doses together with half doses of the EPF *Beauveria caledonica* Bisset and Widden (1.75×10^6 IJs + 5×10^8 conidiospores/500 mL per stump), displaying additive effects of these BCAs (Williams *et al.*, 2013b). Later, Mc Namara *et al.* (McNamara *et al.*, 2018a) also found additive effects for various pairs of EPNs and EPFs that provided different levels of reduction in the emergence of adult *H. abietis* up to 93% with respect to the control.

The aim of this paper is to build on this line of work by studying in clear-felled woodlands the effects of new wild EPFs that provided good control of adult *H. abietis* in the laboratory, examining them alone and in combination with the commercial EPN *S.*

carpocapsae. We are interested not only in the effectiveness but also in the safety of these BCAs in relation to non-target stump-dwelling insects.

Materials and Methods

Sites for the Field Trials

The field trials were conducted in two clear-felled spruce plantations managed by the Forestry Commission, which had been felled for 15 to 18 months. The presence of *H. abietis* larval stages was confirmed through destructive sampling of stumps during July 2021 for the first site trial and during May 2022 for the second field trial. The first site, used for the 2021 trials, was felled around February 2020 in Abergynolwyn, Gwynedd, Wales (grid reference: SH675054), and the second site, used for the 2022 trials, was felled between October 2020 and February 2021 in Clatteringshaws, Scotland (grid reference: NX545791). Both sites were treated with wild entomopathogenic fungi (EPFs). The Welsh site, on 23/07/2021, was treated with *Metarhizium* sp. 15G (RMK-2011b according to BLAST likelihood analysis), and the Scottish site with *Beauveria bassiana* isolate 35G (DAOM210087 according to BLAST likelihood analysis) on 16 June 2022. These wild fungi were obtained from nature reserves in Lancashire (Chapter 3). Additionally, both sites received treatments with the commercial entomopathogenic nematode (EPN) *Steinernema carpocapsae* (Nemasys® C), supplied by BASF, Widnes, UK.

The treatments on each site were arranged in a randomised block design comprising twenty blocks, with ten replicates allocated to emergence trapping and ten to destructive sampling. Each block consisted of four tree stumps, representing three treatments (EPN full dose, EPF full dose and EPF + EPN half doses) and an untreated control stump. In total, 80 tree stums were used per site, with 40 assigned to emergence trapping and 40 to destructive sampling.

Treatments and Application

The mass production of EPF conidiospores was achieved by plating the selected wild fungi in twenty sterile 9 cm Petri dishes containing potato dextrose agar (PDA) medium (Oxoid Ltd., Basingstoke, Hampshire, UK). The plates were incubated in dark conditions at 27 °C for two weeks prior to field application. The conidiospores were rinsed out of the plates the day before the application using sterile distilled water mixed with 0.03% (*v*/*v*) viscous Tween-80 (Sigma-Aldrich, Dorset, UK), and the content of four plates at a time was collected into 50 mL Falcon™ tubes (Scientific Laboratory Supplies, Nottingham, UK) and

kept at 4 °C overnight. The conidiospores were counted using a haemocytometer (Hawksley Ltd, Lancing, West Sussex, UK) placed under a light microscope (400× magnification), and their concentrations were obtained following Lacey (Lacey, 1997).

At the start of the field application, Nemasys® powder containing *S. carpocapsae* was suspended in 1 L measuring cylinders using distilled water. The active EPNs were then counted from five 200 µl aliquots using a binocular stereo microscope to estimate their concentrations per litre. The suspensions were finally diluted into 5 L containers to the desired concentrations and aerated with aquarium pumps (Williams *et al.*, 2013b).

The full-dose treatments included commercial nematodes at a rate of 3.5×10^6 nematodes per 500 mL and wild entomopathogenic fungi at a rate of 10^9 conidiospores per 500 mL. The half-dose treatment (HalfMix) combined 1.75×10^6 nematodes with 5×10^8 conidiospores per 500 mL. The control treatment consisted of distilled water. All the treatments were diluted in distilled water, and when the conidiospores were included, the aqueous treatments contained 0.03% (*v*/*v*) viscous Tween-80. The treatments were applied by drenching the top perimeter of each stump, above soil level, allowing the biological agents to penetrate between the bark and the stump effectively.

Assessment of Efficacy

The efficacy of the treatments was evaluated by destructive sampling and emergence trapping, complemented by a mark–recapture experiment associated with the latter. Three weeks after treatment application, 10 blocks where destructively sampled following a similar approach to that of Dillon *et al.* (Dillon *et al.*, 2006). All the bark from the main stump bole and its roots up to 50 cm, was carefully removed using a chisel, while determining in situ the developmental stage of *H. abietis* (larva, pupa and adult) and the infection status (alive, nematode-killed, fungus-killed or indeterminate). For each *H. abietis*, the depth and distance from the bole of the stump were recorded. The collected weevils were placed in 24-well plates (Greiner Bio-One Ltd, Stonehouse, Gloucestershire, UK) using clean forceps and monitored in the laboratory for two weeks at room temperature (22 °C) to check for the presence of EPF mycelium or EPN.

Modified emergence traps designed by Moore (Moore, 2001) and a parallel mark–recapture experiment were deployed four weeks after the application of the treatments on the remaining 10 blocks. For the mark–recapture test, three freshly cut Sitka spruce billets (approximately 30 cm long by 15 cm diameter) were stacked in order to attract adult *H. abietis* from the surrounding area and placed in the centre of each block on top of a PDA Petri dish (9 cm diameter) containing the sporulated wild *Metarhizium* sp. 17G (RMK-2011b

according to BLAST likelihood analysis) used as a bioinsecticide (Chapter 3). The aim of the mark–recapture experiment was to see if those weevils escaping control in the treated stumps could be attracted to billets in the field and subsequently controlled with the EPF, which was placed under each billet. The traps and billets were checked every week until *H. abietis* emergence ceased in November. Adults of *H. abietis* from the control emergence traps were collected and taken to the laboratory, while the ones collected from the treatment traps were colour-marked on the right elytra with quick-dry nail polish (Sally Hansen, New York, NY, USA) for identification (if recaptured) and released back into the field.

For each collection of weevils from the emergence traps in the second field trial (Clatteringshaws), non-target invertebrates that emerged were also collected. Initially, for all of these invertebrates, the Order or another higher classification level was determined. These taxa comprised Coleoptera, Diptera, Hymenoptera, Lepidoptera, Myriopoda, Arachnida, Opilionidae, Auchenorhyncha, and Collembola.

Following this initial classification, all Diptera were determined to the family level, and ground beetles (Carabidae) were determined to the species level to provide a second, more detailed, taxon list. The Diptera were identified using Unwin's guide (Unwin *et al.*, 1981), and the Carabidae were identified using Luff's guide (Luff, 2007). Following the identification and tabulation of the non-targets, total abundance, broad taxon richness and narrow taxon richness were compared among the treatments, statistically by principal co-ordinates analysis (PCoA).

Statistical Analysis

The emergence data were analysed with R v.4.4.1 (R Core Team, Vienna, Austria) (R Team, 2024); all other univariate analyses were performed on SPSS Statistics 29.0 (IBM Corp., Armonk, NY, USA), and all multivariate analyses were performed on PC-Ord v.7 (MjM Software Design, Gleneden Beach, OR, USA).

The emergence data from Wales (2021) and Scotland (2022) were analysed using a separate negative binomial generalised linear mixed model (GLMM) suitable for count data using the *lme4* R package (Bates *et al.*, 2015), with treatment included as a fixed factor and replicate block as a random factor. Model fit was checked using the *performance* R package (Lüdecke *et al.*, 2021), and likelihood ratio tests (LRTs) were used to determine the effect of each treatment. The *emmeans* R package (Lenth, 2025) was used to calculate the estimated marginal means, and Dunnett's contrasts to compare each treatment group against the control and identify statistically significant reductions in emergence.

The chi² test was performed over destructively measured data from Scotland (2022) using cross-tabulation analysis to evaluate the relationships between the infection types across the different treatments, where standardised residuals (Z scores) were calculated to identify significant deviations from the expected frequencies by random distribution, with a threshold of ±1.96 indicating statistical significance. Additionally, the relationship between *H. abietis* developmental stage and infection type was analysed using the same chi² test and Z scores.

The Bliss independence model (Demidenko and Miller, 2019) was applied to destructive data to evaluate the interaction between EPF and EPN in the HalfMix treatment. The expected infection rate (I_{Bliss}) was calculated using the formula $I_{Bliss} = I_{EPF} + I_{EPN} - (I_{EPF} \times I_{EPN})$, where I_{EPF} is the infection rate in the half-dose EPF treatment, and I_{EPN} is the infection rate in the half-dose EPN treatment. The chi-square value (Chi^2_{Bliss}) was calculated using the formula $Chi^2_{Bliss} = (I_{NF} - I_{Bliss})^2/I_{Bliss}$, where I_{NF} is the observed infection rate for the HalfMix treatment. The *p*-value for each Chi^2_{Bliss} (with 1df) was determined using R (version 4.4.0) with the command >1-pchisq(Chi^2_{Bliss},df). Synergy (S) was calculated as $S = I_{NF} - I_{Bliss}$, where $S > 0$ indicates a synergistic effect, $S = 0$ indicates an additive effect, and $S < 0$ indicates an antagonistic effect between the agents.

The impact of the treatments on non-target invertebrates was assessed with one-way ANOVA. The raw data for the broad and narrow taxa are shown in Appendix Table S4.1 and Appendix Table S4.2, respectively. To further investigate compositional differences among the treatments, two ordinations (PCoA) were performed: one on the broad taxa, and one on the narrow taxa. Additionally, to assess the effect of the grouping variables (treatment and block) on the composition, the multi-response permutation procedure (MRPP) was performed again on the broad taxa and narrow taxa, separately.

Results

Emergence

The GLMM analysis found a significant effect of the treatment on the data for weevil emergence in Scotland in 2022 (LRT Chi² = 12.274, df = 3, $p = 0.007$), and Dunnett's contrasts showed significant reductions in emergence for all treatments compared to the control (for all, $p < 0.05$, Figure 4.1), with the most pronounced reduction in relative emergence observed for the HalfMix treatment (Table 4.1). However, there was no statistically significant overall effect of the treatments on weevil emergence in the GLMM analysis of the 2021 data from Wales (LRT $Chi^2 = 5.544$, df = 3, $p = 0.136$).

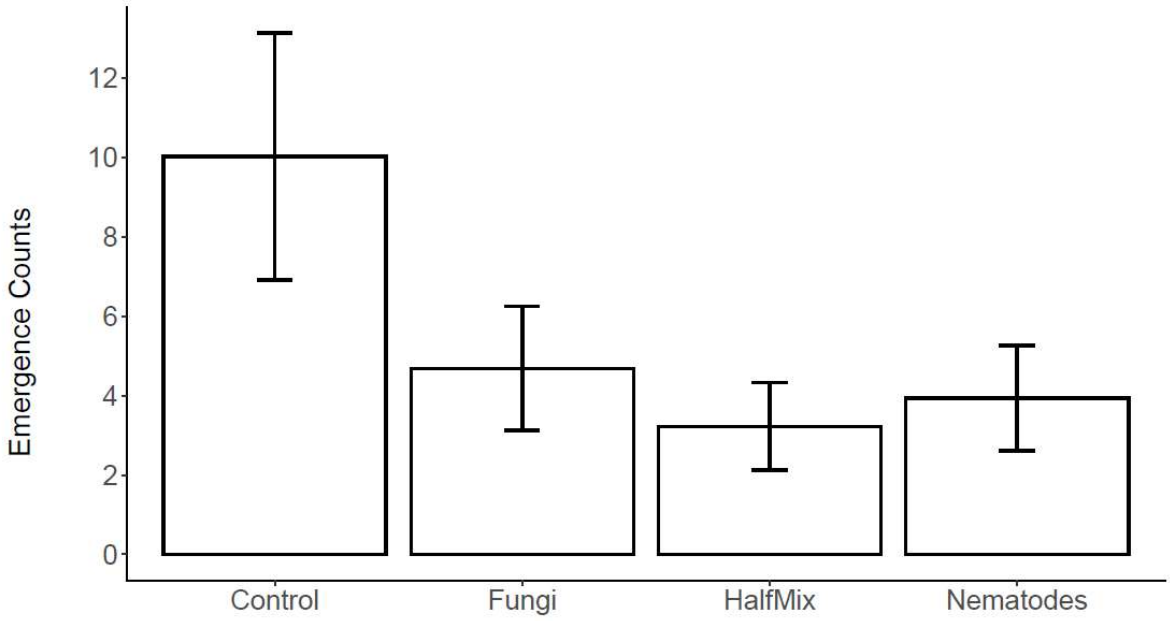


Figure 4.1. Emergence of adult *Hylobius abietis* from treated stumps in the Scotland field trial (2022): Estimated marginal means of adult weevil emergence (counts per trap) are shown for four treatments: entomopathogenic nematodes (EPN; *Steinernema carpocapsae*, full dose), entomopathogenic fungi (EPF; *Beauveria bassiana* isolate 35G, full dose), a combined half-dose treatment (HalfMix: EPF + EPN), and an untreated control (Control). Data were collected weekly from July to October 2022 using emergence traps in a randomised block design (n = 10 replicates per treatment). Counts are estimated marginal means from a negative binomial generalised linear mixed model, with treatment as a fixed factor, and replicate block as a random factor; error bars represent ± SE.

Table 4.1. Effects of entomopathogenic treatments on adult *Hylobius abietis* emergence in the Scotland field trial (2022), analysed using a negative binomial generalised linear mixed model (GLMM): The model included treatment (entomopathogenic fungi [Fungi; *Beauveria bassiana* 35G], entomopathogenic nematodes [Nematodes; *Steinernema carpocapsae*], combined half-dose treatment [HalfMix: EPF + EPN], and untreated control [Control]) as a fixed effect and block as a random effect (n = 10 replicates per treatment).The likelihood ratio test (LRT) reports the overall effect of treatment on emergence (Chi^2 and associated *p*-value). Pairwise comparisons between each treatment and the control were conducted using Dunnett's contrasts. The relative emergence ratio represents the estimated proportional emergence in each treatment relative to the control (values < 1 indicate reduced emergence). Standard errors (SE) are shown for each contrast. Statistically significant differences from the control are indicated by an asterisk (*) at $p < 0.05$.

LRT		Dunnett's Contrasts (vs. Control)			
Chi²	***p*-value**	**Contrast**	**Relative Emergence Ratio**	**SE**	**Sig.**
12.274	0.007*	Fungi vs Control	0.468	0.149	0.046 *
		HalfMix vs Control	0.322	0.107	0.002 *
		Nematodes vs Control	0.393	0.130	0.013 *

Destructive Sampling

The chi-square test revealed a highly significant association between treatment and infection type targeting developing and adult *H. abietis*, indicating a real difference between those categories ($Chi^2 = 186.23$, $p < 0.001$) (Table 4.2). The EPF treatment significantly increased the number of fungus-infected *H. abietis* (Z = 7.6), while the EPN and HalfMix treatments increased the number of nematode-infected weevils (Z = 4.7 and Z = 3.7, respectively). The proportion of alive (non-infected) *H. abietis* in the control treatment was statistically higher than expected by random infection (Z = 4.4), while the non-infected proportions in the HalfMix and nematode treatments were significantly lower than expected (Z = −2.4 and Z = −2.4, respectively). The relationship between developmental stage of *H. abietis* and infection type was also significant, suggesting that different infection types affect each developmental stage in the field ($Chi^2 = 13.84$, $p = 0.031$) (Table 4.2).

The population structure of the weevils in the Scottish field trial is shown in Table 4.3. As can be seen, the majority of the targeted individuals were larvae, followed by pupae and then a small proportion of callow adults.

Table 4.2. Distribution of infection outcomes and developmental stages of *Hylobius abietis* following biological control treatments in the Scotland field trial (2022), destructive sampling analysed by chi-square (χ^2) cross-tabulation: Data are presented for four treatments: entomopathogenic fungi (fungi; *Beauveria bassiana* 35G), entomopathogenic nematodes (nematodes; *Steinernema carpocapsae*), combined half-dose treatment (halfMix: EPF + EPN), and untreated control (control), within a randomised block design (n = 10 replicates per treatment). For each treatment, counts of individuals are shown according to infection category (alive, nematode-killed, fungus-killed, or indeterminate). Percentages are expressed both within treatment (proportion of individuals per treatment assigned to each infection category) and within infection (proportion of each infection category contributed by each treatment). Standardised residuals (Z-scores) are provided to identify deviations from expected frequencies under random distribution, with values

exceeding ±1.96 indicating statistically significant over- or under-representation of a category. Chi-square (χ^2) tests evaluate the association between treatment and infection type, and between developmental stage (larvae, pupae, adults) and infection outcome. Cramer's V is reported as a measure of effect size for significant associations. Statistically significant results are indicated by an asterisk (*) at $p < 0.05$.

		Infection					
		Alive	**Nematodes**	**Fungi**	**Indeterminate**	**Chi²**	**Cramer**
Treatment	**control**	113	0	2	2		
	% within treatment	96.60%	0.00%	1.70%	1.70%		
	% within infection	44.30%	0.00%	10.00%	18.20%		
	Z test (1.96)	4.4	−5.8	−1.6	−0.7		
	fungi	37	0	15	1	Value	Value
	% within treatment	69.80%	0.00%	28.30%	1.90%	186.23	0.394
	% within infection	14.50%	0.00%	75.00%	9.10%		
	Z test (1.96)	0.5	−3.90	7.6	−0.40		
	halfMix	49	51	2	6	***p*-value**	***p*-value**
	% within treatment	45.40%	47.20%	1.90%	5.60%	<0.001 *	<0.001 *
	% within infection	19.20%	45.10%	10.00%	54.50%		
	Z test (1.96)	−2.40	3.7	−1.50	1.8		
	nematodes	56	62	1	2		
	% within treatment	46.30%	51.20%	0.80%	1.70%		
	% within infection	22.00%	54.90%	5.00%	18.20%		
	Z test (1.96)	−2.40	4.7	−2.10	−0.70		
Developmental stage	**adults**	26	4	1	1		
	% within development	81.30%	12.50%	3.10%	3.10%		
	% within infection	10.20%	3.50%	5.00%	9.10%		
	Z test (1.96)	1.2	−1.70	−0.50	0.1	Value	Value
	larvae	142	79	13	10	13.84	0.132
	% within development	58.20%	32.40%	5.30%	4.10%		

% within infection	55.70%	69.90%	65.00%	90.90%		
Z test (1.96)	−1.10	1.2	0.2	1.3	***p*-value**	***p*-value**
pupae	87	30	6	0	0.031 *	0.031 *
% within development	70.70%	24.40%	4.90%	0.00%		
% within infection	34.10%	26.50%	30.00%	0.00%		
Z test (1.96)	0.9	−0.80	−0.10	−1.80		

Table 4.3. Population structure of *Hylobius abietis* recorded during destructive sampling in the Scotland field trial (2022): Values represent the total number of individuals (n) and their relative proportions (%) across developmental stages (larvae, pupae, and adults) recovered from destructively sampled stumps (10 blocks) three weeks after treatment application. Percentages are calculated relative to the total number of individuals recorded across all treatments. The dataset is dominated by larval stages, followed by pupae and a smaller proportion of adults, reflecting the developmental composition of the field population at the time of sampling.

Population Structure		
	Total	**%**
Adults	32	8.0%
Larvae	244	61.2%
Pupae	123	30.8%

In terms of effect size, according to Cohen (Cohen, 1988), the relationship between infection type and treatment type was medium and statistically significant (Cramer's V = 0.394, $p < 0.001$). Additionally, a small but statistically significant relationship was found between *H. abietis* developmental stage and type of infection (Cramer's V = 0.132 with $p = 0.031$).

The analysis of the interaction between EPF and EPN revealed that the total observed infection rate ($I_{NF} = 0.5463$) exceeded the Bliss model prediction ($I_{Bliss} = 0.3790$), indicating a synergistic interaction between EPN and EPF in the HalfMix treatment. However, the low chi^2 value ($Chi^2_{Bliss} = 0.0739$) and the high p-value (p-value = 0.7858) suggested that the deviation was not statistically significant. A block-level interaction analysis found

predominantly synergistic effects, with some antagonistic effects, although none was statistically significant (Table 4.4).

Table 4.4. Interaction between entomopathogenic fungi (EPF) and entomopathogenic nematodes (EPN) in the HalfMix treatment, evaluated using the Bliss independence model in the Scotland field trial (2022): The observed infection rate (I_{NF}) for the combined treatment (HalfMix: EPF + EPN) is compared with the expected infection rate under independent action (I_{Bliss}), calculated as: $I_{Bliss} = I_{EPF} + I_{EPN} - (I_{EPF} \times I_{EPN})$, where I_{EPF} and I_{EPN} represent infection rates in the corresponding single-agent half-dose treatments. The deviation between observed and expected infection rates was assessed using a chi-square statistic (Chi^2_{Bliss}) with 1 degree of freedom, and associated p-values. Synergy (S) was calculated as $S = I_{NF} - I_{Bliss}$, where $S > 0$ indicates synergistic interaction, $S = 0$ additive interaction, and $S < 0$ antagonistic interaction. Results are presented for each replicate block (n = 10) and for the total dataset. Positive S values indicate higher than expected infection under combined treatment, while negative values indicate lower than expected infection. No statistically significant deviations from Bliss independence were detected ($p > 0.05$).

	I_{NF}	I_{Bliss}	Chi^2_{Bliss}	*p*-Value	Synergy (S)	
Total	0.546296296	0.37899579	0.073851637	0.785809	0.167301	Synergy
Block5	0.285714286	0.5	0.091836735	0.761855	−0.21429	Antagonist
Block9	0.764705882	0.6125	0.03782307	0.845799	0.152206	Synergy
Block10	1	0.5	0.5	0.4795	0.5	Synergy
Block11	0.8	0.409090909	0.373535354	0.541084	0.390909	Synergy
Block12	0	0.352941176	0.352941176	0.552453	−0.35294	Antagonist
Block13	0.285714286	0.428571429	0.047619048	0.827259	−0.14286	Antagonist
Block16	0.647058824	0.340697674	0.275485161	0.599676	0.306361	Synergy
Block18	0.296296296	0.25	0.49	0.483927	0.35	Synergy
Block19	0.5	0.25	0.25	0.617075	0.25	Synergy
Block20	0.9	0.3125	1.1045	0.293281	0.5875	Synergy

Nematode- and fungus-infected *H. abietis* organisms were found at different depths and distances from the stump bole. *S. carpocapsae* actively infected at depths of 36 cm underground and spread longitudinally along superficial stump roots up to 49 cm. In the case

of *H. abietis* infected by *B. bassiana* 35G, the maximum infection depth was 23 cm underground and 19 cm longitudinally (Table 4.5).

Table 4.5. Spatial distribution of *Hylobius abietis* individuals by infection type relative to stump position in the Scotland field trial (2022): Distances are presented for individuals classified as alive, nematode-infected (*Steinernema carpocapsae*), fungus-infected (*Beauveria bassiana* 35G), or indeterminate, based on destructive sampling of stumps (n = 10 blocks). Depth (cm) represents the vertical position of individuals relative to the soil surface, with negative values indicating positions below ground level and positive values above ground. "cm to bole" represents the horizontal distance from the centre of the stump bole along roots or surrounding soil. Summary statistics (mean, median, minimum, maximum) are provided for each infection category. Nematode-infected individuals were recovered at greater depths and distances from the bole compared to fungus-infected individuals, indicating deeper vertical penetration and wider horizontal dispersal of EPN relative to EPF under field conditions.

		Infection		
	Alive	**Nematodes**	**Fungi**	**Indeterminate**
Depth (cm)				
Mean	−16.359	−15.681	−9.4	−13.364
Median	−17	−15	−9	−15
Minimum	−40	−36	−23	−21
Maximum	23	−3	2	−1
cm to Bole				
Mean	5.86	5.03	2.15	3
Median	0	0	0	0
Minimum	0	0	0	0
Maximum	34	49	19	15

Mark–Recapture

The mark–recapture experiment results were limited due to the low numbers of recaptures, which meant that neither blocks nor treatments could be statistically tested for differences in recaptures. Billets attracted adult *H. abietis*, and it is notable that the field trial in Scotland attracted more weevils overall than did the billets in Wales. Only a very small number of insects were adults released from the emergence traps (Table 4.6). Death could not be confirmed, as this experiment was intended to allow the weevils to freely feed and

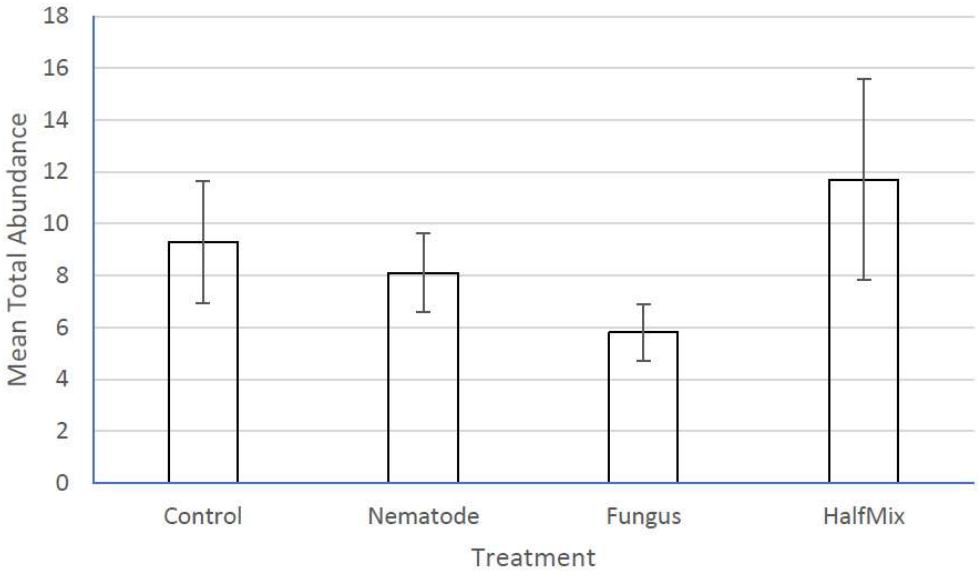
move to enter in contact with the conidiospores placed under the billets. For both field trials, no weevil adults were observed to have died as the result of EPF infection.

Table 4.6. Mark–recapture outcomes for adult *Hylobius abietis* in field trials conducted in Wales (2021) and Scotland (2022): For each block, the number of adults marked and released from emergence traps (Marked–Released), the total number of weevils observed on billets (Total on Billets), and the number of marked individuals subsequently recaptured (Recaptured) are shown. Recaptured individuals are annotated where applicable to indicate evidence of infection (fungal or nematode). Billets (Sitka spruce logs) were deployed to attract adult weevils and positioned above entomopathogenic fungal inoculum to assess potential secondary infection following dispersal. However, recapture rates were low across both field trials, preventing statistical comparison among treatments or blocks. Although billets attracted adult *H. abietis*, particularly in the Scotland trial, very few marked individuals were recaptured, and no confirmed mortality attributable to entomopathogenic fungi was observed under these experimental conditions.

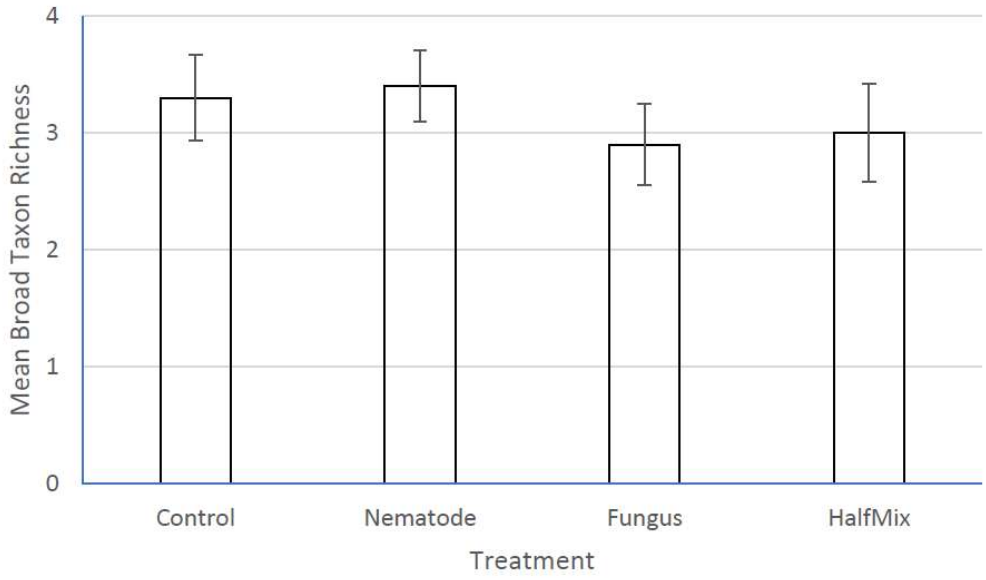
	Wales 2021			Scotland 2022		
	Marked–Released	Total on Billets	Recaptured	Marked–Released	Total on Billets	Recaptured
Block1	1	3	0	6	47	0
Block2	2	1	0	8	35	0
Block3	20	13	5Fungi	13	38	0
Block4	0	1	0	6	27	2Fungi
Block5	0	5	0	20	38	1Nematode
Block6	0	3	0	25	56	0
Block7	3	3	0	0	36	0
Block8	2	3	0	34	27	1Fungi
Block9	3	6	0	16	18	0
Block10	1	0	0	6	18	0

Effects of the Treatments on Non-Targets

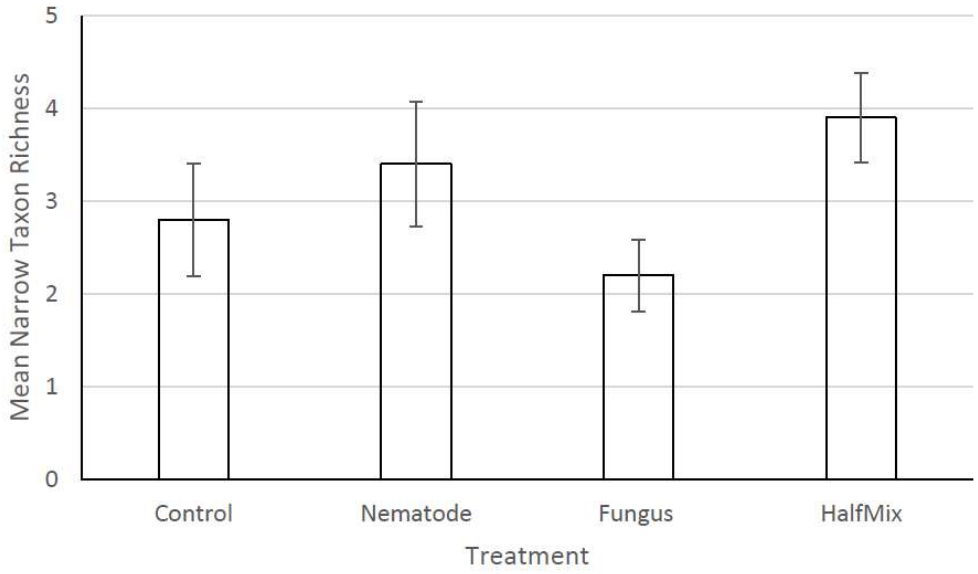
There was no significant effect of the treatments on the total abundance (Figure 4.2a), broad taxon richness (Figure 4.2b, Appendix Table S4.1) or narrow taxon richness (Figure 4.2c, Appendix Table S4.2) of non-targets. In all cases, one way ANOVA returned *p* values greater than 0.05.


18
16
14
12
10
8
6
4
2
0
Mean Total Abundance
Control
Nematode
Fungus
HalfMix
Treatment

(**a**)


4
3
2
1
0
Mean Broad Taxon Richness
Control
Nematode
Fungus
HalfMix
Treatment

(**b**)



(**c**)

Figure 4.2. Effects of biological control treatments on non-target invertebrate communities in the Scotland field trial (2022): Effects of the treatment (nematode [EPN; *Steinernema carpocapsae*], fungus [EPF; *Beauveria bassiana* 35G], HalfMix [mixed half doses EPF+EPN] and control) on (**a**) non-target total abundance, (**b**) broad taxon richness and (**c**) narrow taxon richness. None of the differences are significant (one-way ANOVA, $p > 0.05$ in all cases, see Appendix Tables S4.1 and S4.2). Error bars are +/− SE. For definitions of broad and narrow taxa, see Materials and Methods.

Two ordinations are presented: firstly, for the broad taxa, and secondly for the narrow taxa (Figures 4.3a and 4.3b, respectively). These are multidimensional scaling plots (also called principal co-ordinates analysis [PCoA]). As can be seen in Figure 4.3a,b, there is no clustering of traps with respect to the treatment from which they were drawn. This indicates that compositional effects of treatments were not evident in the dataset. This was further borne out by the results of the multi-response permutation procedure. In all cases, both treatment and block effects were low (see Chance-Corrected Within-Group Agreements [*A*], which were low for all metrics). The *p* values simulated using Monte Carlo randomisation never reached significance for either the broad taxa or the narrow taxa with respect to treatment effects or block effects (Table 4.7).

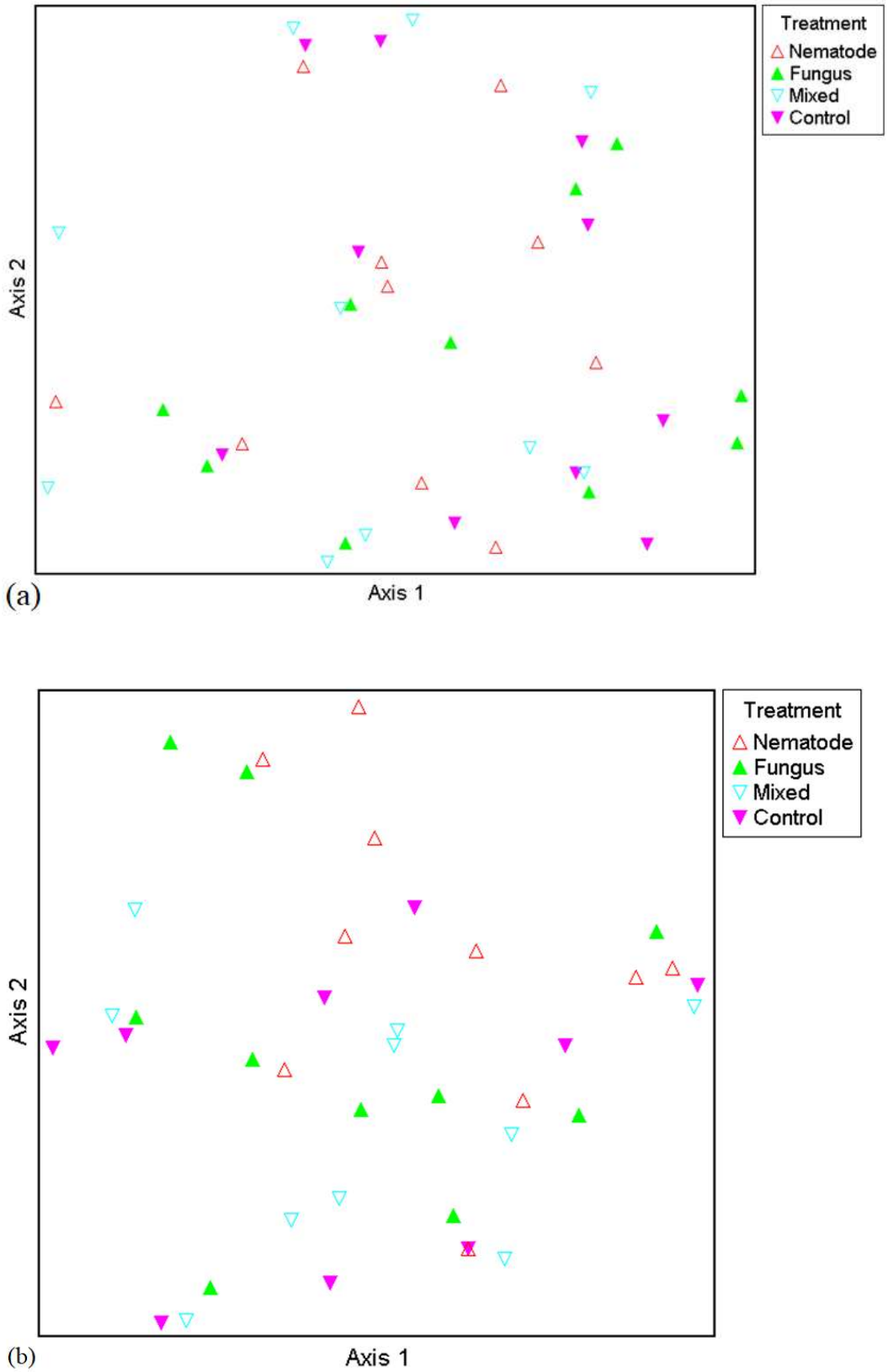


Figure 4.3. Principal coordinates analysis (PCoA) of non-target invertebrate community composition in the Scotland field trial (2022): Ordinations are shown for (a) broad taxonomic groups and (b) narrow taxonomic groups, based on community composition data from emergence traps. Each point represents an individual trap sample,

and distances between points reflect compositional dissimilarity among samples. Points are grouped by treatment: entomopathogenic nematodes (EPN; *Steinernema carpocapsae*), entomopathogenic fungi (EPF; *Beauveria bassiana* 35G), mixed treatment (HalfMix: EPF + EPN), and untreated control (Control). No clear clustering of samples by treatment is observed in either ordination. These patterns were formally tested using the multi-response permutation procedure (MRPP), which detected no significant differences in community composition among treatments or blocks (see Table 4.7). Definitions of broad and narrow taxonomic groupings are provided in the Materials and Methods section.

Table 4.7. Multi-response permutation procedure (MRPP) analysis of non-target invertebrate community composition at two taxonomic resolutions (broad and narrow taxa) in the Scotland field trial (2022): MRPP was used to test for differences in community composition with respect to two grouping variables: treatment (entomopathogenic nematodes, entomopathogenic fungi, combined treatment, and control) and block. The chance-corrected within-group agreement (A) provides a measure of within-group homogeneity relative to that expected by chance, where $A = 1$ indicates complete agreement, $A \approx 0$ indicates no group structure, and $A < 0$ indicates lower similarity within groups than expected by chance. P-values were generated using Monte Carlo randomisation. No statistically significant differences were detected for any grouping variable or dataset (all $p > 0.05$), and A values were consistently low or negative, indicating weak or absent structuring of communities by treatment or block.

Dataset	Grouping Variable	Chance-Corrected Within-Group Agreement (A)	p-Value
Broad Taxa	Treatment	−0.011	0.653
Broad Taxa	Block	−0.024	0.694
Narrow Taxa	Treatment	−0.011	0.860
Narrow Taxa	Block	0.012	0.261

Discussion

The results from the present field studies provide compelling evidence for the effectiveness of the wild EPF *B. bassiana* 35G alone and together with the commercial EPN *Steinernema carpocapsae* as BCAs against *H. abietis* in clear-felled spruce plantations in

Scotland. These findings contribute to the growing body of literature advocating for sustainable and environmentally benign pest management strategies in forestry (van Lenteren *et al.*, 2017).

The data clearly indicate a statistically significant reduction in weevil emergence caused by all treatment regimens (EPFs alone, EPNs alone or a combination of both) compared to the control. Notably, the application of *B. bassiana* 35G at half of recommended operational dose (HalMix) in conjunction of half of the operational dose *of S. carpocapsae* demonstrated the most substantial impact on weevil emergence, thereby affirming the potential of this wild EPF as a potent standalone treatment. This killing potential against adult *H. abietis* was also previously demonstrated under controlled laboratory conditions at different concentrations (*Chapter 3*). The combination treatment (HalfMix) and the full dose EPN treatment also showed considerable efficacy, albeit to a slightly lesser extent than the full EPF treatment. This suggests that mixed doses could provide adequate control, as documented in previous studies (Williams *et al.*, 2013b, McNamara *et al.*, 2018a). Interaction analysis using the Bliss independence model indicated overall synergistic effects in the HalfMix treatment between half doses of B. *bassiana* 35G and *S. carpocapsae*. However, these effects were not statistically significant, and block-level analyses revealed some variability, including occasional antagonistic interactions. Such variability highlights the potential influence of environmental conditions and experimental consistency on the interaction dynamics of EPFs and EPNs against *H. abietis*. Nonetheless, the overall results support the additive nature of combined treatments, demonstrating their potential to enhance pest control efficacy while reducing the required dosages.

The significant reduction in weevil emergence in the Scottish trial underscores the adaptability and potential success of these biological agents under varying environmental conditions. This finding is particularly relevant considering the ongoing challenges posed by climate change, which is likely to alter the lifecycle and dispersal patterns of *H. abietis* and other forest pests (Inward *et al.*, 2012). The observed efficacy across different sites further bolsters the argument for a wider adoption of these BCAs in forest management practices across the U.K. and potentially in other regions facing similar pest challenges (van Lenteren *et al.*, 2017).

It may be suggested that the higher number of adult *H. abietis* attracted to billets in the field trial in Scotland compared to the trial in Wales may be a result of the closer proximity in Scotland of mature undisturbed conifer plantations, which feasibly supply a spillover of adult weevils (Skrzecz and Moore, 1997). This highlights a danger that attract-and-kill approaches could lure in more weevils and make matters worse. Alternatively, it could

simply be that weevil populations are larger in Scotland. The low numbers of recaptures from treatment-released stumps highlights the limitation of relying only on billets as attractants for adult weevils without traps to contain them (Fedderwitz *et al.*, 2022). The development of lures including alpha-pinene and EPF carriers may provide more effective alternatives in lure-and-kill approaches for the management of adult *H. abietis* (Lalík *et al*., 2021, Fedderwitz *et al.*, 2022).

A critical aspect of deploying BCAs in the field is ensuring that they do not adversely affect non-target organisms (Smagghe *et al.*, 2023). Previous work has shown that whereas site and tree species (of the stump) have a significant effect on the species richness, abundance and community composition of non-target Coleoptera, nematode treatment had no effect on these variables(Dillon *et al.*, 2012). Our results, from a more limited dataset, support these conclusions for invertebrates in general and further show that also entomopathogenic fungi have no effect on these variables aligning with findings of recent studies (Chiew *et al.*, 2022). The results of the present study are particularly encouraging in this regard. The statistical analyses revealed no significant impact of any of the treatments on the abundance or taxon richness of non-target invertebrates. The ordination analyses and subsequent MRPP tests further corroborated the absence of compositional changes in the invertebrate community structure, reinforcing the conclusion that wild EPFs and commercial EPNs can be integrated into pest management strategies without detrimental effects on biodiversity (Dillon *et al.*, 2012, Chiew *et al.*, 2022). These findings are of paramount importance as they support the safety profile of these BCAs, thereby addressing a major concern associated with the use of synthetic insecticides, which are often non-selective and can cause collateral damage to beneficial organisms within the ecosystem (Desneux *et al.*, 2007, Saha and Kaviraj, 2008, Merivee *et al.*, 2015).

The relationship between infection type (fungal or nematode) and the developmental stage of *H. abietis* indicated a statistically significant association in infecting and reducing the population of *H. abietis* across different developmental stages as seen in previous research (Ansari *et al*., 2012, Kapranas *et al*., 2017). This is important as, to date, the wild EPF *B. bassiana* 35G had only been shown to infect adult *H. abietis*, while in this study, we proved it efficient against pupae and larvae. The differential infection rates observed suggest that the timing and method of application could be critical in maximising the impact of these BCAs (Moore *et al*., 2004). For instance, studying how this wild EPF targets the different developmental stages of *H. abietis* could help to target those stages most susceptible to infection and thereby enhance the overall treatment efficacy and reduce the likelihood of pest resurgence.

These findings have important implications for the future of integrated pest management (IPM) in forestry (Fedderwitz *et al.*, 2022). The demonstrated effectiveness of wild EPFs and EPNs suggests them as a viable alternative to synthetic insecticides, aligning with broader environmental goals to reduce chemical inputs in agriculture and forestry (Kapranas et al., 2016, McNamara et al., 2018a). The use of BCAs could be particularly beneficial in managed forest ecosystems, where the risk of pest outbreaks is exacerbated by practices such as clear-felling.

However, while the results are promising, further research is warranted to optimise the application protocols for these or new BCAs. This includes investigating the optimal timing, dosage and combination of EPFs and EPNs to ensure maximum efficacy across different forest types and climatic conditions. Additionally, long-term studies are needed to assess the persistence of these agents in the environment and their potential cumulative effects on both target and non-target organisms over time.

Conclusion

In conclusion, the data from this study provide strong support for the use of EPFs and EPNs as effective and environmentally safe tools in the management of *H. abietis* populations. Their integration into IPM strategies could significantly enhance the sustainability of forest management practices, reducing reliance on synthetic insecticides and mitigating their associated environmental risks. This research contributes to the growing evidence base that supports a paradigm shift towards more ecologically sound pest management solutions in forestry.

Author Contributions:

Conceptualization, L.M.Q.-O., R.M., R.R. and C.D.W.; methodology, C.D.W. and R.M.; validation, L.M.Q.-O. and C.D.W.; formal analysis, L.M.Q.-O., W.T.S. and C.D.W.; investigation, L.M.Q.-O. and C.D.W.; resources, C.D.W. and R.M.; data curation, L.M.Q.-O.; writing—original draft preparation, L.M.Q.-O.; writing—review and editing, L.M.Q.-O., W.T.S., C.D.W., R.M. and R.R.; visualization, L.M.Q.-O. and W.T.S.; supervision, R.M., R.R. and C.D.W.; project administration, C.D.W.; funding acquisition, C.D.W. and R.M. All authors have read and agreed to the published version of the manuscript.

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CHAPTER 5:

General discussion

Overview

Overpopulation of the large pine weevil (*Hylobius abietis*) is one of the most economically significant pests of coniferous forests across Europe and Asia, particularly affecting managed plantations where clear-felling practices provide optimal breeding conditions affecting restocking (Leather *et al.*, 1999). Its feeding activity severely hampers the establishment of young conifer seedlings, leading to substantial economic losses and threatening the sustainability of managed forests (Långström and Day, 2007).

Current control measures predominantly rely on synthetic insecticides, which, despite their efficacy, are increasingly subject to regulatory restrictions due to environmental concerns and offer limited long-term efficacy as seen by the persistence of weevil populations. Achieving a more sustainable reforestation is in urgent need for alternative control strategies that align with sustainable forestry practices. As a result, there is growing interest in developing biological control agents (BCAs) that offer sustainable and environmentally safe alternatives.

This thesis builds upon existing research into the potential of entomopathogenic fungi (EPFs) and entomopathogenic nematodes (EPNs) as promising BCA for *H. abietis* management (Williams *et al.*, 2013b, McNamara *et al.*, 2018a). Previous studies have demonstrated the efficacy of commercial BCAs, highlighting their ability to provide effective pest control while minimising non-target effects and environmental contamination. However, their field performance has often been inconsistent due to variations in environmental conditions, soil composition, application methods and fungal strain virulence.

In response to these challenges, increasing attention has been directed towards non-commercial, native EPFs, such as *Beauveria caledonica*, which may offer enhanced virulence and adaptability to local conditions (McNamara *et al.*, 2018a). Additionally, research has explored the potential additive effects of *B. caledonica* and commercial EPFs when combined with commercial EPNs, aiming to improve control efficacy against *H. abietis*. This research aims to build on this line of research by:

1 Bioprospecting to isolate native EPF strains using a dual-baiting method to maximise the diversity of isolates and identify highly virulent fungal strains with potential application against *H. abietis*.

2 Assessing the pathogenicity and infection effects of the isolated EPFs under laboratory conditions to identify strains with high virulence and desirable behavioural modifications in *H. abietis*.

3 Testing the best performing EPFs in field trials, evaluating their efficacy in practical forestry settings both as standalone treatments and in combination with the commercial EPN *Steinernema carpocapsae*.

Synthesis of results

The research objectives set for this project were successfully achieved through a systematic assessment of the diversity and distribution of wild EPFs (*Chapter 2*), a detailed evaluation of their virulence and infection effects under controlled conditions (*Chapter 3*), and their subsequent validation in field applications (*Chapter 4*). Collectively, these findings provide a strong scientific foundation for integrating EPFs and EPNs into an effective biological control strategy for *H. abietis* management.

The outcomes of this research contribute significantly to the growing body of literature supporting the use of BCAs in forestry management. Furthermore, they establish a robust scientific basis for optimising the deployment of EPFs and EPNs, paving the way for their incorporation into integrated pest management (IPM) strategies. By providing viable solutions for mitigating the damage caused by *H. abietis* while maintaining ecosystem balance, this study underscores the potential of biological control as a sustainable alternative to conventional pest management methods. Beyond *H. abietis* control, these findings contribute to broader efforts in biological pest control and sustainable forestry, offering valuable insights into the development of environmentally responsible pest management strategies.

Bioprospecting for Wild Entomopathogenic Fungi: Implications for Biocontrol Development

The first objective of this research was to expand biological control efforts by identifying novel, locally adapted BCAs that are ecologically suited to their target environments. The rationale behind this approach was that native strains are more likely to persist in field conditions, minimising environmental impact on local microfauna and enhancing long-term efficacy.

Chapter 2 examined the environmental and methodological factors influencing EPF diversity across different habitats in Lancashire nature reserves. The study confirmed that sampling site was the most influential factor shaping fungal communities, with certain

strains exhibiting strong associations to specific sites. *Beauveria bassiana* was prevalent in grasslands, *Beauveria brogniartii* in grassland quarries, and *Metarhizium anisopliae* var. *anisopliae* was predominantly isolated from grassland quarries and woodland soils. The isolation of variety of strains of *Beauveria bassiana* and *Metarhizium anisopliae*—two of the most widely studied entomopathogens—highlights their ecological importance in regulating insect populations.

One of the key methodological advancements in this study was the use of a dual baiting approach with *Galleria mellonella* and *Tenebrio molitor*. Although bait type did not significantly influence community composition, the combined used of both insect baits provided a broader spectrum approach capturing strains that might have been overlooked due to host specificity or low number of baits used on each sample. In this context, dual baiting functions as a complementary sampling strategy that improves recovery probability rather than selectively targeting specific EPF taxa (Sharma *et al*., 2018). The diversity of novel EPF strains obtained, suggests that this approach is effective for capturing ecologically relevant EPF diversity with potential application against *H. abietis* and other forestry pests that need to be further investigated.

The study also explored the role of soil depth in EPF distribution, where results indicated that while soil depth had no statistically significant effect on species richness or community composition, indicating that EPF assemblages were not vertically structured within the sampled soil strata. Although a slightly higher number of isolates was recovered from the upper soil layer (0–10 cm), this pattern was not associated with consistent compositional differences and should be therefore interpreted cautiously.

Rather than indicating a general preference for surface soils, these findings likely reflect the complex and context-dependent nature of EPF ecology (Quesada-Moraga *et al*., 2007). As discussed in *Chapter 2*, EPF distribution and persistence are shaped by interacting abiotic and biotic factors, including soil properties, microbial competition, plant associations, and host availability, which can obscure the effect of individual variables such as depth (Lacey *et al*., 2015). In particular, the role of organic matter remains ambiguous. While organic-rich soils may support greater insect host availability, potentially enhancing EPF persistence, they may also harbour higher microbial activity and antagonistic organisms that limits fungal survival (Uzman *et al*., 2019).

Edaphic factors, particularly pH and carbon fractions content, were significantly correlated with EPF composition, emphasising the role of soil chemistry in shaping fungal communities. It has been observed that nutritional conditions and the carbon-to-nitrogen

ratios influence fungal growth, sporulation, and virulence, further complicating predictions of EPF distribution based solely on soil characteristics (Uzman *et al*., 2019).

Further research to better understanding these soil-fungal interactions could enhance bioprospecting efforts by enabling the creation of predictive models of EPF distribution, improving the likelihood of recovering strains with high potential for biological control applications.

From an applied perspective, these findings suggest that future bioprospecting efforts could benefit of prioritising targeted sampling strategies considering soil chemistry as a key component of sampling design and including the use of multiple bait species and ecologically diverse sites. While topsoil collection may improve recovery efficiency, it should be regarded as a pragmatic strategy rather than evidence of ecological stratification, and inclusion of multiple soil depths may still be necessary to capture the full extent of EPF diversity. The identification of novel, locally adapted fungi is particularly relevant for forestry management, as indigenous strains may have enhanced environmental persistence and adaptability compared to commercial strains. Furthermore, to ensure the sustainability of selected strains in forestry settings, long-term persistence studies beyond two years are strongly recommended (McNamara *et al.*, 2018a).

Lethal and infection effects of wild EPFs on *Hylobius abietis*: Implications on screening of new native biopesticides.

The second objective of this study was to assess the virulence and infection effects of the 12 most promising EPF isolates from the first soil baiting carried on 2020 (*Chapter 2*) against adult *H. abietis* under controlled laboratory conditions to determine their suitability as BCAs. The findings revealed significant variation in both mortality rates and infection effects among the tested strains. Notably, *Beauveria bassiana* 35G and *Metarhizium* sp. 17G emerged as the most effective isolates, exhibiting LT50 values of 9 and 10 days, respectively, confirming their potential as highly effective biological control agents.

Beyond direct mortality effects, this study investigated the effects of different EPF dosage applications on *H. abietis*, an often-overlooked aspect in biological control research. The results demonstrated that infection with highly virulent EPF strains led to reduced bark consumption by adult weevils, potentially mitigating immediate damage to young conifer saplings. This finding aligns with previous research showing that fungal infections can trigger behavioural and physiological changes in insect hosts, leading to reduced feeding activity (Barta *et al.*, 2019).

Interestingly, exposure to lower EPF concentrations of highly virulent strains resulted in increased feeding activity compared to individuals treated with higher doses, although feeding levels did not exceed those observed in control treatments. This suggests that mild infections may allow weevils to maintain relatively normal feeding behaviour.

This finding has important implications for field applications, as suboptimal fungal applications may fail to prevent tree damage if fungal concentrations are not carefully optimized, although they may still contribute to modest, non-significant reductions in feeding (Barta *et al.*, 2019). The observed negative correlation between LT50 and feeding inhibition underscores the importance of selecting highly virulent EPF strains, not only for their lethal effects but also for their ability to suppress feeding in *H. abietis*.

Additionally, weight change analyses indicated complex physiological responses in infected weevils, but no consistent relationship between EPF virulence and reduction in body weight was observed across isolates. While the two most virulent isolates showed a tendency towards reduced weight at intermediate concentrations (Figures 3.6 and 3.7), these differences were not statistically significant compared to the control. These patterns suggests that infection-induced stress may Influence host physiology under certain conditions, but the effects appear to be dependent on EPF isolates and its doses rather than directly linked to virulence alone. Consequently, any potential impact of EPF infection on host metabolism or nutrient utilisation remains uncertain. These findings highlight the multifaceted impact of EPFs on *H. abietis* and reinforce the need for further research into the mechanisms underlying these infection effects.

Taken together, these results underscore the importance of evaluating both lethal and suboptimal infection effects when selecting EPFs for biocontrol applications. While virulence (LT50) remains a key criterion, behavioural modifications such as feeding suppression may play an important role in pest suppression under field conditions.

Moreover, as demonstrated in *Chapter 4*, lower concentrations of the highly virulent *B. bassiana* 35G proved efficacious when combined with *S. carpocapsae* in the field experiments, exhibiting additive interactions against early developmental stages of *H. abietis*. These findings are consistent with previous studies (Williams *et al.*, 2013b, McNamara *et al.*, 2018a) and highlight the importance of conducting preliminary laboratory assays to explore EPN and EPF interactions across all *H. abietis* developmental stages before field application. A more thorough understanding of these interactions could enhance the efficacy of biological control strategies, optimising their potential in forestry pest management.

It is important to note, however, that the unexpectedly high mortality observed in control treatments necessitates caution in interpreting these results. Ideally, control groups should exhibit minimal mortality, yet external stress factors—such as handling, environmental conditions, or pre-existing vulnerabilities in test individuals—may have contributed to these unexpected losses. To enhance the reliability of future experiments, we recommend refining rearing protocols to minimise handling-induced stress and implementing more rigorous screening procedures to exclude individuals with potential pre-existing conditions. These refinements will ensure that control mortality remains at an acceptable level, thereby improving the accuracy of EPF efficacy assessments and strengthening the scientific basis for their application in biological control strategies.

Field performance of wild EPFs, its additive and synergistic effects with EPNs and, interactions with non-target species

The final objective covered in this thesis was to evaluate the field performance of the most promising EPFs and their interactions with EPNs in real-world forestry conditions.

Chapter 4 demonstrated that all biological treatments significantly reduced *H. abietis* emergence, with EPF-alone applications effectively infecting all developmental stages, up to 23 cm underground. However, to further develop *B. bassiana* 35G as a BCA, it is necessary to expand research on its infectivity in larvae and pupae of *H. abietis* and evaluate different application methods to optimise infection rates on weevils. This approach could also be applied to newly isolated EPF strains in future bioprospection projects, ensuring that bioassays assess their efficacy across all developmental stages of *H. abietis* or other forestry pest targets.

The most effective suppression of *H. abietis* emergence was achieved through the combined application of EPFs and EPNs at half of their recommended operational doses, suggesting potential additive or synergistic interactions between the two BCAs. This was further examined using the Bliss independence model, which provided evidence for an overall additive effect between *B. bassiana* 35G and *S. carpocapsae*, as synergy was observed but statistical significance was not reached. At the block level, different types of interactions, including antagonistic effects, were observed across sites. These findings underscore the complexity of EPF-EPN interactions in variable field conditions and highlight the need for further optimisation to enhance their collaborative effects.

Given the potential operational advantages of combining EPFs and EPNs, further research should focus on refining application techniques and environmental conditions to maximise their synergy. Previous studies (Ansari *et al.*, 2006, Ansari *et al.*, 2008) have demonstrated

synergistic interactions in the control of other Coleopteran forestry pests, highlighting the need for targeted investigations to refine EPF-EPN combinations. Notably, other studies (Dillon *et al.*, 2007, Williams *et al.*, 2013b) have reported that reducing the dosage and water volume of certain EPN applications does not compromise their efficacy in controlling *H. abietis* in field trials. This suggests that similar evaluations could be conducted for EPFs such as *B. bassiana* 35G. Successfully identifying highly effective pairings could lead not only to improve biological control outcomes, but also significantly reduce operational costs and logistical challenges by minimising the required quantities of BCAs and optimising water usage for effective *H. abietis* management.

A crucial component of this study was the assessment of potential non-target impacts. The data indicated no significant effects on the overall abundance or taxon richness of non-target invertebrates, reinforcing the environmental safety of the BCAs tested in this work. This finding is particularly important when considering the broader applicability of this approach, not only for *H. abietis* control but also for the management of other forestry pests. The results support the use of novel, native EPFs as a viable alternative to synthetic insecticides, which often have unintended consequences on beneficial organisms. By demonstrating both efficacy and ecological safety, this study strengthens the case for integrating EPFs into sustainable pest management strategies in forestry.

Critical evaluation of project design and limitations

A critical evaluation of the methodological decisions and limitations of this project is necessary for interpreting its findings appropriately and identifying priorities for future work.

Sampling scope and site selection

Although approximately 40 sites were initially identified as potentially suitable for bioprospecting, only a subset was sampled and later analyses focused on four sites. This reduction was driven primarily by practical constraints, including limited soil storage space, restricted incubator capacity, and the time-intensive nature of soil baiting and insect processing. Delays in processing also resulted in loss of isolate viability, limiting downstream molecular identification.

Focusing subsequent work on the four most productive sites improved efficiency and enabled robust ecological analyses, but it may also have biased the study towards EPF-rich environments. As a consequence, the findings may not fully represent broader landscape-

level variation in EPF occurrence. Future studies would benefit from maintaining wider spatial coverage, even if sampling intensity per site must be reduced.

Sampling design and spatial representativeness

The use of a standardised 5 × 5 m grid provided fine-scale information on EPF community structure within each sampled location. However, this design captured only a small fraction of each reserve and therefore cannot be assumed to represent the fungal diversity of the entire site.

The sampling pattern was originally selected in part because it was compatible with approaches used for entomopathogenic nematode surveys (Orozco *et al*., 2014), and the broader project initially aimed to recover both EPFs and EPNs. Although EPNs were not recovered, the design still proved suitable for isolating EPFs. Nevertheless, other EPF-focused studies have used broader or more composite sampling strategies (e.g. Sharma *et al*., 2018; Bueno-Pallero *et al*., 2020), which may capture wider spatial heterogeneity more effectively. The present design therefore offers strong local ecological resolution but limited site-level representativeness. A future multi-scale sampling framework combining fine-scale grids with broader site coverage would likely strengthen inference.

Molecular identification using ITS

Identification of fungal isolates was based on the ITS region, which has been widely used in previous EPF studies (McNamara *et al*., 2018a; Sharma *et al*., 2018; Bueno-Pallero *et al*., 2020) and was appropriate given that the taxonomic composition of the fungal community was unknown at the outset.

However, ITS does not always provide optimal resolution within genera such as *Metarhizium* and *Beauveria*, where alternative markers such as EF1-α or Bloc may improve species-level identification (Korosi *et al*., 2019). The exclusive use of ITS therefore likely limited taxonomic resolution in some cases. This choice was influenced partly by budgetary constraints, as molecular work required substantial resources. Future studies should consider multi-locus approaches where feasible.

Environmental variables and ordination analyses

Only soil chemical variables were included as quantitative environmental predictors in ordination analyses. They were the only site-level quantitative data available, but they are unlikely to represent the full set of ecological drivers shaping EPF communities. Plant

composition, insect host abundance, and rhizosphere interactions (Quesada-Moraga *et al*., 2022), may all be highly influential but were not quantified directly in this study.

This limitation is particularly relevant because EPFs are now recognised not only as insect pathogens, but also as rhizosphere-competent, endophytic, and epiphytic organisms whose persistence and ecological roles may depend strongly on plant associations and multitrophic interactions (Quesada-Moraga *et al*., 2022). The absence of insect survey data also restricts interpretation of host-related structuring of EPF communities.

A further limitation is that soil chemistry was characterised across deeper strata (including 20–40 cm and 40–60 cm), whereas EPF baiting targeted only the upper 20 cm. Although deeper horizons may still influence nutrient movement and the wider soil environment, a closer depth match between biological and chemical sampling would strengthen ecological interpretation in future studies.

Habitat choice for bioprospecting

Bioprospecting was conducted across a range of habitats, including grasslands, quarries, and woodland, despite the ultimate application target being forestry. This was intentional. In the context of augmentative biological control, it is not necessarily desirable to identify strains that are maximally persistent in the target habitat, because limited persistence after pest suppression may reduce unintended long-term ecological effects (van Lenteren *et al*., 2017). Nevertheless, bioprospecting outside forestry habitats may also reduce the likelihood of identifying strains optimally adapted to forestry environments. Future programmes could therefore benefit from combining wider exploratory sampling with more targeted sampling in forestry-relevant habitats.

Use of adults rather than juvenile stages in laboratory assays

Laboratory bioassays in *Chapter 3* were conducted using adult *H. abietis*, primarily because adults were the only life stage that could be obtained consistently and handled reliably under laboratory conditions. The maintenance of larvae and pupae proved logistically challenging, as their collection required destructive sampling of stumps across distant field sites, often yielding low, inconsistent, or null recoveries.

Adults were therefore selected as a practical and conservative screening stage, particularly given evidence that all developmental stages are susceptible to *Beauveria* and *Metarhizium*, with adults often regarded as relatively resistant (Ansari and Butt, 2012). Under this rationale, strains capable of infecting adults were considered likely candidates for activity against juvenile stages. In practice, this approach led to identification of field-relevant

isolates, but the assumption of cross-stage efficacy was not tested directly under laboratory conditions and should therefore be treated cautiously. Future studies should prioritise the inclusion of larval and pupal stages to confirm stage-specific virulence.

Selection of isolates for field trials

The isolates used in field trials were not selected solely on biological performance but were also constrained by culture viability and contamination. Soil baiting and screening were time-consuming, and some cultures lost viability or became contaminated before they could be advanced into later experiments.

This explains, for example, why isolate 15G was used in the initial Wales trial despite not ultimately emerging as one of the strongest candidates in later screening work. At the time, it was among the most suitable viable cultures available for field deployment (Appendix Table S5.1, and Appendix Figure S5.1). Later experiments (*Chapter 3*), conducted under improved conditions and with better culture recovery, identified *B. bassiana* 35G as the stronger field candidate. This highlights the importance of establishing stable long-term culture collections early in a project.

Limitations of the Wales field trial

The Wales field trial was limited by unexpectedly low *H. abietis* population density, resulting in poor emergence and reduced power to assess treatment effects. Site selection was constrained by time and by difficulty locating sufficiently infested stumps within the treatment window. As a result, the final site proved less suitable than anticipated.

Although this trial contributed operational experience, its inferential value was limited compared with the later Scottish trial, where improved site selection and timing produced a more informative dataset. This contrast emphasises the importance of thorough pre-validation of pest density prior to field experimentation.

Absence of commercial EPF comparisons

The project did not include a commercial EPF strain as a benchmark comparator. Inclusion of a commercial standard such as Met52 would have strengthened the practical interpretation of the results by allowing direct comparison between native and existing commercial BCAs as seen in other studies (Williams *et al*., 2013b).

Its absence does not undermine the internal comparisons made within the thesis, but it does limit how directly the performance of native isolates can be evaluated in applied commercial terms. Future trials would benefit from including at least one commercial fungal standard.

Future research directions and considerations

The findings of this thesis provide a solid foundation for the continued development of *Hylobius abietis* biocontrol strategies using native EPFs and their interactions with EPNs. However, several critical aspects require further investigation to refine and optimise these biocontrol agents for operational implementation in forestry. Future research should focus on enhancing strain selection, refining application methods, understanding microbial interactions, and assessing long-term ecological impacts.

Optimisation of EPF-EPN Combinations and Application Techniques

One of the most significant findings from this research is the potential for additive or synergistic interactions between *B. bassiana* 35G and *Steinernema carpocapsae*, which achieved the highest levels of *H. abietis* suppression when applied together at half of their recommended, alike to results from previous research on EPN+EPF additive effects on LPW (McNamara *et al.*, 2018a), and EPN+EPF synergistic effects on the black vine weevil *Otiorhynchus sulcatus* Fabricius 1775 (Ansari *et al*., 2010). However, variation in efficacy was observed across experimental units, including cases of antagonistic interactions. It is important to note that these differences were detected at the block level within site and should therefore be interpreted cautiously. The experimental design incorporates replication to account for inherent special variability, and it may not be meaningful to attribute biological significance to block-to-block differences in isolation.

Rather than indicating consistent site-level patterns, these results highlight the inherent variability and complexity of multi-agent biocontrol systems in field conditions (Gao et al., 2026). To refine the practical application of these biological control agents, further research should prioritise:

- Optimisation of application strategies by refining the timing, concentration, and environmental conditions under which EPFs and EPNs are applied is essential to consistently promote synergy. Factors such as temperature, soil moisture, and microbial competition may influence their combined effectiveness.
- Investigation of potential competition between EPFs and EPNs under varying temperature, moisture, and soil conditions to determine how environmental factors influence their combined efficacy.
- Evaluation of additional fungal strains that could complement *B. bassiana* 35G, as different fungal species or strains may exhibit superior compatibility with EPNs field conditions with specific soil types or climates.

- Assessment of novel delivery mechanisms and its influence on the efficacy of BCAs. Several studies have explored oil-based applications (Reji Rani and Nithya, 2017), included gel formulations (Fallet *et al.*, 2022), encapsulation technologies (Sharma *et al.*, 2019), and biopolymer carriers (Saberi Riseh *et al.*, 2022, Friuli *et al.*, 2023), all of which have shown potential to improve the persistence and even distribution of EPFs and EPNs in the field.

A promising avenue for future work is the optimisation of lower application doses, particularly considering previous studies indicating that reduced water volumes and lower EPN concentrations do not necessarily compromise efficacy (Dillon *et al.*, 2007, Williams *et al.*, 2013b). If similar reductions can be applied to EPFs such as *B. bassiana* 35G, this could lead to substantial operational savings, particularly in large-scale forestry applications. Furthermore, integrating predictive models to tailor EPF-EPN application rates based on site-specific environmental parameters could enhance efficiency while reducing overall resource consumption.

Further investigations into infection dynamics and pest behaviour

This thesis demonstrated that fungal infections not only cause direct mortality in *H. abietis*, but also influence feeding behaviour, with highly virulent strains reducing bark consumption. However, the feeding responses observed at lower infection doses indicate that sublethal effects are complex and not necessarily associated with consistent reductions in feeding relative to untreated controls. This highlights the need for a more nuanced understanding of how infection intensity influences host behaviour.

Previous studies have already established the susceptibility of multiple developmental stages to entomopathogens under laboratory conditions (Ansari and Butt, 2012, Williams *et al*., 2015). In this context, the results from *Chapter 4* extend existing knowledge by demonstrating the potential for native EPFs alone and in combination with EPFs to targeting adult weevils under field conditions. The results presented here therefore support the inclusion of adults as a relevant target stage within integrated control strategies, particularly given their central role in seedling damage.

Recent studies have highlighted the feasibility of incorporating EPFs into “lure-and-kill” or contact-based delivery systems targeting adult *H. abietis*, where attractants or substrates are used to maximise contact between the pest and fungal inoculum. For example, Lalík *et al*., (2021) demonstrated that carriers colonised with *Beauveria bassiana* (strain AMEP20) can effectively infect adult weevils following brief contact, resulting in substantial mortality under laboratory, semi-natural, and field conditions. Their findings showed that even short

exposure periods were sufficient to initiate infection, supporting the potential of passive transmission systems for adult control (Lalík *et al*., 2021).

In this context, the results obtained in *Chapter 4* with *B. bassiana* 35G further reinforce the applicability of such strategies. While the current study employed direct soil-based applications, the demonstrated efficacy of 35G against adult weevils suggests that this strain could be adapted to similar carrier-based or lure-and-kill approaches. Given that adult *H. abietis* are highly mobile and responsible for the majority of feeding damage to seedlings (Leather *et al.,* 1999), integrating highly virulent local strains such as 35G into contact-based delivery systems could enhance both exposure rates and control efficiency.

Accordingly, further research is required to refine both the mechanistic understanding and applied implementation of EPF-based control strategies.

Future research should:

- Examine infection dynamics across all developmental stages of *H. abietis*, particularly larvae and pupae, to better understand how fungi and nematodes interact within the pest's life cycle.
- Investigate feeding responses under varying infection intensities, including whether fungal metabolites influence weevil behaviour and whether these compounds could be harnessed for pest deterrence.
- Assess host immune responses to EPF infection, as variations in susceptibility between individuals could impact biocontrol reliability.
- Evaluate the suitability of *B. bassiana* 35G for integration into lure-and-kill or carrier-based delivery systems, including its ability to sporulate on artificial substrates, persist under environmental stressors (e.g. UV exposure and desiccation), and effectively infect adult weevils under field-relevant conditions.

Furthermore, while laboratory experiments provided controlled insights into infection effects, the high mortality rates observed in these trials warrant careful interpretation. Future studies should refine rearing protocols, improve handling procedures to minimise stress, and ensure that test individuals are free from pre-existing conditions, which could otherwise skew virulence assessments.

Long-term persistence, environmental impact, and non-target effects

A key advantage of using native EPFs as BCAs is their potential for environmental persistence, allowing for long-term pest suppression without repeated applications (van Lenteren *et al*., 2017). However, the longevity of introduced fungal strains in forestry soils

remains uncertain, and their interactions with natural microbial communities require further study. Future research should:

- Conduct long-term persistence trials to assess whether applied EPFs establish within forestry ecosystems (McNamara *et al*., 2018a), how their populations fluctuate over multiple seasons, and whether they exhibit lasting biocontrol effects.
- Investigate potential non-target impacts over extended periods (Chiew *et al.*, 2022), as while short-term assessments indicated no significant effects on invertebrate communities, longer-term studies are needed to confirm ecological safety.
- Explore the influence of soil chemistry and microbial interactions on EPF persistence (Uzman *et al*., 2019), particularly how different soil compositions might enhance or inhibit fungal proliferation.

The integration of genomic tools and metagenomic analyses could further enhance these investigations, allowing researchers to track EPF establishment and assess their ecological interactions at a microbial level.

Scaling up and field validation across different forestry environments

While this study successfully demonstrated the efficacy of BCAs under determined field conditions, scaling up biocontrol applications requires further validation across diverse forestry environments. This should include comparisons with existing control methods for *H. abietis* across diverse coniferous plantations in the UK (Hardy *et al*., 2020, Moore *et al*., 2021). Future trials should:

- Test EPF and EPN combinations across different climatic regions to evaluate their robustness under varying environmental conditions, pest infestation levels, and coniferous plantation types. Understanding how these combinations perform across such interacting factors will be increasingly important for predicting outcomes under future climate scenarios (Inward *et al.,* 2012).
- Assess practical implementation strategies, such as integrating BCAs into existing forestry pest management programmes and determining cost-effective deployment strategies (Willoughby *et al.,* 2017).
- Collaborate with forestry stakeholders, including commercial growers and policymakers, to ensure that biocontrol solutions are both scientifically sound and operationally viable (van Lenteren *et al*., 2017).

Such large-scale validation studies are crucial for bridging the gap between experimental research and real-world implementation, ultimately ensuring that BCAs can be integrated into sustainable forestry practices.

Conclusion

This thesis has advanced the field of biological control of *Hylobius abietis* by systematically evaluating the potential of native EPFs and their interactions with EPNs as sustainable alternatives to conventional insecticide-based management strategies. Through bioprospecting, laboratory pathogenicity assessments, and field trials, this research has identified *Beauveria bassiana* 35G as a promising biocontrol agent with high virulence against *H. abietis*. Moreover, its additive effects when applied alongside *Steinernema carpocapsae* highlight the potential for multi-agent strategies in integrated pest management (IPM).

Field trials demonstrated that EPF-EPN combinations, applied at reduced doses, can enhance pest suppression while lowering operational costs. However, site-specific variations in efficacy and interactions—ranging from synergistic to antagonistic—underscore the complexity of multi-agent biocontrol systems and the need for further refinement. Additionally, the high control mortality observed in laboratory trials suggests that external stress factors may have influenced results, reinforcing the necessity of improving rearing protocols to ensure reliable assessments of fungal pathogenicity. The persistence of EPFs in forestry environments remains another critical aspect requiring further long-term ecological studies to optimise their efficacy and sustainability.

Future research should focus on refining application techniques, improving formulation strategies such as encapsulation or gel-based carriers to enhance fungal persistence, and expanding field validation across diverse forestry environments. Investigating the broader ecological interactions of these BCAs will be essential to maximising their effectiveness while ensuring environmental safety. The findings of this research contribute significantly to the development of sustainable pest management strategies for *H. abietis* control. By identifying highly virulent native EPFs and demonstrating their potential synergy with EPNs, this study provides a pathway toward reducing reliance on chemical insecticides while promoting environmentally responsible forestry practices. While further optimisation and large-scale validation are necessary, the results presented in this thesis reinforce the viability of biological control as a key component in the future of sustainable forestry pest management.

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APPENDICES

Appendix Table S2.1. List of nature reserves considered for bioprospecting across North West England: The table summarises site name, site code (where applicable), grid reference, habitat description, and field observations used to guide site selection. Sites were prioritised based on reported biodiversity, presence of insect and insectivorous communities, and habitat conservation status. The "Samples" column indicates sites from which soil samples were collected. Three sampling campaigns were conducted: (i) July–September 2020 (eight sites), (ii) March 2022 (four sites: HM, FX, SQ and CQ; samples subsequently lost due to incubator failure), and (iii) March 2023 (four sites: HM, FX, SQ and CQ), which provided the dataset used for downstream analyses. Site codes: FF = Freshfield Dune Heath; HM = Highfield Moss; CM = Cadishead & Little Woolden Moss; BN = Boilton–Nab–Redscar–Tunbrook Woods; FX = Foxhill Bank Local Nature Reserve; SQ = Salthill Quarry Local Nature Reserve; CQ = Cross Hill Quarry Local Nature Reserve; MD = Middleton Nature Reserve.

Site	Samples	Location	Vegetation communities	Observations
Lunt Meadows		SD 355 021	Wetlands	Insect spotting, Lichens and Mosses
Seaforth Nature Reserve		SD 318 971	Wetlands	Insect spotting, Lichens and Mosses
Freshfield Dune Heath	FF	SD 298 081	Dune Heath and Woodland	Insect spotting zone
Haskayne Cutting Nature Reserve		SD 353 091	Grassland and Wetlands	Wildlife corridor with butterflies
Holiday Moss		SD 488 010	Woodland and Raised bog	Insect spotting zone
Wigan Flashes Local Nature Reserve		SD 585 030	Wetlands, Woodland and Grassland	Insect spotting zone
Abram Flash		SD 612 002	Wetlands	Links Wigan Flashes to Lightshaw Meadows
Lightshaw Meadows		SJ 6152 9997	Wetlands	Biodiverse meadow grassland

Highfield Moss	HM	SJ 609 961	Lowland heath	Insect spotting zone, Lichens and Mosses
Cutacre		SD 710 022	Wetlands, Grassland and Ponds	Biodiverse rich in birds and wildflowers
Astley Moss		SJ 692 975	Mossland/Wetland	Lichens and Mosses, diverse in invertebrates, Delicate state of recovery
Cadishhead & Little Woolden Moss	CM	SJ698 951	Raised bog	Lichens and Mosses, diverse in invertebrates, Delicate state of recovery
Moston Fairway		SD 884 016	Wetlands, Woodland and Grassland	Insect spotting, Lichens and Mosses
Mere Sands Wood Nature Reserve		SD 456 157	Woodland and Lakes	Insect spotting
Freemans Pasture Local Nature Reserve		SD 564 154	Grassland	Insect spotting, species-rich grassland
Dean Wood		SD 629 153	Woodland	Lichens and Mosses
Longworth Clough Nature Reserve		SD 695 102	Woodland	Butterflies
Summerseat Nature Reserve		SD 793 145	Wetlands and Woodlands	Insect spotting, Scots pine
Cop Lane		SD 524 276	Grassland	Wildflowers and insects, Bramble and hawthorn

Willow Farm Wood Nature Reserve		SD 589 258	Woodland	Insect spotting, Ancient woodland
Pleasington Old Hall Wood and Wildlife Garden		SD 643 260	Grassland and Woodlands	Insects, birds and anphibians
Summerseat Nature Reserve		SD 793 146	Wetlands and Woodlands	Wildflowers and Insects
Foxhill Bank Local Nature Reserve	FX	SD 739 278	Grassland and Woodlands	Insects, birds and anphibians. Mosses and Lichens
Brockholes Nature Reserve		SD 584 305	Wetlands, Woodland and Grassland	Insect spotting, damselflies.
Boilton, Nab, Redscar and Tunbrook Woods	BN	SD 580 313	Woodlans	Insectivorous Shrew, Moles, Birds, Bats.
Upper Coldwell Reservoir		SD 847 364	Grassland	Butterflies
Salthill Quarry Local Nature Reserve	SQ	SD 755 426	Grassland	Wildflowers and Insects
Cross Hill Quarry Local Nature Reserve	CQ	SD 747 436	Grassland	Insectivorous species
Moor Piece Nature Reserve		SD 692 446	Woodland	Scots pine, Birch, peat bog patch, insectivorous species, High Biodiversity.
Winmarleigh & Cockerham Moss		SD 446 479	Raised bog	Insect spotting, Insectivorous plants and animals.

Barnaby's Sands and Burrows Marsh		SD 361 456	Saltmarsh and mudflats	Extensive ungrazed saltmarsh
Middleton Nature Reserve	MD	SD 415 591	Grasslands and Wetlands	Pollinators and more than 250 lepidoptera species
Heysham Nature Reserve		SD 407 601	Wetland, Grassland and Woodland	Highly Biodiverse
Heysham Moss		SD 423 603	Raised bog	Butterflies, Lichens and Mosses, highly biodiverse
Freeman's Pools		SD 456 615	Wetlands	Grassland Invertebrates, Populus nigra.
Aughton Woods		SD 543 663	Woodland	Small-leaved lime trees (indicators of ancient woodland)
Over Kellet Pond		SD 525 698	Wetlands	Important Invertebrates and protected amphibians
Warton Crag		SD 494 730	Grassland and Woodlands	Ancient trees, and high insect diversity.
Red Rocks Marsh		SJ 205 881	Coastal dunes, Grassland	Coastal sand dunes.

Appendix Table S2.2. Vegetation survey of the Salthill Quarry site (SQ; SD 75508 42490) conducted on 25 September 2023: Vegetation was recorded within a 2 m × 2 m plot on an 13° slope using three quadrats (Q1–Q3). For each taxon, percentage cover is shown for each quadrat. The table also includes total vegetation layer cover (%) and mean vegetation height (cm) for each quadrat. Scientific names are provided for all recorded plant species. These data were used to support National Vegetation Classification (NVC) assignment of the site..

Salt quarry (SQ)	**Species List**	**Scientific name**	**Q1 (%)**	**Q2 (%)**	**Q3 (%)**
Grid reference:	Betony	*Betonica officinalis*	2	<1	<1
SD 75508 42490	Lady's bedstraw	*Galium verum*	40	30	20
Date:25/09/2023	Wavy hair grass	*Deschampsia flexuosa*	70	80	60
Slope: 13°	Creeping velvet grass	*Holcus mollis*	10	10	10
Sample area:	Wood-sedge	*Carex sylvatica*	5	10	5
2m x 2m	Common knapweed	*Centaurea nigra*	30	40	25
	Common eyebright	*Euphrasia nemorosa*	2	0	<1
	Liverwort	*Agrimonia eupatoria*	10	5	5
	Hawthorn	*Crataegus monogyna*	1	<1	<1
	Bramble	*Rubus fruticosus*	1	2	0
	Pale wood violet	*Viola reichenbachiana*	25	10	5
	Creeping buttercup	*Ranunculus repens*	<1	0	0
	Willow feather moss	*Amblystegium serpens*	50	55	40
	St John's wort	*Hypericum perforatum*	2	10	10
	Bluebell	*Campanula rotundifolia*	0	2	0
	Salad burnet	*Sanguisorba minor*	0	30	25
		Layers cover (%)	100	100	100
		Layers mean height (cm)	15.667	39.334	18.33

Appendix Table S2.3 Vegetation survey of the Middleton site (MD; SD 41740 59239) conducted on 14 September 2023: Vegetation was recorded within a 2 m × 2 m plot on an 5° slope using three quadrats (Q1–Q3). For each taxon, percentage cover is shown for each quadrat. The table also includes total vegetation layer cover (%) and mean vegetation height (cm) for each quadrat. Scientific names are provided for all recorded plant species. These data were used to support National Vegetation Classification (NVC) assignment of the site.

Middleton (MD)	**Species List**	**Scientific name**	**Q1 (%)**	**Q2 (%)**	**Q3 (%)**
Grid reference:	Ribwort Plantain	*Plantago lanceolata*	20	5	1
SD 41740 59239	Geranium	*Geranium pratense*	<1	<1	
Date:14/09/2023	Horsetail	*Equisetum palustre*	3	10	15
Slope: 5°	Rush	*Juncus effesus*	30	30	60
Sample area:	Hairy tare	*Vicia hirsuta*	8	<1	
2m x 2m	Great tussock sedge	*Carex paniculata*	25	5	20
	Wavy hair grass	*Deschampsia flexuosa*	85	85	5
	Yarrow	*Achillea millefolium*	<1	0	
	Ragwort	*Senecio jacobaea*	<1	0	25
	Mouse-ear chickweed	*Cerastium fontanum*	<1	0	
	Common fleabane	*Pulicaria dysenterica*	30	<50	30
	Lesser knapweed	*Centaurea jacea*	<5	20	2
	Greater Bird's-foot-trefoil	*Lotus pedunculatus*	50-60	40	30
	Meadow pea	*Lathyrus pratensis*	5	10	<5
	Woodsy Thyme-moss	*Plagiomnium cuspidatum*	30		
	Delicate/Philibert's Tamarisk-moss	*Thuidium assimile*			
	Swan's-neck Thyme-moss	*Mnium hornum*	100	95	100
	St. Peter's wort	*Hypericum tetrapterum*	5	2	10
	Rough Hawkbit	*Leontodon hispidus*	<10		
	Common knapweed	*Centaurea nigra*	<10	25	2

Rosebay willowherb	*Chamaenerion angustifolium*	0	2	
Braken	*Pteridium aquilinum*	0	0	3
Velvet grass	*Holcus lanatus*	0	0	5
Hairy willowherb	*Epilobium hirsutum*	0	0	2
Creeping Thistle	*Cirsium arvense*	0	0	1
Hogweed	*Heracleum spondylium*	0	0	15
Bramble	*Rubus fruticosus*	0	0	1
	Layers cover (100%)	100	95	100
	Layers mean height (cm)	55	53.667	52.33

Appendix Table S2.4. Vegetation survey of the Cross Hill Quarry site (CQ; SD 74471 43356) conducted on 14 September 2023: Vegetation was recorded within a 2 m × 2 m plot on an 17° slope using three quadrats (Q1–Q3). For each taxon, percentage cover is shown for each quadrat. The table also includes total vegetation layer cover (%) and mean vegetation height (cm) for each quadrat. Scientific names are provided for all recorded plant species. These data were used to support National Vegetation Classification (NVC) assignment of the site.

Cross Hill quarry (CQ)	**Species List**	**Scientific name**	**Q1 (%)**	**Q2 (%)**	**Q3 (%)**
Grid reference:	Geranium	*Geranium pratense*	20		10
SD 74471 43356	Field horsetail	*Equisetum arvense*	25	25	40
Date:14/09/2023	Creeping Thistle	*Cirsium arvense*	20	60	20
Slope: 17°	Velvet grass	*Holcus lanatus*	50	30	80
Sample area:	Wood speedwell	*Veronica montana*	5	<20	2
2m x 2m	Rosebay willowherb	*Chamaenerion angustifolium*	30	20	20
	Bramble	*Rubus fruticosus*	35	30	30
	Hogweed	*Heracleum spondylium*	<1	2	
	Common feather moss	*Kinderbergia praelonga*			
	Yellow feather moss	*Homalothecium lutescens*			
	Common striated feathermoss	*Eurhynchium striatum*	80	85	90
	Lesser knapweed	*Centaurea jacea*	<1	<1	
	Meadow pea	*Lathyrus pratensis*	0	<1	
	Meadowsweet	*Filipendula ulmaria*	0	5	2
		Layers cover (100%)	90	90	90
		Layers mean height (cm)	82	75	42

Appendix Table S2.5. Vegetation survey of the Foxhill Bank site (FX; SD 74059 27978) conducted on 25 September 2023: Vegetation was recorded within a 4 m × 4 m plot on an 11° slope using three quadrats (Q1–Q3). For each taxon, percentage cover is shown for each quadrat. The table also includes total vegetation layer cover (%) and mean vegetation height (cm) for each quadrat. Scientific names are provided for all recorded plant species. These data were used to support National Vegetation Classification (NVC) assignment of the site.

Foxhill (FX)	**Species List**	**Scientific name**	**Q1 (%)**	**Q2 (%)**	**Q3 (%)**
Grid reference:	Ash	*Fraxinus excelsior*	5	5	5
SD 74059 27978	Bramble	*Rubus fruticosus*	30	30	40
Date:25/09/2023	Herb robert	*Geranium robertianum*	30	20	5
Slope: 11°	broad leaved willowherb	*Epilobium montanum*	5	<1	<1
Sample area:	Sycamore	*Acer pseudoplatanus*	2	0	0
4m x 4m	Male fern	*Dryopteris filix mas*	<1	2	0
	Lady fern	*Athyrium filix femina*	<1	0	0
	Opposite-leaved Golden-saxifrage	*Chrysosplenium oppositifolium*	10	0	0
	Nettle	*Urtica dioica*	<1	<1	0
	Tree moss	*Climacium dendroides*	50	20	30
	Creeping buttercup	*Ranunculus repens*	<1	2	2 - 5
	Spinulose Wood Fern	*Dryopteris carthusiana*	0	<1	0
	Wood avens	*Geum urbanum*	0	2	2
	Broad-leaved helleborine	*Epipactis helleborine*	0	<1	<1

Layers cover (100%)	90	70	80
Layers mean height (cm)	80.66	37.3	45

Appendix Table S2.6. Molecular identification of the 197 EPF isolates recovered during the 2023 soil survey: Isolates were obtained from soil samples collected on 1 March 2023 and identified by sequencing of the ITS region followed by BLAST® comparison against GenBank. For each isolate, the table reports site and soil fraction of origin, laboratory identification code, closest BLAST match, percentage sequence identity, and corresponding GenBank accession number. Topsoil (T) refers to 0–10 cm depth and bottom soil (B) to 10–20 cm depth. Sequences were submitted to GenBank under submission number SUB15201310. Site codes: FX = Foxhill Bank; CQ = Cross Hill Quarry; SQ = Salthill Quarry; MD = Middleton; HM = Highfield Moss.

Origin / Soil Fraction	Lab ID	BLAST Consensus	Percent Identity	GenBank Accession
FX/T	C 1G 4	*Metarhizium anisopliae* var. *anisopliae* isolate PL52-1	98.98%	PV382668
FX/T	C 1T 1	*Metarhizium anisopliae* strain 687	98.34%	PV382669
FX/T	C 1T 2	*Metarhizium anisopliae* strain 687	98.32%	PV382670
FX/T	C 1T 4	*Metarhizium anisopliae* strain 687	99.47%	PV382671
FX/B	C 2G 4	*Metarhizium anisopliae* strain 687	97.99%	PV382672
FX/B	C 2G 5	*Metarhizium anisopliae* strain 687	98.66%	PV382673
FX/B	C 2T 1	*Metarhizium anisopliae* strain 687	98.66%	PV382674
FX/B	C 2T 3	*Metarhizium anisopliae* strain 687	98.99%	PV382675
FX/B	C 2T 4	*Metarhizium anisopliae* strain 687	99.00%	PV382676
FX/B	C 2T 5	*Metarhizium sp.* RMK-2011b	99.15%	PV382677
FX/T	C 3T 4	*Metarhizium anisopliae* strain 687	98.67%	PV382678
FX/T	C 3T 5	*Metarhizium anisopliae* strain 687	97.81%	PV382679
FX/T	C 5G 1	*Metarhizium anisopliae* strain 687	98.57%	PV382680
FX/T	C 5G 2	*Metarhizium anisopliae* strain 687	98.16%	PV382681
FX/T	C 5G 3	*Metarhizium anisopliae* strain 687	98.16%	PV382682
FX/T	C 5G 5	*Metarhizium anisopliae* strain 687	98.50%	PV382683
FX/T	C 5T 1	*Metarhizium anisopliae* strain 687	98.66%	PV382684
FX/T	C 5T 2	*Metarhizium anisopliae* strain 687	98.17%	PV382685
FX/T	C 5T 3	*Metarhizium anisopliae* var. *anisopliae* strain LRC 142	97.67%	PV382686
FX/T	C 5T 4	*Metarhizium sp.* RMK-2011b	98.81%	PV382687
FX/B	C 6G 1	*Metarhizium anisopliae* strain 687	98.17%	PV382688

FX/B	C 6G 3	*Metarhizium anisopliae* strain 687	98.33%	PV382689
FX/B	C 6G 5	*Metarhizium anisopliae* var. *anisopliae* isolate PL52-1	88.74%	PV382690
FX/B	C 6T 3	*Metarhizium anisopliae* strain 687	98.50%	PV382691
FX/B	C 6T 5	*Metarhizium anisopliae* var. *anisopliae* isolate PL52-1	98.29%	PV382692
FX/T	C 7G 1	*Metarhizium sp*. RMK-2011b	98.48%	PV382693
FX/T	C 7G 2	*Metarhizium sp*. RMK-2011b	98.45%	PV382694
FX/T	C 7T 5	*Metarhizium anisopliae* strain 687	97.66%	PV382695
FX/B	C 8G 1	*Metarhizium anisopliae* strain 687	99.16%	PV382696
FX/B	C 8G 4	*Metarhizium anisopliae* strain 687	98.83%	PV382697
FX/B	C 8G 5	*Metarhizium anisopliae* strain 687	98.01%	PV382698
FX/B	C 8T 3	*Metarhizium anisopliae* strain 687	99.00%	PV382699
FX/B	C 8T 4	*Metarhizium anisopliae* strain 687	98.32%	PV382700
FX/B	C 8T 5	*Clonostachys rosea* f. *catenulata*	98.37%	PV382701
FX/T	C 9G 1	*Metarhizium anisopliae* strain 687	97.84%	PV382702
FX/T	C 9G 2	*Metarhizium anisopliae* strain 687	98.50%	PV382703
FX/T	C 9G 3	*Metarhizium anisopliae* strain 687	97.98%	PV382704
FX/T	C 9T 1	*Clonostachys rosea* f. *catenulata*	98.37%	PV382705
FX/T	C 9T 2	*Metarhizium sp.* RMK-2011b	98.14%	PV382706
FX/T	C 9T 3	*Metarhizium anisopliae* var. *anisopliae* isolate PL52-1	98.64%	PV382707
FX/T	C 9T 4	*Metarhizium anisopliae* strain 687	98.32%	PV382708
FX/T	C 11G 1	*Metarhizium anisopliae* strain 687	98.49%	PV382709
FX/T	C 11G 2	*Metarhizium anisopliae* strain 687	98.33%	PV382710
FX/T	C 11G 3	*Metarhizium anisopliae* strain 687	97.99%	PV382711
FX/T	C 11G 4	*Metarhizium anisopliae* strain 687	98.50%	PV382712
FX/T	C 11G 5	*Metarhizium sp*. RMK-2011b	98.32%	PV382713
FX/T	C 11T 2	*Metarhizium anisopliae* isolate T5R2	98.59%	PV382714

FX/T	C 11T 3	*Metarhizium anisopliae* strain 687	99.29%	PV382715
FX/B	C 12G 2	*Metarhizium anisopliae* var. *anisopliae* strain LRC 142	97.14%	PV382716
FX/B	C 12G 3	*Metarhizium anisopliae* strain 687	98.83%	PV382717
FX/B	C 12G 4	*Metarhizium anisopliae* strain 687	98.49%	PV382718
FX/B	C 12G 5	*Metarhizium sp*. RMK-2011b	98.48%	PV382719
FX/B	C 12T 1	*Metarhizium anisopliae* strain 687	98.49%	PV382720
FX/B	C 12T 2	*Metarhizium anisopliae* strain 687	97.84%	PV382721
FX/B	C 12T 3	*Metarhizium anisopliae* strain 687	98.79%	PV382722
FX/B	C 12T 5	*Metarhizium sp*. RMK-2011b	98.06%	PV382723
FX/T	C 13G 1	*Metarhizium anisopliae* strain 687	98.01%	PV382724
FX/T	C 13G 2	*Metarhizium anisopliae* strain 687	98.67%	PV382725
FX/T	C 13G 4	*Metarhizium anisopliae* strain 687	98.33%	PV382726
FX/T	C 13T 2	*Metarhizium anisopliae* strain 687	98.66%	PV382727
FX/T	C 13T 3	*Metarhizium sp*. RMK-2011b	98.31%	PV382728
FX/T	C 13T 5	*Metarhizium anisopliae* strain 687	98.83%	PV382729
FX/B	C 14G 2	*Clonostachys rosea* f. *catenulata*	98.19%	PV382730
FX/B	C 14T 4	*Metarhizium sp*. RMK-2011b	98.62%	PV382731
FX/T	C 15G 1	*Metarhizium anisopliae* strain 687	98.99%	PV382732
FX/T	C 15G 2	*Metarhizium sp.* RMK-2011b	97.96%	PV382733
FX/T	C 15G 3	*Metarhizium sp*. RMK-2011b	99.14%	PV382734
FX/B	C 16G 2	*Metarhizium sp.* RMK-2011b	98.48%	PV382735
FX/B	C 16T 1	*Metarhizium sp*. RMK-2011b	98.62%	PV382736
FX/B	C 16T 2	*Metarhizium sp*. RMK-2011b	98.80%	PV382737

FX/B	C 16T 5	*Metarhizium anisopliae* var. *anisopliae* isolate PL52-1	99.48%	PV382738
FX/T	C 17G 2	*Metarhizium anisopliae* strain 687	98.81%	PV382739
FX/T	C 17T 1	*Metarhizium sp.* RMK-2011b	99.14%	PV382740
FX/T	C 17T 2	*Metarhizium sp*. RMK-2011b	98.63%	PV382741
FX/B	C 18G 4	*Metarhizium sp*. RMK-2011b	98.81%	PV382742
FX/B	C 18T 1	*Metarhizium anisopliae* isolate T5R2	98.22%	PV382743
FX/B	C 18T 2	*Metarhizium anisopliae* strain 687	98.49%	PV382744
FX/B	C 18T 3	*Metarhizium anisopliae* strain 687	99.16%	PV382745
FX/B	C 18T 4	*Metarhizium sp.* RMK-2011b	99.14%	PV382746
CQ/T	C 19G 2	*Metarhizium anisopliae* strain 687	98.50%	PV382747
CQ/T	C 19G 3	*Metarhizium anisopliae* var. *anisopliae* isolate PL52-1	98.65%	PV382748
CQ/T	C 19T 1	*Metarhizium sp*. RMK-2011b	98.64%	PV382749
CQ/T	C 19T 2	*Metarhizium anisopliae* strain ARSEF5851	98.78%	PV382750
CQ/T	C 19T 3	*Metarhizium anisopliae* isolate T5R2	98.57%	PV382751
CQ/T	C 19T 4	*Metarhizium sp*. RMK-2011b	99.32%	PV382752
CQ/T	C 19T 5	*Metarhizium anisopliae* var. *anisopliae* isolate F4	90.72%	PV382753
CQ/B	C 20G 3	*Metarhizium anisopliae* isolate T5R2	99.47%	PV382754
CQ/B	C 20G 4	*Beauveria bassiana* strain KK2	96.20%	PV382755
CQ/B	C 20T 1	*Metarhizium sp*. RMK-2011b	99.15%	PV382756
CQ/B	C 22T 4	*Metarhizium anisopliae* var. *anisopliae* isolate PL52-1	99.31%	PV382757
CQ/T	C 23G 2	*Metarhizium sp*. RMK-2011b	98.64%	PV382758
CQ/T	C 23G 5	*Metarhizium anisopliae* var. *anisopliae* isolate PL52-1	98.29%	PV382759
CQ/T	C 23T 1	*Metarhizium sp.* RMK-2011b	99.15%	PV382760

CQ/T	C 23T 4	*Clonostachys rosea* f. *catenulata*	98.51%	PV382761
CQ/T	C 25G 2	*Metarhizium anisopliae* var. *anisopliae* isolate GMX10.2-3	98.31%	PV382762
CQ/T	C 25G 3	*Metarhizium anisopliae* var. *anisopliae* isolate PL52-1	99.15%	PV382763
CQ/T	C 25T 1	*Metarhizium sp*. RMK-2011b	98.47%	PV382764
CQ/T	C 25T 2	*Metarhizium sp*. RMK-2011b	98.29%	PV382765
CQ/B	C 26G 1	*Metarhizium sp*. RMK-2011b	98.45%	PV382766
CQ/B	C 26G 2	*Metarhizium sp.* RMK-2011b	98.31%	PV382767
CQ/B	C 26G 3	*Metarhizium anisopliae* var. *anisopliae* isolate PL52-1	99.15%	PV382768
CQ/T	C 27G 1	*Metarhizium sp*. RMK-2011b	98.64%	PV382769
CQ/T	C 27G 2	*Metarhizium sp*. RMK-2011b	98.63%	PV382770
CQ/T	C 27G 3	*Metarhizium sp*. RMK-2011b	98.47%	PV382771
CQ/T	C 27G 4	*Metarhizium sp*. RMK-2011b	97.97%	PV382772
CQ/T	C 29G 1	*Metarhizium sp.* RMK-2011b	98.81%	PV382773
CQ/T	C 29G 2	*Metarhizium anisopliae* var. *anisopliae* strain LRC 142	98.18%	PV382774
CQ/T	C 29T 1	*Metarhizium anisopliae* strain 687	98.64%	PV382775
CQ/T	C 29T 2	*Metarhizium anisopliae* strain 687	98.50%	PV382776
CQ/T	C 29T 3	*Metarhizium sp*. RMK-2011b	98.63%	PV382777
CQ/T	C 29T 4	*Metarhizium anisopliae* strain 687	97.97%	PV382778
CQ/T	C 29T 5	*Metarhizium anisopliae* strain 687	97.82%	PV382779
CQ/B	C 30G 2	*Metarhizium sp*. RMK-2011b	97.80%	PV382780
CQ/B	C 30G 3	*Metarhizium anisopliae* strain 687	98.33%	PV382781

CQ/B	C 30G 4	*Metarhizium anisopliae* var. *anisopliae* isolate PL52-1	98.46%	PV382782
CQ/B	C 30T 4	*Metarhizium sp*. RMK-2011b	98.81%	PV382783
CQ/T	C 31G 2	*Metarhizium sp*. RMK-2011b	98.82%	PV382784
CQ/T	C 31G 3	*Metarhizium sp.* RMK-2011b	98.45%	PV382785
CQ/T	C 31T 1	*Metarhizium anisopliae* var. *anisopliae* isolate PL52-1	98.81%	PV382786
CQ/T	C 31T 2	*Metarhizium sp*. RMK-2011b	98.28%	PV382787
CQ/T	C 31T 5	*Metarhizium anisopliae* strain 687	98.50%	PV382788
CQ/B	C 32G 4	*Metarhizium sp*. RMK-2011b	98.14%	PV382789
CQ/T	C 33G 1	*Metarhizium anisopliae* strain 687	90.37%	PV382790
CQ/T	C 33G 3	*Clonostachys rosea* f. *catenulata*	97.84%	PV382791
CQ/T	C 33G 5	*Clonostachys rosea* f. *catenulata*	96.72%	PV382792
CQ/T	C 33T 1	*Metarhizium sp.* RMK-2011b	98.64%	PV382793
CQ/B	C 34T 3	*Metarhizium anisopliae* strain 687	98.31%	PV382794
CQ/B	C 34T 5	*Metarhizium anisopliae* strain 687	98.66%	PV382795
SQ/T	C 91G 4	*Clonostachys rosea* f. *catenulata*	98.52%	PV382796
SQ/T	C 91T 1	*Metarhizium anisopliae* strain 687	99.00%	PV382797
SQ/T	C 91T 2	*Metarhizium anisopliae* isolate T5R2	94.13%	PV382798
SQ/T	C 91T 3	*Metarhizium sp*. RMK-2011b	98.64%	PV382799
SQ/T	C 91T 4	*Metarhizium sp.* RMK-2011b	98.63%	PV382800
SQ/T	C 91T 5	*Metarhizium sp*. RMK-2011b	99.13%	PV382801
SQ/B	C 92G 1	*Metarhizium anisopliae* strain 687	98.16%	PV382802
SQ/B	C 92T 2	*Metarhizium anisopliae* strain 687	98.66%	PV382803
SQ/B	C 92T 3	*Metarhizium sp*. RMK-2011b	99.15%	PV382804
SQ/B	C 92T 4	*Metarhizium sp*. RMK-2011b	98.30%	PV382805

SQ/T	C 93T 4	*Beauveria brongniartii* (Sacc.) Petch, 1926	98.67%	PV382806
SQ/T	C 93T 5	*Metarhizium anisopliae* strain 687	98.99%	PV382807
SQ/T	C 95G 4	*Beauveria bassiana* isolate DAOM195005	98.36%	PV382808
SQ/T	C 95T 2	*Metarhizium anisopliae* strain 687	97.99%	PV382809
SQ/T	C 95T 3	*Metarhizium sp.* RMK-2011b	98.31%	PV382810
SQ/B	C 96G 2	*Metarhizium anisopliae* isolate T5R2	97.35%	PV382811
SQ/T	C 97G 1	*Metarhizium anisopliae* strain 687	99.16%	PV382812
SQ/T	C 97G 2	*Metarhizium anisopliae* strain 687	98.66%	PV382813
SQ/T	C 97G 4	*Metarhizium anisopliae* strain 687	98.33%	PV382814
SQ/T	C 97T 3	*Metarhizium anisopliae* strain 687	98.65%	PV382815
SQ/B	C 98G 1	*Metarhizium sp*. RMK-2011b	98.63%	PV382816
SQ/B	C 98T 1	*Metarhizium anisopliae* strain 687	98.82%	PV382817
SQ/B	C 98T 2	*Metarhizium anisopliae* strain 687	98.47%	PV382818
SQ/B	C 98T 3	*Metarhizium anisopliae* strain 687	98.50%	PV382819
SQ/B	C 98T 5	*Clonostachys rosea* f. *catenulata*	98.36%	PV382820
SQ/T	C 99T 5	*Metarhizium anisopliae* strain 687	97.84%	PV382821
SQ/B	C 100G 2	*Beauveria brongniartii* (Sacc.) Petch, 1926	98.52%	PV382822
SQ/B	C 100G 3	*Metarhizium sp.* RMK-2011b	98.47%	PV382823
SQ/T	C 101T 4	*Beauveria brongniartii* (Sacc.) Petch, 1926	99.02%	PV382824
SQ/B	C 102G 1	*Beauveria brongniartii* (Sacc.) Petch, 1926	98.21%	PV382825
SQ/B	C 102G 3	*Metarhizium anisopliae* strain 687	98.67%	PV382826
SQ/B	C 102T 4	*Metarhizium anisopliae* strain 687	98.50%	PV382827

SQ/T	C 103G 4	*Metarhizium sp*. RMK-2011b	98.98%	PV382828
SQ/T	C 103G 5	*Metarhizium sp.* RMK-2011b	99.15%	PV382829
SQ/T	C 103T 1	*Metarhizium anisopliae* strain 687	98.48%	PV382830
SQ/T	C 103T 3	*Metarhizium anisopliae* strain 687	98.83%	PV382831
SQ/B	C 104G 1	*Metarhizium anisopliae* strain 687	98.63%	PV382832
SQ/B	C 104G 3	*Metarhizium anisopliae* strain 687	98.00%	PV382833
SQ/B	C 104G 4	*Metarhizium sp*. RMK-2011b	98.98%	PV382834
SQ/B	C 104G 5	*Metarhizium sp.* RMK-2011b	98.80%	PV382835
SQ/B	C 104T 1	*Metarhizium sp*. RMK-2011b	98.62%	PV382836
SQ/B	C 104T 2	*Metarhizium anisopliae* strain 687	98.00%	PV382837
SQ/B	C 104T 3	*Metarhizium sp.* RMK-2011b	98.13%	PV382838
SQ/B	C 106G 2	*Metarhizium anisopliae* strain 687	99.16%	PV382840
SQ/T	C 107G 1	*Metarhizium anisopliae* strain 687	98.16%	PV382841
SQ/T	C 107G 4	*Metarhizium anisopliae* strain 687	98.33%	PV382842
SQ/B	C 108G 1	*Metarhizium anisopliae* strain 687	98.32%	PV382843
MD/T	C 127G 1	*Metarhizium anisopliae* var. *anisopliae* isolate PL52-1	99.32%	PV382844
MD/T	C 127G 2	*Metarhizium anisopliae* strain 687	98.49%	PV382845

MD/T	C 127G 3	*Metarhizium anisopliae* strain 687	98.48%	PV382846
MD/T	C 127G 4	*Metarhizium anisopliae* strain 687	98.49%	PV382847
MD/T	C 127G 5	*Metarhizium anisopliae* strain 687	98.67%	PV382848
MD/B	C 128G 1	*Metarhizium anisopliae* strain 687	98.82%	PV382849
MD/B	C 128G 2	*Metarhizium anisopliae* strain 687	98.66%	PV382850
MD/B	C 128G 5	*Metarhizium anisopliae* strain 687	98.50%	PV382851
MD/B	C 128T 1	*Metarhizium anisopliae* strain 687	98.32%	PV382852
MD/T	C 129G 4	*Metarhizium anisopliae* strain 687	98.83%	PV382853
MD/T	C 129T 1	*Metarhizium anisopliae* strain 687	98.66%	PV382854
MD/T	C 129T 3	*Metarhizium anisopliae* strain 687	98.33%	PV382855
MD/T	C 129T 4	*Metarhizium anisopliae* strain 687	98.50%	PV382856
MD/B	C 130T 1	*Metarhizium anisopliae* strain 687	98.49%	PV382857
MD/B	C 130T 5	*Metarhizium anisopliae* strain 687	99.17%	PV382858
MD/T	C 131G 1	*Metarhizium anisopliae* strain 687	99.49%	PV382859
MD/T	C 131G 2	*Metarhizium anisopliae* strain 687	98.16%	PV382860
MD/T	C 131T 1	*Metarhizium anisopliae* strain 687	98.66%	PV382861
MD/B	C 132G 1	*Metarhizium sp*. RMK-2011b	98.47%	PV382862

MD/T	C 133T 2	*Beauveria bassiana* isolate DAOM210087	98.35%	PV382863
MD/T	C 137G 5	*Metarhizium anisopliae* strain 687	98.49%	PV382864
MD/T	C 141G 2	*Beauveria bassiana* isolate DAOM210087	97.39%	PV382865

Appendix Table S2.7. Frequency (prevalence) and abundance of EPF strains recovered during the 2023 bioprospecting survey (unpooled dataset): Values represent the number of soil samples in which each strain was detected (sample frequency; prevalence) and the total number of isolates recovered per strain (abundance). Data are partitioned by bait insect (*Galleria mellonella* and *Tenebrio molitor*) and summarised across all sites and soil depths (site × sample × soil depth × bait insect; n = 87). Abbreviations: Ma = Metarhizium anisopliae; Msp = Metarhizium sp.; Bb = Beauveria bassiana; B. brongniartii = Beauveria brongniartii; Cr f. catenulata = ***Clonostachys rosea*** f. ***catenulata***.

Strain	Galleria samples	Tenebrio samples	Total samples	Galleria isolates	Tenebrio isolates	Total isolates
Ma 687	21	18	39	58	42	100
Msp RMK-2011b	17	14	31	29	27	56
Ma PL52-1	7	5	12	7	6	13
Ma T5R2	3	3	6	3	3	6
Cr f. catenulata	4	3	7	5	3	8
B. brongniartii	2	2	4	2	2	4
Ma LRC 142	2	1	3	2	1	3
Bb DAOM210087	1	1	2	1	1	2
Bb DAOM195005	1	0	1	1	0	1
Bb KK2	1	0	1	1	0	1
Ma ARSEF5851	0	1	1	0	1	1
Ma F4	0	1	1	0	1	1
Ma GMX10.2-3	1	0	1	1	0	1

Appendix Table S2.8. Distribution, prevalence, and abundance of entomopathogenic fungal (EPF) strains recovered from soil-pooled samples during the 2023 bioprospecting survey: Topsoil (0–10 cm) and bottom soil (10–20 cm) samples were combined within each sampling point to generate pooled soil samples (n = 57). "Samples" indicate the number of pooled samples in which each strain was detected (prevalence), whereas "isolates" indicate the total number of recoveries per strain (abundance). Values are presented separately for each bait insect (*Galleria mellonella* and *Tenebrio molitor*) and as totals across both bait types. Abbreviations: Ma = *Metarhizium anisopliae*; Msp = *Metarhizium* sp.; Bb = *Beauveria bassiana*; B. brongniartii = *Beauveria brongniartii*; Cr f. catenulata = *Clonostachys rosea* f. *catenulata*.

Strain	Galleria samples	Tenebrio samples	Galleria isolates	Tenebrio isolates	Total samples	Total isolates
Ma 687	19	21	19	21	40	40
Msp RMK-2011b	14	17	14	17	31	31
Ma PL52-1	7	5	7	5	12	12
Cr f. catenulata	3	4	3	4	7	7
Ma T5R2	2	4	2	4	6	6
B brongniartii	2	2	2	2	4	4
Ma LRC 142	2	1	2	1	3	3
Bb DAOM210087	1	1	1	1	2	2
Bb DAOM195005	1	0	1	0	1	1
Bb KK2	1	0	1	0	1	1
Ma ARSEF5851	0	1	0	1	1	1
Ma F4	0	1	0	1	1	1
Ma GMX10.2-3	1	0	1	0	1	1

Appendix Table S2.9. Eigenvalues and variance explained by principal coordinate axes (PCoA) derived from Bray–Curtis dissimilarities of EPF strain composition: Results are presented for the unpooled dataset (site × sample × soil depth × bait insect; n = 87) and the soil-pooled dataset (site × pooled sample × bait insect; n = 57). Variance explained (%) is calculated from positive eigenvalues only, following standard practice for Bray–Curtis-based ordination. Cumulative variance represents the total proportion of variation explained by successive axes.

Dataset	Axis	Eigenvalue	Variance explained (%)	Cumulative (%)
Unpooled	PCoA1	11.862	40.73	40.73
	PCoA2	4.157	14.28	55.01
	PCoA3	2.883	9.90	64.91
	PCoA4	2.674	9.18	74.09
Soil pooled	PCoA1	5.952	33.24	33.24
	PCoA2	2.628	14.68	47.92
	PCoA3	1.881	10.51	58.42
	PCoA4	1.786	9.98	68.40

Appendix Table S2.10: Indicator Species Analysis (IndVal.g; 5,000 permutations) for EPF strains associated with bait insect type in the soil-pooled dataset (n = 57): Analysis tested for preferential association of EPF strains with bait insects (*Galleria mellonella* vs *Tenebrio molitor*). Columns "Galleria" and "Tenebrio" indicate presence (1) or absence (0) of each strain within samples associated with each bait type. The IndVal statistic (IndVal.g) quantifies the strength of association between each strain and bait group. Permutation-based p-values are reported where applicable. NA indicates that permutation-based p-values could not be computed for strains occurring in both bait groups without exclusivity. No strains showed statistically significant association with either bait insect ($p < 0.05$). Abbreviations: Ma = *Metarhizium anisopliae*; Msp = *Metarhizium* sp.; Bb = *Beauveria bassiana*; B. brongniartii = *Beauveria brongniartii*; Cr f. catenulata = *Clonostachys rosea* f. *catenulata*.

Strain	Galleria	Tenebrio	IndVal	p-value
Ma ARSEF5851	0	1	0.189	0.485
Ma F4	0	1	0.189	0.485
Bb KK2	1	0	0.186	1.000
Ma GMX10.2-3	1	0	0.186	1.000
Bb DAOM195005	1	0	0.186	1.000
Ma 687	1	1	0.838	NA
Ma T5R2	1	1	0.324	NA
Ma PL52-1	1	1	0.459	NA
Msp RMK-2011b	1	1	0.737	NA
Cr f. catenulata	1	1	0.350	NA
Ma LRC142	1	1	0.229	NA
Bb DAOM210087	1	1	0.187	NA
B. brongniartii	1	1	0.265	NA

Appendix Table 3.1. Survival analysis of fungal isolates screened for pathogenicity against their original bait host species: Twelve fungal isolates recovered during the 2020 soil-baiting survey were screened for pathogenicity against the same bait host species from which they were originally isolated. Survival was monitored daily for 14 days following inoculation in two assays. Assay 1 (10 insects per treatment, in February 2021) and Assay 2 (20 insects per treatment, in March 2021). Values shown are the number of insects tested (n), number of deaths recorded during the assay, number censored at the end of follow-up, mean survival time ± standard error, 95% confidence interval, and median survival time. Control insects were handled identically but were not exposed to fungal inoculum**.**

	isolate	host	n	deaths	censored	mean_survival	sd_survival
Assay							
1	6G2	Galleria	10	10	0	6	1.15470054
	15G1	Galleria	10	10	0	7.4	1.0749677
	17G1	Galleria	10	10	0	5.6	2.22111083
	22G3	Galleria	10	10	0	4.7	1.94650684
	23T1	Tenebrio	10	10	0	4.7	1.88856206
	30G4	Galleria	10	10	0	5.4	1.8973666
	33G2	Galleria	10	10	0	5.1	2.02484567
	35G2	Galleria	10	10	0	5.8	2.0976177
	93G2	Galleria	10	10	0	4.2	2.34757558
	102G4	Galleria	10	10	0	5.3	2.66874919
	139G2	Galleria	10	10	0	4.6	2.06559112
	143G2	Galleria	10	10	0	5.6	1.71269768
	Control	Galleria	10	6	4	9.3	4.00138865
Assay							
2	6G2	Galleria	20	20	0	6.2	0.83350875
	15G1	Galleria	20	20	0	6.05	0.39403446
	17G1	Galleria	20	20	0	4.65	1.46089374
	22G3	Galleria	20	20	0	6.45	0.99868334
	23T1	Tenebrio	20	20	0	6.15	1.03998988
	30G4	Galleria	20	20	0	6.6	0.50262469
	33G2	Galleria	20	20	0	6.15	0.87509398
	35G2	Galleria	20	17	3	9.3	3.24605023
	93G2	Galleria	20	20	0	7.8	1.05630936

102G4	Galleria	20	17	3	7.95	2.7429335
139G2	Galleria	20	20	0	6.8	0.7677719
143G2	Galleria	20	20	0	6.3	0.65694669
Control	Galleria	20	9	11	10.95	2.66507628

Appendix Table 3.2. Survival analysis parameters for *Hylobius abietis* adults exposed to fast-acting EPF isolates at two conidial concentrations: Adult weevils (n = 10 per treatment) were exposed to EPF isolates at 10^7 conidia ml^{-1} (Med) and 10^6 conidia ml^{-1} (Low) and monitored for 21 days under laboratory conditions (November 2022) with access to food and water. The table reports restricted mean survival time (days ± standard error, S.E.), 95% confidence intervals (C.I.), and estimated time (days) to reach specific mortality thresholds (25%, 50%, 75%, 90%, and 100%). Median lethal time (LT_{50}) corresponds to the time to 50% mortality. The "95% Median C.I." represents the confidence interval of the median survival estimate (fiducial limits). The control group consisted of untreated insects maintained under identical conditions. Treatment labels: 17 and 35 refer to EPF isolates; Med = 10^7 conidia ml^{-1}; Low = 10^6 conidia ml^{-1}.

		Restricted Mean			Age in days at % mortality					
Name	**No. of subjects**	**Days**	**S.E.**	**95% C.I.**	**25%**	**50%**	**75%**	**90%**	**100%**	**95% Median C.I.**
17 Med	10	13.40	2.60	8.31 ~ 18.49	4.00	12.00				4.0 ~ 21.0
35 Med	10	9.90	1.40	7.15 ~ 12.65	6.00	9.00	10.00	14.00		6.0 ~ 9.0
17 Low	10	15.30	1.96	11.45 ~ 19.15	9.00	16.00				9.0 ~ 21.0
35 Low	10	17.70	1.26	15.24 ~ 20.16	14.00	20.00				14.0 ~ 21.0
Control	10	21.00	0.00	21.00 ~ 21.00						- ~ -

Appendix Table 3.3. Pairwise Log-rank tests comparing survival distributions of *Hylobius abietis* adults exposed to EPF isolates: Pairwise comparisons were conducted between treatments using Kaplan–Meier survival data, with results reported as Chi-square (χ^2) statistics and associated p-values. Comparisons include (i) each EPF isolate versus the control (CTRL), (ii) pairwise comparisons among EPF isolates tested at 10^8 conidia ml^{-1} (April 2022), and (iii) comparisons between the two fastest-acting isolates (17G and 35G) at medium (10^7 conidia ml^{-1}) and low (10^6 conidia ml^{-1}) concentrations (November 2022). CTRL = untreated control insects. Isolate codes (e.g., 6G, 17G, 35G) correspond to EPF strains described in Table 3.1. "Med" and "Low" refer to conidial concentrations of 10^7 and 10^6 conidia ml^{-1}, respectively. Significant differences in survival between groups are indicated by p-values < 0.05.

12EPF	X2	P-value
CTRL/6G	0.69	0.4059
CTRL/15G	0.09	0.7652
CTRL/17G	6.49	0.0108
CTRL/22G	0	0.9569
CTRL/23T	1.63	0.2015
CTRL/30G	2.81	0.0935
CTRL/33G	2.14	0.1435
CTRL/35G	3.78	0.052
CTRL/93G	0	0.9511
CTRL/102G	0.03	0.8609
CTRL/139G	3.86	0.0494
CTRL/143G	3.49	0.0617

12EPF	X2	P-value
17G/15G	7.67	0.0056
17G/22G	5.42	0.0199
17G/33G	5.38	0.0204
17G/35G	0.03	0.8588
17G/93G	7.15	0.0075
17G/102G	10.86	0.001
17G/139G	20.13	7.2E-06
17G/143G	15.23	0.0001
35G/15G	6.1	0.0135
35G/33G	3.86	0.0493
35G/93G	6.44	0.0112
35G/102G	8.11	0.0044
35G/139G	15.7	0.0001
35G/143G	11.2	0.0008

12EPF	X2	P-value
6G/139G	7.9	0.005
6G/143G	6.75	0.0094
15G/139G	5.65	0.0175
15G/143G	4.58	0.0323
22G/139G	3.92	0.0477
23T/139G	10.32	0.0013
23T/143G	8.91	0.0028
30G/102G	5.67	0.0172
30G/139G	13.6	0.0002
30G/143G	11.21	0.0008
33G/139G	13.26	0.0003
33G/143G	11.1	0.0009

2 Best	X2	P-value
CTRL/17G med	6.39	0.0115
CTRL/ 35G med	16.62	0.000046
CTRL/17G low	6.39	0.0115
CTRL/35G low	6.39	0.0115
35G med/17G low	3.86	0.0496
35G med/35G low	8.82	0.003

Appendix Table 3.4. Statistical analyses of feeding and weight-change responses of *Hylobius abietis* following exposure to EPF treatments: Data were first assessed for normality using the Kolmogorov–Smirnov (K–S) test. Variables with normally distributed residuals ($p > 0.05$) were analysed using one-way ANOVA, with results reported as F-statistics and associated p-values. Variables that deviated from normality ($p < 0.05$) were analysed using the Kruskal–Wallis test, with results reported as H statistics (χ^2 approximation), degrees of freedom (df), and p-values. Analyses were conducted on two datasets: (i) all twelve EPF isolates applied at full dose (10^8 conidia ml^{-1}; "12 EPF"), and (ii) the two fastest-acting isolates (17G and 35G) tested at medium (10^7 conidia ml^{-1}) and low (10^6 conidia ml^{-1}) concentrations ("Med_Low"). Response variables include total weight change, weight change per day, and feeding activity measured as area consumed at different time points (T = 4, 7, and 14 days) and per day. Significant effects are indicated by an asterisk (*) where $p < 0.05$.

Normally distributed variables	**K-S**	**ANOVA**	
	p-value	**F**	**p-value**
Weight difference 12EPF	0.2	1.128	0.344
Weight difference/day 12EPF	0.2	1.773	0.06
Weight difference Med_Low	0.2	4.838	0.002*
Weight difference/day Med_Low	0.183	4.556	0.004*
Area consumed T=7 Med_Low	0.2	1.444	0.235
Area consumed T=14 Med_Low	0.199	6.123	<0.001*
Area consumed/day Med_Low	0.2	4.092	0.007*
Non-normally distributed variables	**K-S**	**Kruskal-Wallis Test**	
	p-value	**H (df=12) H (df=4)**	**p-value**
Area consumed T=4 12EPF	<0.001	23.5	0.024*
Area consumed T=4 Med_Low	0.028	1.441	0.837
Area consumed T=7 12 EPF	<0.001	24.32	0.018*
Area consumed T=14 12EPF	0.002	22.229	0.035*
Area consumed/day 12EPF	0.04	13.355	0.344

Appendix Table 3.5. Results of post hoc multiple comparisons for weight change and feeding responses of *Hylobius abietis* following exposure to entomopathogenic fungal (EPF) treatments: Post hoc tests were performed following ANOVA to identify pairwise differences between treatments. Tukey's HSD test was applied where assumptions of homogeneity of variance were met, and Dunnett's T3 test was used where variances were unequal. The table reports statistically significant and marginal pairwise comparisons ($p \leq 0.05$) for weight change, weight change per day, and bark consumption (area consumed) after 14 days of exposure under laboratory conditions with access to food and water. Analyses are presented for two datasets: (i) twelve EPF isolates tested at full dose (10^8 conidia ml^{-1}; "12 EPF", April 2022), and (ii) the two fastest-acting isolates (17G and 35G) tested at medium (10^7 conidia ml^{-1}; M) and low (10^6 conidia ml^{-1}; L) concentrations ("Med_Low", November 2022). Treatment labels indicate pairwise comparisons between groups (e.g., 17G M vs 35G L). CTRL = untreated control insects. Only comparisons showing statistically significant or near-significant differences are presented.

	Tuckey's HSD post-hoc test	
	Treatments	P value
Weight difference Med_Low	17G M vs 35G L	0.015
	35G M vs 35G L	0.004
Weight difference/day 12 EPF	15G vs 17G	0.049
Weight difference/day Med_Low	CTRL vs 35G M	0.056
	17G M vs 35G L	0.037
	35G M vs 35G L	0.007
	Dunnett T3 post-hoc test	
	Treatments	P value
Area T=14 Med_Low	CTRL vs 17G M	0.012
	CTRL vs 35G M	<0.001
	35G M vs 35G L	0.021
Area/day Med_Low	CTRL vs 17G M	0.016
	CTRL vs 35G M	0.007

Appendix Table 3.6. Pairwise comparisons of bark consumption using Dunn's post hoc test following Kruskal–Wallis analyses for non-normally distributed data: Dunn's test was applied to identify pairwise differences between treatments after significant Kruskal–Wallis tests of bark consumption (area eaten) at 4, 7, and 14 days (T = 4, 7, 14) following exposure to twelve entomopathogenic fungal (EPF) isolates at 10^8 conidia ml^{-1} (April 2022). Results are reported as test statistics (Z) and associated p-values for significant pairwise comparisons. Comparisons include EPF isolates versus the untreated control (CTRL) and between isolates. Treatment labels (e.g., 23T, 17G, 35G) correspond to EPF strains described in Table 3.1. Negative test statistic values indicate reduced bark consumption relative to the comparator group, whereas positive values indicate increased consumption. Only statistically significant comparisons ($p < 0.05$) are presented.

Pairwise Comparisons (Dunn's test)											
	Treatment pairs	Test Statistic	p value		Treatment pairs	Test Statistic	p value		Treatment pairs	Test Statistic	p value
Area T=4	23T/CTRL	-40.4	0.016	Area T=7	23T/CTRL	-33.7	0.045	Area T=14	17G/CTRL	-35.55	0.035
12 EPF	30G/CTRL	-41	0.015	12 EPF	30G/CTRL	-40.05	0.017	12 EPF	35G/CTRL	-38.45	0.022
	139G/CTRL	-44.7	0.008		33G/CTRL	-38.45	0.022		35G/102G	34.2	0.042
	143G/CTRL	-36	0.033		93G/CTRL	-34.75	0.039		35G/143G	35.1	0.037
	139G/6G	-38.6	0.022		139G/CTRL	-39.75	0.018		35G/15G	55.3	0.001
	139G/15G	-53.8	0.001		143G/CTRL	-35.35	0.036		17G/15G	52.4	0.002
	30G/6G	-34.9	0.038		30G/15G	54.8	0.001		30G/15G	47.7	0.005
	30G/15G	50.1	0.003		139G/15G	-54.5	0.001		23T/15G	44.55	0.008
	23T/6G	-34.3	0.042		33G/15G	53.2	0.002		33G/15G	42.9	0.011
	23T/15G	49.5	0.003		143G/15G	-50.1	0.003		93G/15G	38.4	0.023
	143G/15G	-45.1	0.007		93G/15G	49.5	0.003				
	93G/15G	41.3	0.014		23T/15G	48.45	0.004				

33G/15G	40.3	0.017	35G/15G	46.7	0.006
35G/15G	37.2	0.027	17G/15G	42.7	0.011
22G/15G	34.5	0.041	22G/15G	34.75	0.039

Appendix Table 3.7. Summary of feeding activity, weight change, and virulence metrics for *Hylobius abietis* exposed to EPF treatments: The table presents mean bark consumption (area consumed, mm²) at 4, 7, and 14 days (T = 4, 7, 14), mean daily consumption (area/day), total weight change, and weight change per day for adult weevils exposed to (i) twelve EPF isolates at full dose (10^8 conidia ml^{-1}; April 2022) and (ii) the two fastest-acting isolates (17G and 35G) at medium (10^7 conidia ml^{-1}, "med") and low (10^6 conidia ml^{-1}, "low") concentrations (November 2022). Pearson's correlation coefficients (r) and associated two-tailed significance values (p-values) are reported to assess relationships between feeding activity or weight change variables and LT_{50} across treatments. Correlation strength is interpreted as weak ($|r| < 0.3$), moderate ($0.3 \leq |r| < 0.7$), and strong ($|r| \geq 0.7$). Statistically significant correlations are indicated by an asterisk (*) where $p < 0.05$. Treatment labels correspond to EPF isolates described in Table 3.1. Median lethal time (LT_{50}, days) is included as a measure of virulence for each treatment.

EPF	Area (mm²) T=4	Area (mm²) T=7	Area (mm²) T=14	Area/day (mm²)	Weight change (g)	Weight change/day (g)	LT50
6G	186.6742	252.4507	350.0549	27.91437	0.00819	0.000764	13
15G	262.4034	342.4332	448.5599	37.02597	0.00267	0.000136	18
17G	124.6657	165.6966	191.2077	20.86385	0.00664	0.000939	10
22G	121.062	187.4243	285.7726	22.63469	0.005	0.000421	18
23T	108.939	180.5595	242.74	24.1288	0.0065	0.000721	11
30G	88.9534	134.7717	210.253	19.79037	0.00741	0.000677	12
33G	97.0758	135.2949	229.3447	18.06889	0.00363	0.000269	17
35G	113.6664	158.1119	183.1018	20.87167	0.00515	0.000569	9
93G	107.7096	148.981	250.4346	20.58196	0.00808	0.000744	
102G	166.337	226.9836	403.3046	29.45316	0.00775	0.000613	19
139G	83.7784	148.4317	313.4036	22.38597	0.0054	0.000386	
143G	94.6463	142.4422	329.8806	25.36843	0.00924	0.00067	
Control	248.7053	341.6187	408.0618	29.79523	0.00588	0.000465	21
Pearsons	0.596	0.616	0.776	0.534	-0.297	-0.649	
Significance	0.069	0.058	0.008*	0.112	0.405	0.042*	
17G med	162.9637	303.7066	486.8891	40.969	0.00012	-0.0002	12
35G med	200.6779	329.6928	405.9393	44.00543	-0.00154	-0.00041	9
17G low	197.5568	380.0981	605.2921	49.34048	0.00498	0.000386	16
35G low	229.3362	464.1046	881.3304	66.78741	0.01091	0.000795	20
Control	217.1198	497.9739	1091.791	80.47555	0.00702	0.000372	
Pearsons	0.626	0.906	0.972	0.888	0.987	0.994	
Significance	0.374	0.094	0.028*	0.112	0.013*	0.006*	

Appendix Table S4.1: Broad taxonomic composition of non-target invertebrates recorded from emergence traps in the Scotland field trial (2022): Counts represent the number of individuals collected per trap, organised by block (1–10) and treatment: entomopathogenic nematodes (Nematode; *Steinernema carpocapsae*), entomopathogenic fungi (Fungi; *Beauveria bassiana* 35G), combined treatment (Mixed; EPF + EPN), and untreated control (Control). Taxa are grouped at a broad taxonomic level (typically order or higher), including Coleoptera, Hymenoptera, Diptera, Myriapoda, Lepidoptera, Arachnida, Auchenorrhyncha, Opilionidae, and Collembola. "Total Abundance" represents the sum of all individuals recorded per trap across all taxa. These data were used to calculate total abundance and broad taxon richness (Figure 4.2), and to construct multivariate community composition analyses (PCoA and MRPP; Figure 4.3, Table 4.7).

	Coleoptera	Hymenoptera	Diptera	Myriapoda	Lepidoptera	Arachnida	Auchenorrhyncha	Opilionidae	Collembola	Total Abundance
1 Nematode	3	0	1	0	0	0	0	0	0	4
1 Fungi	1	0	2	0	0	0	1	0	0	4
1 Mixed	0	0	6	0	0	1	0	3	0	10
1 Control	1	3	1	0	0	1	0	0	0	6
2 Nematode	5	3	4	0	0	0	0	0	0	12
2 Fungi	2	0	0	0	0	0	1	0	0	3
2 Mixed	31	2	1	0	0	1	0	3	0	38
2 Control	1	1	0	0	0	0	0	0	0	2
3 Nematode	7	0	1	0	0	0	0	2	0	10
3 Fungi	2	1	1	0	0	0	0	0	0	4
3 Mixed	1	4	2	0	0	0	0	0	0	7
3 Control	2	1	0	0	0	1	0	0	0	4
4 Nematode	1	1	4	0	0	0	0	1	0	7
4 Fungi	0	0	0	0	0	0	1	0	0	1
4 Mixed	0	0	3	0	0	0	0	0	0	3
4 Control	5	0	1	0	0	0	0	0	0	6
5 Nematode	4	0	1	0	0	1	1	0	1	8
5 Fungi	6	2	0	0	0	0	0	1	0	9
5 Mixed	3	2	5	0	1	0	0	0	1	12
5 Control	12	1	2	0	0	2	0	6	0	23
6 Nematode	4	1	3	0	0	0	0	1	0	9

6 Fungi	2	1	0	0	0	0	0	0	0	3
6 Mixed	20	0	6	0	0	1	0	0	3	30
6 Control	2	3	1	0	0	0	0	0	0	6
7 Nematode	1	0	1	0	0	0	0	1	0	3
7 Fungi	0	1	2	0	0	1	0	1	0	5
7 Mixed	4	0	0	0	0	0	0	3	0	7
7 Control	12	1	7	0	0	0	0	2	1	23
8 Nematode	1	2	1	1	0	0	0	0	0	5
8 Fungi	0	3	1	0	0	1	1	6	0	12
8 Mixed	2	0	1	0	0	1	0	0	0	4
8 Control	1	1	5	0	0	1	0	0	0	8
9 Nematode	11	0	5	0	0	1	0	0	2	19
9 Fungi	7	1	1	0	0	0	0	0	0	9
9 Mixed	2	0	1	0	0	0	0	0	0	3
9 Control	3	0	0	0	0	0	0	4	0	7
10 Nematode	0	0	3	0	0	0	0	1	0	4
10 Fungi	1	0	6	0	0	1	0	0	0	8
10 Mixed	1	0	2	0	0	0	0	0	0	3
10 Control	4	0	3	0	0	0	0	1	0	8

Appendix Table S4.2 Narrow taxonomic composition of non-target invertebrates recorded from emergence traps in the Scotland field trial (2022): Counts represent the number of individuals collected per trap, organised by block (1–10) and treatment: entomopathogenic nematodes (Nematode; *Steinernema*

carpocapsae), entomopathogenic fungi (Fungi; *Beauveria bassiana* 35G), combined treatment (Mixed; EPF + EPN), and untreated control (Control). Taxa are identified at a finer resolution than in Appendix Table S4.1. Diptera are resolved to family level (e.g. Stratiomyidae, Rhagionidae, Mycetophilidae, Ceratopogonidae, Tipulidae, Tachinidae), while ground beetles (Coleoptera: Carabidae) are identified to species level (e.g. *Patrobus atrofus*, *Pterostichus niger*, *Carabus problematicus*, *Nebria brevicollis*, *Trechus quadristriatus*). Remaining taxa are presented at the lowest practical taxonomic resolution achieved during identification."Total abundance" represents the sum of all individuals recorded per trap across all taxa. These data were used to calculate narrow taxon richness (Figure 4.2) and to perform multivariate community composition analyses (PCoA and MRPP; Figure 4.3, Table 4.7).

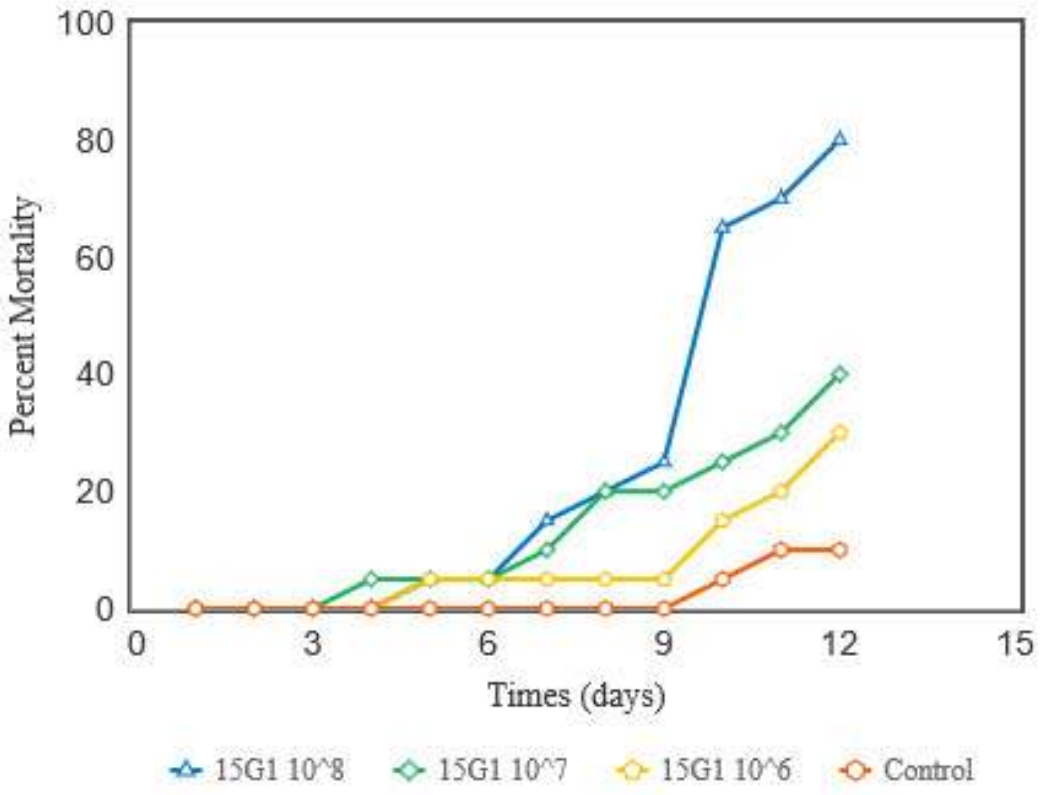
	Stratiomyidae	**Rhagionidae**	**Mycetophilidae**	**Periscelididae**	**Syrphidea**	**Ceratopogonidae**	**Lauxaniidae**	**Dolichopodidae**	**Bibionidae**	**Tipulidae**	**Tachinidae**	**Patrobus atrofus**	**Bradycellus harpalinus**	**Notiophilus biguttatus**	**Pterostichus niger**	**Carabus problematicus**	**Nebria brevicollis**	**Trechus quadristriatus**	**Trichoceridae**	**Pterostichus madidus**	**Carabus glabratus**	**Total abundance**
1 Nematode	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	4
1 Fungi	0	0	0	0	0	0	0	0	1	1	0	0	0	0	1	0	0	0	0	0	3
1 Mixed	0	0	1	0	1	2	0	0	0	1	1	0	0	0	0	0	0	0	0	0	6
1 Control	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	2
2 Nematode	0	1	0	0	0	3	0	0	0	0	0	0	0	0	1	0	1	0	3	0	9
2 Fungi	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	2
2 Mixed	0	0	1	0	0	0	0	0	0	0	0	0	0	0	2	1	0	0	0	0	4
2 Control	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3 Nematode	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	2

3 Fungi	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	2
3 Mixed	0	0	0	0	1	0	0	0	0	1	0	0	0	0	2	0	0	0	1	1	0	6
3 Control	1	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3
4 Nematode	0	0	1	1	0	1	0	0	0	0	0	0	0	0	1	1	0	0	0	1	0	6
4 Fungi	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1
4 Mixed	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	3
4 Control	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1
5 Nematode	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	2	0	4
5 Fungi	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	3
5 Mixed	0	2	0	0	0	1	0	0	0	3	1	0	0	0	0	0	1	0	0	0	0	8
5 Control	0	1	0	0	0	0	0	0	1	0	0	0	0	9	0	0	0	0	0	1	0	12
6 Nematode	0	0	0	0	0	1	0	0	1	0	0	1	0	1	0	0	0	0	1	1	0	6
6 Fungi	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	3
6 Mixed	0	2	0	0	1	3	0	0	0	0	0	0	1	0	1	0	0	2	0	1	0	11
6 Control	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	3
7 Nematode	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1
7 Fungi	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	2
7 Mixed	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	2	0	0	1	0	4
7 Control	0	0	0	3	1	2	0	0	0	0	0	0	0	0	3	0	1	0	1	4	0	15
8 Nematode	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1
8 Fungi	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
8 Mixed	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	3
8 Control	0	0	0	0	0	2	2	0	0	0	0	0	0	0	0	0	0	0	1	0	0	5
9 Nematode	0	0	0	0	3	1	1	0	0	0	0	0	0	4	0	0	1	0	0	2	0	12
9 Fungi	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	3	0	6
9 Mixed	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	3
9 Control	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	2
10 Nematode	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	3
10 Fungi	0	0	0	0	4	1	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	7
10 Mixed	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	3
10 Control	0	0	0	0	1	1	0	1	0	0	0	0	0	0	0	3	0	0	0	0	0	6

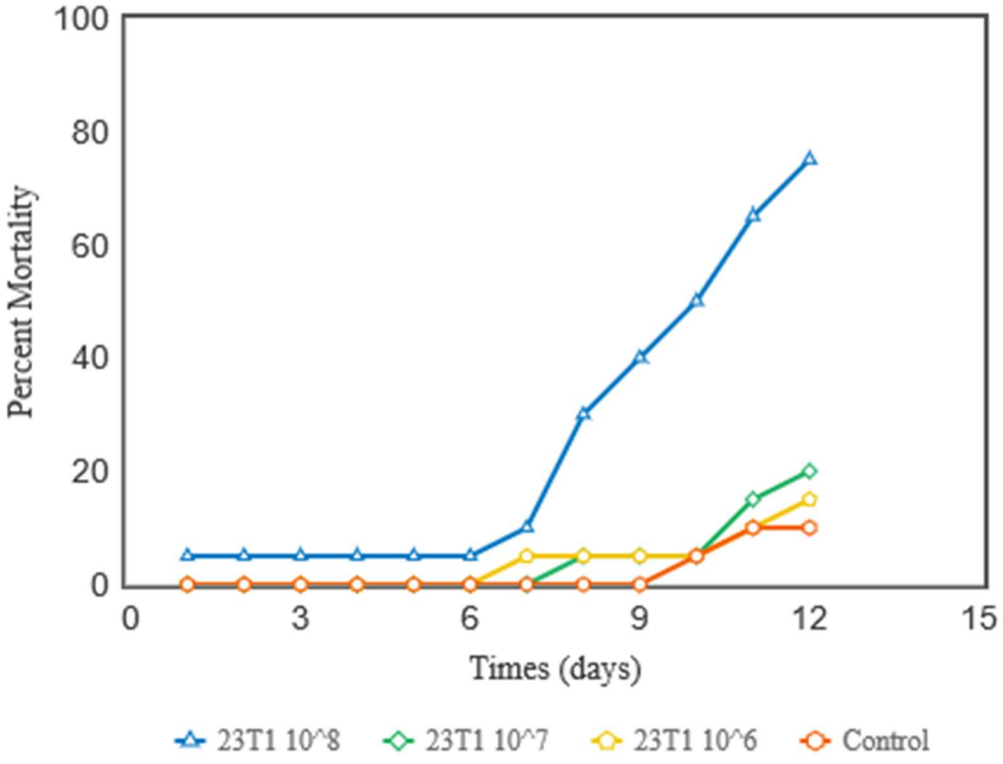
Appendix Table S5.1. Mean and median survival metrics of four selected entomopathogenic fungal (EPF) isolates obtained during the 2020 bioprospecting survey, tested against adult *Hylobius abietis* at three concentrations (10^8 [Full], 10^7 [Med], and 10^6 [Low] conidia ml^{-1}) in June 2021: For each treatment, values are presented as the number of insects tested (n), restricted mean survival time (days ± SE), 95% confidence interval (C.I.), and age in days at specified mortality percentiles (25%, 50%, 75%, 90%, and 100%). Median survival time (LT50) and its 95% C.I. (fiducial limits) are also reported. Control insects were handled identically but were not exposed to fungal inoculum. Survival was monitored daily for 12 days following inoculation under controlled laboratory conditions. These assays were conducted on a subset of four EPF isolates selected from the original 12 strains screened in early 2021 (Appendix Table 3.1), due to loss of viability and contamination in long-term cultures. The results informed the selection of *Metarhizium* sp. 15G for the first field trial in Wales (2021), representing the most effective isolate available at the time of application.

		Restricted Mean			Age in days at % mortality					
Name	**No. of subjects**	**Days**	**S.E.**	**95% C.I.**	**25%**	**50%**	**75%**	**90%**	**100%**	**95% Median C.I.**
15G 10^8	20	9.95	0.43	9.11 ~ 10.79	9	10	12	-	-	10.0 ~ 11.0
15G 10^7	20	10.80	0.49	9.83 ~ 11.77	10	-	-	-	-	11.0 ~ 12.0
15G 10^6	20	11.40	0.36	10.70 ~ 12.10	12	-	-	-	-	12.0 ~ 12.0
23T1 10^8	20	9.75	0.59	8.60 ~ 10.90	8	11	12	-	-	8.0 ~ 11.0
23T1 10^7	20	11.70	0.20	11.31 ~ 12.09	-	-	-	-	-	- ~ -
23T1 10^6	20	11.70	0.25	11.22 ~ 12.18	-	-	-	-	-	- ~ -
30G4 10^8	20	10.70	0.39	9.94 ~ 11.46	8	11	-	-	-	11.0 ~ 12.0
30G4 10^7	20	10.70	0.45	9.82 ~ 11.58	9	-	-	-	-	9.0 ~ 12.0
30G4 10^6	20	11.60	0.16	11.28 ~ 11.92	11	-	-	-	-	12.0 ~ 12.0
143G2 10^8	20	10.20	0.60	9.02 ~ 11.38	8	11	-	-	-	9.0 ~ 12.0
143G2 10^7	20	11.10	0.37	10.38 ~ 11.82	10	-	-	-	-	12.0 ~ 12.0
143G2 10^6	20	11.65	0.16	11.33 ~ 11.97	11	-	-	-	-	12.0 ~ 12.0
Control	20	11.85	0.11	11.64 ~ 12.06	-	-	-	-	-	- ~ -

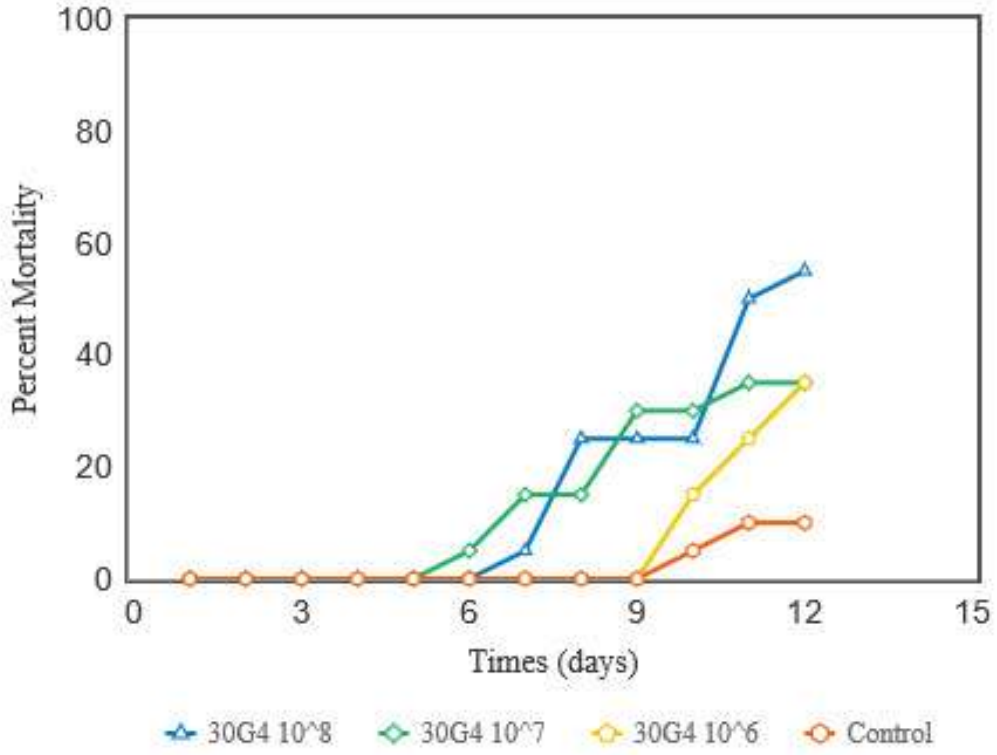
(A)


100
80
60
40
20
0
Percent Mortality
0
3
6
9
12
15
Times (days)
15G1 10^8
15G1 10^7
15G1 10^6
Control

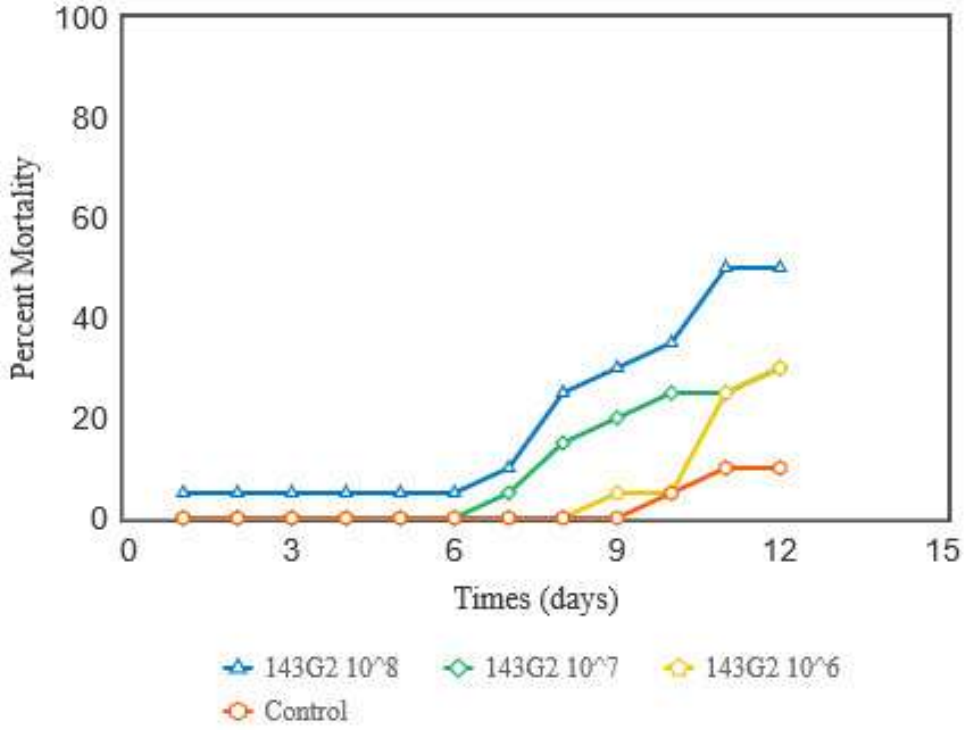
(B)


100
80
60
40
20
0
Percent Mortality
0
3
6
9
12
15
Times (days)
23T1 10^8
23T1 10^7
23T1 10^6
Control

(C)



(D)



Appendix Figure S5.1. Cumulative mortality curves of adult *Hylobius abietis* exposed to four selected entomopathogenic fungal (EPF) isolates obtained from the 2020 bioprospecting survey, tested at three concentrations: full (10^8 conidia ml^{-1}), medium (10^7 conidia ml^{-1}), and low (10^6 conidia ml^{-1}). The figure is presented as four panels (A–D), each corresponding to one EPF isolate. Within each panel, survival curves for the three concentrations are shown alongside the untreated control. Mortality was recorded daily over

a 12-day period following inoculation under laboratory conditions. These assays were conducted in June 2021 on a reduced subset of isolates due to the loss of viability and contamination of several original cultures obtained in late 2020. The isolate 15G was selected for the 2021 field trial based on its relative performance among the viable candidates available at the time, rather than its ranking in the full screening dataset presented in *Chapter 3*. Steeper curves indicate higher virulence and faster host mortality.