Molecular analysis of subterranean detritivore food webs

A thesis presented for the degree of Doctor of Philosophy

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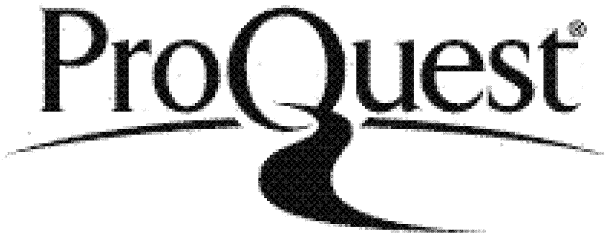

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Abstract

Insect- and mollusc-parasitic nematodes are widely used for the biological control of soil-dwelling pests. Despite being used successfully to control a broad range of pest species in a variety of different crops, there have been many examples of pest control failures. Although the abiotic factors that influence the short-term persistence of nematodes are well understood, there is a lack of knowledge about the biotic factors.

PCR-probes were designed and tested for the detection of three species of mollusc- and insect-parasitic nematodes: *Phasmarhabditis hermaphrodita*, *Heterorhabditis megidis* and *Steinernema feltiae*. These probes were used successfully to amplify nematode DNA from the gut of microarthropod predators. Potential sources of error and variation in prey detection times were tested and quantified, including nematode phoresy, scavenging, changes in temperature and predator body size. Field trials and molecular screening were used to identify predators of nematodes in the field, including the surface-dwelling collembola *Isotoma viridis* and *Isotomurus palustris*. Positive numerical responses by the surface-dwelling collembola indicated that immigration into nematode-sprayed (and hence food-rich) habitats was occurring.

Terminal Restriction Fragment Length Polymorphism (T-RFLP) was tested for monitoring the effects of nematode biopesticide application on soil-dwelling nematode abundance and community structure. Using this technique, it was possible to create a nematode 'community profile'. Furthermore, it was possible, using T-RFLP, to identify changes in the nematode community after the application *P. hermaphrodita* to the soil.

The nematode-specific PCR primers were used in conjunction with primers designed to amplify DNA from mollusc and insect hosts, including *Deroceras reticulatum*, *Tenebrio molitor* and *Steinernema feltiae*. Using these primers, it was possible to detect nematode parasitism within the host and intraguild predation by the carabid *Pterostichus melanarius* on infected hosts. Choice-test feeding trials with *P. melanarius* were used to investigate the production of antifeedants by the nematode-bacterium complex within the host.

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Publications

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Harper GL, Sheppard SK, Harwood JD, Read DS, Glen DM, Bruford MW, Symondson WOCS (2006) Evaluation of temperature gradient gel electrophoresis for the analysis of prey DNA within the guts of invertebrate predators. *Bulletin of Entomological Research* **96**, 295-304.

King RA, Read DS, Traugott M, Symondson WOC (2007) Molecular analysis of predation: a review of best practice for DNA-based approaches. *Molecular Ecology* (*In Press*).

Read DS, Glen DM, Bruford MW, Symondson WOC (2005) Molecular detection of the nematode *Phasmarhabditis hermaphrodita* within slugs and predators of slugs. IBOC-WPRS Bulletin.

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Chapter 1

Introduction

1. Introduction

This thesis describes an investigation into the interactions of parasitic nematode biological control agents within the soil food-web. This introduction provides a summary of information about soil-dwelling nematodes and in particular the life-cycle and use of mollusc- and insect-parasitic nematodes from the families Heterorhabditidae, Steinernematidae and Phasmarhabditidae for the biological control of soil-dwelling pests. This is followed by a description of the issues associated with the poor persistence of introduced nematodes in the soil ecosystem and how this affects the efficacy of pest control. The known interactions of parasitic nematodes with other soil-dwelling biota are summarised and the literature is reviewed for known biotic antagonists of nematodes in the soil and in their hosts. The issues associated with describing trophic links in below-ground food-webs are discussed and the techniques that have been used in the past to determine these links are reviewed. The use of DNA techniques for the detection of predation, parasitism and the presence of nematodes in substrates are then summarised. This is followed by a description of nematode genetics, focussing on the diversity of nematodes from the families Heterorhabditidae, Steinernematidae and Phasmarhabditidae. An appraisal of the most appropriate target genes for designing species and group-specific probes is discussed. Finally, the aims, rationale and objectives of the study are outlined.

1.1. Soil-dwelling nematodes

One of the most numerous and diverse groups of soil-dwelling organisms are the nematodes. Estimates of the number of species range from 400,000 to 10 million (Hammond *et al.* 1995), making them the most numerous multicellular organisms found on earth. Soils and sediments in particular are home to large numbers of nematodes in mixed communities of great diversity; farmland topsoil can contain millions of nematodes per m^2 (Yeates 1979) with over 3 million per m^2 at some sites (Yeates 2003). Even in temperate soils, nematode species diversity at a single site can be high, with up to 154 species (Hodda and Wanless 1994) and 21 genera (Yeates and Bongers 1999) recorded in an English grassland.

Nematodes within soils are known to exhibit a range of feeding behaviours. Yeates (1993) categorized free-living nematodes in the soil into eight functional groups, comprising the herbivores (plant feeders), fungal feeders, bacterial feeders, substrate feeders (detritivores), predators, algal feeders, omnivores and the dispersal stages of parasites. This diverse range of feeding habits and the high abundance found in soils around the world, means that nematodes play an important role in many soil processes: including nutrient cycling and mineralization (Anderson *et al.* 1983), grazing bacteria and fungi (Bakhtiar *et al.* 2001; De Mesel *et al.* 2004), decomposition of organic material (Ingham *et al.* 1985) and plant growth (Ferris *et al.* 2004). In some ecosystems, up to 40 % of nutrient mineralisation may be due to nematodes and other microbial organisms as they feed on bacteria (De Ruiter *et al.* 1993).

1.2. Entomopathogenic and mollusc parasitic nematodes

Parasitic nematodes display a wide range of life-cycles and life-histories, parasitizing almost all groups of animals and plants and occur in almost all marine, terrestrial and freshwater habitats (Blouin *et al.* 1999). Entomopathogenic and mollusc-parasitic nematodes, in the families Steinernematidae, Heterorhabditidae and Rhabditidae, are widely used for the biological control of soil-dwelling pests. These nematodes are ideally suited for biological control in that they possess the attributes of predators, parasites and microbial pathogens (Kaya and Gaugler 1993). They have the ability to find hosts within the soil matrix, following chemical and vibrational cues (O'Halloran and Burnell 2003), they kill the host quickly (Smart 1995), inhibit feeding (Glen *et al.* 2000) and can recycle within the environment causing lasting pest suppression (Smits 1996). These nematodes have also proven to be safe towards vertebrates (Bathon 1996), can be applied with existing spraying equipment (Koppenhofer and Kaya 2001) and can be applied in conjunction with many existing chemical pesticides (Barbara and Buss 2005).

The diversity of invertebrates that can be infected by nematodes in the laboratory has been shown to be considerably higher than the diversity that can be successfully controlled in the field (Bathon 1996). Despite this, parasitic nematodes have been used to control a range of pests, including lepidopterans such as winter moths (*Operophtera brumata*) (see Tomalak 2003), codling moths (*Cydia pomonella*) (see Lacey *et al.* 2006)

and oriental fruit moths (*Grapholita molesta*) (see Riga *et al.* 2006). Pest Coleoptera controlled with nematodes include; flour beetles (*Tribolium castaneum*) (see Ramos-Rodriguez *et al.* 2007), black vine weevils (*Otiorhynchus sulcatus*) (see Lola-Luz and Downes 2007) and Japanese beetles (*Popillia japonica*) (see Mannion *et al.* 2001). Parasitic nematodes have also been used to control Diptera (Simard *et al.* 2006; Kim *et al.* 2004), Orthoptera (Parkman and Smart 1996) and Mollusca (Wilson *et al.* 1993).

The range of crops in which nematodes have successfully been used as biological control agents is also high, although commercial use tends to be restricted to crops with a high economic value (Georgis *et al.* 2006). Because of this, the most successful use of entomopathogenic nematodes has been in the protection of nursery and greenhouse plants (Georgis *et al.* 2006), although nematodes have also been successfully applied for the control of pests in orchards (Toledo *et al.* 2006), citrus plantations (Gress and Stock 2007), mushroom houses (Fenton *et al.* 2002), turfgrass (Simard *et al.* 2006), forests (Armendariz *et* al. 2002), vegetable (Nielsen 2003) and cereal crops (Hass *et al.* 1999b).

1.3. Parasitic nematode life-cycle

Nematodes from the families Heterorhabditidae and Steinernematidae are obligate parasites of insects (Gaugler *et al.* 1997). Although not closely related, nematodes from both families have very similar life-cycles, due to convergent evolution (Poinar 1993). Therefore, the life-cycles of both heterorhabditid and steinernematid nematodes are described together in the following section.

For the nematodes in the families Heterorhabditidae and Steinernematidae, only one stage of the life-cycle is not totally dependent upon the host organism for survival (Koppenhofer and Kaya 2001). This stage is described as the third-stage infective juvenile (IJ) (Figure 1.3.1). The IJs are completely non-feeding, relying upon lipid food reserves for nourishment (Mracek *et al* 1999). In this stage they can survive for weeks whilst active, depending upon the quality of the host they emerged from (Smart 1995). They are also able to survive for months in a near-anhydrobiotic state (Smart 1995), although as this stage is non-feeding and the food reserves are limited, a host needs to be found for continued survival. The first stage in the infection process is host searching (Kaya and Gaugler 1993) which begins with habitat selection. Species such as

Steinernema carpocapsae are adapted to search for hosts near the soil surface (Ropek and Jaworska 1994), whereas *Heterorhabditis bacteriophora* is adapted for searching deep within the soil profile (de Doucet *et al.* 1999).

Each species of parasitic nematode utilises one of two methods of host location; ambush or cruise foraging. Ambush foragers utilise a strategy known as 'nictating' (Grewal *et al.* 1994; Campbell and Gaugler 1997). This involves standing vertically near the soil surface on their tails. When a potential host organism passes by, the nematode will attach itself to the surface of the host (Lewis *et al.* 1992). Nematode species known to utilise this technique include *Steinernema feltiae*, *S. scapterisci* and *S. carpocapsae*. Cruise foraging nematodes are highly mobile and utilise the film of water found on the surface of soil particles for mobility (Kaya and Gaugler 1993). They respond to long range volatiles to locate and identify suitable host organisms (Lewis *et al.* 1992; Grewal *et al.* 1994). Carbon dioxide (CO_2) has been shown to elicit a host finding response in nematodes in combination with chemical cues produced by insect larvae (Boff *et al.* 2001). Nematodes known to fall into this category include *S. glaseri* and *H. bacteriophora*.

Once the host has been found, the nematodes invade through the natural openings in the host organism, usually the mouth, anus, or spiracles (Dowds and Peters 2002). Direct penetration through weak areas of the cuticle is also possible with some species of heterorhabditid nematodes (Smart 1995). If nematode entry into the host is through the mouth or anus, it will then proceed to penetrate through the host gut wall (Dowds and Peters 2002). When the nematode IJs reach the haemocoel of the host, symbiotic bacteria are released from the nematode gut, which soon multiply. The host is killed by toxins that are released by the developing bacteria. These toxins are not well understood, but it is thought that an array of different toxins is released, with varying levels of virulence towards the host. The toxin-complexes contain toxins with both injectable and oral activity on the insect host (Ffrench-constant *et al.* 2007).

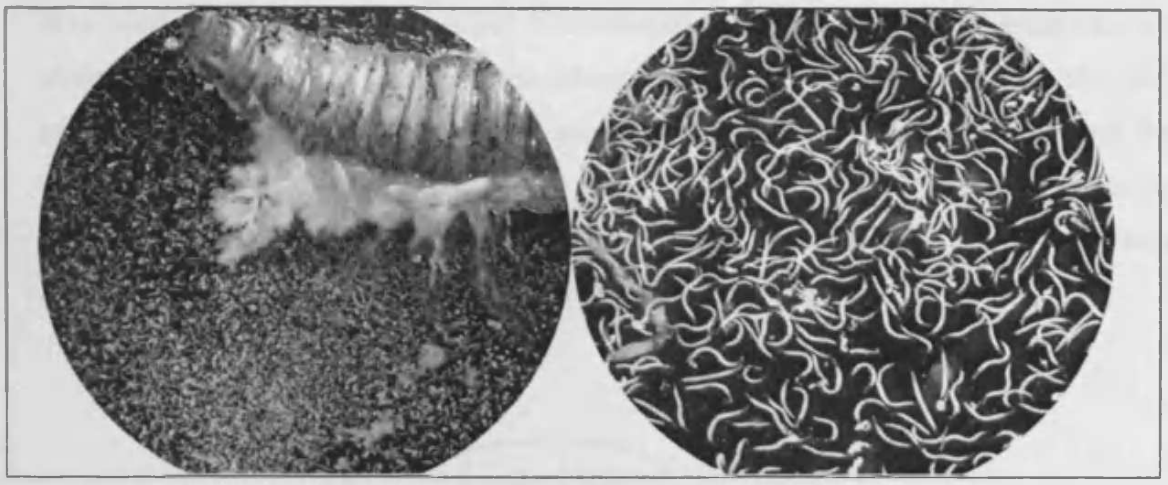


Figure 1.3.1 Larvae of the lepidopteran *Galleria mellonella* infected with the entomopathogenic nematode *Heterorhabditis megidis* (L). Close-up of *H. megidis* infective juveniles (IJs) after emerging from the host (R).

Each entomopathogenic nematode species is associated with just one bacterial symbiont, although one species of bacteria may be associated with more than one species of nematode (Koppenhofer and Kaya 2001). The nematode-bacterium association has been shown to work on a number of different levels, with each organism deriving different benefits. The nematode relies upon the bacteria for rapidly killing the host, creating a suitable environment for the nematode's development and reproduction, and transforming the host tissue into a useable food source (Akhurst and Boemare 1990). The bacteria can also produce chemical inhibitors that protect the cadaver from colonisation by other microorganisms (Walsh and Webster 2003) and may protect it from scavenging by some invertebrates by producing antifeedant chemicals (Baur *et al.* 1998; Zhou *et al.* 2002). The bacterium gains benefit from the association with the nematode in the form of protection from the external environment, penetration into the hosts haemocoel and inhibition of the hosts antibacterial proteins (Ankhurst and Boemare 1990).

The IJs then moult into the fourth stage and feed upon the symbiotic bacteria and other metabolic by-products (Smart 1995). The fourth-stage juveniles then moult into first-stage adults. First stage steinernematid adults are males and females, whereas first stage heterorhabditid adults are hermaphrodites (Adams and Nguyen 2002). If the conditions are right, such as plentiful food resources, the life-cycle will repeat again from first-stage juveniles to second-stage adults within the host cadaver, but with the production

of non-infective third-stage juveniles. Second-stage adult heterorhabditid nematodes are always male and female and not hermaphroditic (Adams and Nguyen 2002). The life-cycle takes approximately 10-14 days depending upon the nematode species and the condition of the host cadaver. After this point, the host cuticle breaks down, releasing the new generation of IJs back into the soil, from where the life-cycle can be completed again (complete life-cycle shown in Figure 1.3.2.).

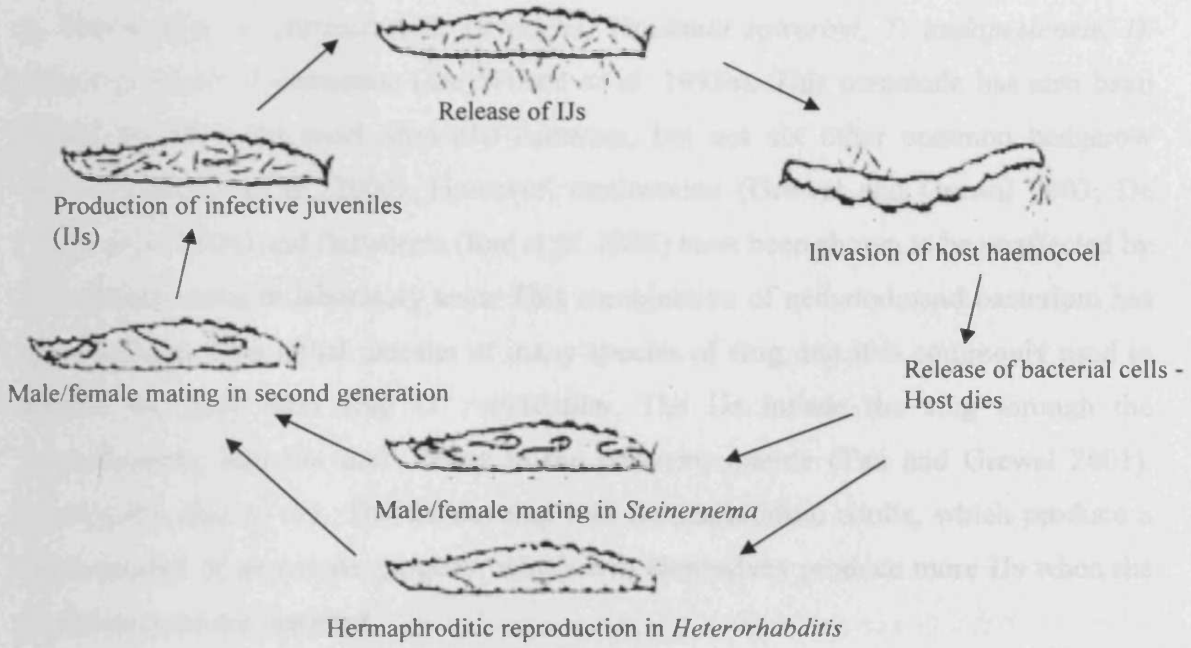


Figure 1.3.2 Complete life-cycle of heterorhabditid and steinernematid nematodes (Modified from Weeden *et al.* 2007).

The life-cycle of phasmarhabditid nematodes is remarkably similar to that of the unrelated entomopathogenic nematodes. *Phasmarhabditid hermaphrodita* is the only nematode parasite of slugs that has been developed as a biological control agent. As with heterorhabditid and steinernematid nematodes, the Phasmarhabditidae are bacterial feeders that only have one free-living stage in the life-cycle which are not dependent on the host organism for survival, the non-feeding third-stage larvae. The infective juvenile (IJ) develops when inhospitable conditions arise and is enclosed by the cuticle of the second-stage larvae, allowing a higher resistance to desiccation and UV light. The IJs of *P. hermaphrodita* have been found to carry up to five bacterial types (Wilson *et al.*

1995b). However, commercially produced *P. hermaphrodita* are reared on a monoculture of *Moraxella osloensis*. As with the bacteria associated with the entomopathogens, the associated bacteria of *P. hermaphrodita* produce a toxin responsible for host death (Tan and Grewal 2002).

P. hermaphrodita has been found to be pathogenic to a wide range of molluscs, including the slugs *Deroceras leave* and *Leidyula floridana* (see Grewal *et al.* 2003), young *Arion lusitanicus* (see Speiser *et al.* 2001; Grimm 2002), *A. ater*, *A. intermedius*, *A. intermedius*, *A. distinctus*, *A. silvaticus*, *Tandonia sowerbyi*, *T. budapestensis*, *D. reticulatum* and *D. caruanae* (see Wilson *et al.* 1993a). This nematode has also been shown to infect the snail *Monacha cantiana*, but not six other common hedgerow species (Wilson *et al.* 2000). However, earthworms (Grewal and Grewal 2003; De Nardo *et al.* 2004) and flatworms (Rae *et al.* 2005) have been shown to be unaffected by *P. hermaphrodita* in laboratory tests. This combination of nematode and bacterium has been found to be a lethal parasite of many species of slug, but it is commonly used to control the grey field slug *D. reticulatum*. The IJs invade the slug through the pneumostome, into the shell cavity in the posterior mantle (Tan and Grewal 2001), causing the slug to die. The IJs develop into hermaphroditic adults, which produce a large number of nematode progeny, which will themselves produce more IJs when the food resources are depleted.

P. hermaphrodita has been tested in a range of different crops, including lettuce (Wilson *et al.* 1995a; Iglesias *et al.* 2001a), winter wheat (Wilson *et al.* 1994, 1996; Hass *et al.* 1999), oilseed rape (Wilson *et al.* 1995b) and asparagus (Ester *et al.* 2003). Although application of nematodes may take up to seven days to kill the slugs, feeding inhibition caused by infection provides rapid protection of the crop (Wilson *et al.* 1994).

1.4. Persistence of parasitic nematodes in the field

The ability of nematodes to recycle in the environment is important for the persistence of nematode populations. This is intrinsically linked with pest control in soils, as the post-application persistence of parasitic nematodes is thought to be one of the key factors affecting nematode efficacy against soil-dwelling pests (Smits 1996; Strong 2002). Baur and Kaya (2001) compiled the data from a number of studies that examined

the persistence of entomopathogenic nematodes in soil in the absence of hosts and calculated the persistence half-lives for each study (i.e. the time for 50% of the original inoculum to remain). The half-lives for *Steinernema* species ranged from 2 to 236 days and for *Heterorhabditis* species it was 2 to 40 days. The average persistence half-life for steinernematid nematodes is approximately 60 days, compared to approximately 34 days for heterorhabditid nematodes (Strong 2002).

Increased persistence of nematode IJs in the soil is thought to increase the 'window of opportunity' in which pest control can occur (Baur and Kaya 2001). The persistence of parasitic nematodes can be divided into two distinct processes: (1) the persistence of free-living nematode stages in the soil and (2) the ability of nematodes to recycle in their hosts. These processes, although linked, are intrinsically different and subject to different factors. These processes play different roles at different stages of nematode application. Immediately after inundation, the persistence of IJs in the soil is the most important factor, as more than 80 % of the introduced IJs can perish within a few hours of application and after this, losses of 5-10 % per day can occur (Smits 1996). After the initial reduction, nematode numbers will reach a baseline in the soil, from which the population will then fluctuate for an undefined period of time due to host infection and subsequent increase in number in the soil (albeit to a lower level than the initial application) (see Figure 1.4.1). This persistence will either continue indefinitely, or the introduced nematode population will decline to a point at which it reaches local extinction.

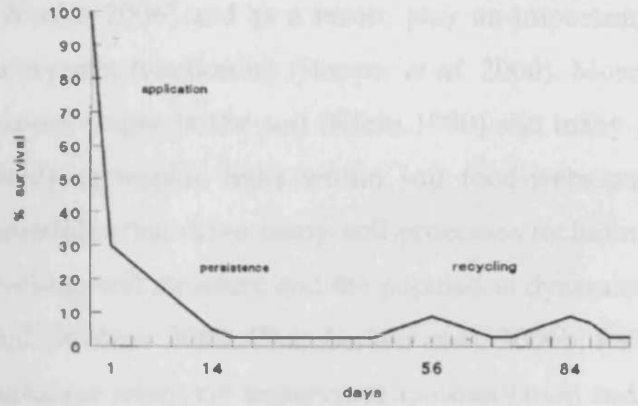


Figure 1.4.1 The short and long-term persistence of nematode infective juveniles (IJs) in the soil after an inundative application for the control of soil dwelling pests (From Smits 1996).

A number of abiotic factors have been shown to influence nematode persistence in soil, including soil structure (Koppenhofer and Fuzy 2006), temperature (Saunders and Webster 1999), soil moisture (Grant and Villani 2003; Koppenhofer and Fuzy 2007), UV light (Nickle and Shapiro 1994) and oxygen availability (Baur and Kaya 2001). In an attempt to determine the factors limiting the short-term persistence of entomopathogenic nematodes, Wilson and Gaugler (2004) conducted field experiments with the entomopathogenic nematode *H. bacteriophora*. The persistence of *H. bacteriophora* in plots was compared when the nematodes were applied to either the turf surface or sub-surface. No difference in persistence was observed between application methods. However, in the sub-surface plots, a negative correlation between the number of mites and collembola and the persistence of the nematodes was observed. This indicates that predation by these microarthropods may constitute a significant mortality factor in this system. Despite the evidence that biotic mortality factors may play an important role in nematode persistence, they have received little attention, probably due to the difficulties in determining trophic interactions in below-ground food-webs.

1.5. Parasitic nematodes in soil food-webs

In contrast to above-ground and aquatic food-webs, the dynamics and structure of below-ground food-webs have received relatively little attention. Below-ground

communities usually support a higher diversity of biota than above-ground ecosystems (Wardle 2006) and as a result, play an important role in maintaining biodiversity and ecosystem functioning (Hooper *et al.* 2000). More than 90 % of insect species have life history stages in the soil (Klein 1990) and many pests attack plant roots (Finch 1993). Studying trophic links within soil food-webs can improve our understanding of the principles that drive many soil-processes including decomposition, energy and nutrient cycling, soil structure and the population dynamics of the organisms that live within the soil (Wolters 2000; Chamberlain *et al.* 2006b; Partsch *et al.* 2006). However, soil food-webs are relatively impervious to observation and, as a result, knowledge about below-ground interaction lags behind the study of above-ground ecology. Because of this, the understanding of the ecology of entomopathogenic nematodes is weak (Strong 2002).

The interactions of both introduced and natural populations of mollusc and entomopathogenic nematodes within the soil food-web (Figure 1.5.1) can be broadly divided up into four categories. The first interaction is between the nematodes and their host organisms. The population dynamics of entomopathogenic nematodes in conjunction with their hosts has been well documented, where natural populations of entomopathogenic nematodes are highly patchy in distribution (Bohan 2000) and where these patches occur they can reduce densities of their hosts (Campbell *et al.* 1995). This interaction has been shown to produce a trophic cascade, in which natural populations of nematodes can suppress insect herbivores to the point at which plant productivity is increased. This has been shown in the field, where the entomopathogenic nematode *H. marelatus* through infection of ghost moth caterpillars, *Hepialus humuli*, reduced herbivory on lupine seedlings (Strong *et al.* 1999). It is this mechanism that is exploited through the introduction and inundation of parasitic nematodes for the control of plant pests.

A second potential link in the soil food-web is through the interaction of infected hosts and host cadavers with invertebrate predators and scavengers. This link has received surprisingly little attention in entomopathogenic nematodes, considering that the ability to recycle in the host organism is vital to the long term persistence of introduced biological control agents (see section 1.6.1). The third link in the soil-food web involving parasitic nematodes is due to predation or parasitism directly on the free-living IJs in the soil. A wide range of organisms are known to feed on, parasitize or

infect nematodes, causing reductions in nematode persistence and increases in the abundance of organisms that benefit from nematophagy (see section 1.6.2).

The fourth and final potential link with the soil food web is the direct interactions with free-living nematodes in the soil. For example, Grewal *et al.* (1997) found that applications of the entomopathogenic nematodes *Steinernema carpocapsae* and *S. riobravis* were capable of reducing the population density of the plant parasitic nematodes, *Meloidogyne* sp., *Belonolaimus longicaudatus* and *Criconemella* sp. A similar result was caused by the application of the nematodes *S. feltiae* and *S. carpocapsae* on the potato cyst nematode *Globodera rostochiensis* (see Perry *et al.* 1998) and after the application of *S. feltiae* on the root knot nematode *Meloidogyne incognita* (see Lewis *et al.* 2001). Jagdale *et al.* (2002) discovered that both live and dead *S. carpocapsae* were capable of reducing numbers of plant-parasitic nematodes, leading to the suggestion that allelochemicals produced by the nematode or the bacterial symbiont were responsible for this effect. Application of two strains of *Heterorhabditis bacteriophora* and one of *H. indica* significantly reduced the abundance, species richness, diversity and maturity of the whole nematode community, although this effect was caused by the reduction of plant-parasitic nematodes only (Somasekhar *et al.* 2002).

Identifying the organisms that link to mollusc and entomopathogenic nematodes in soil food-webs may provide useful information on a number of aspects in the control of soil-dwelling pests. Knowledge of the organisms that act as nematode antagonists may allow for farming practices or nematode application methods that reduce antagonistic interactions and enhance nematode persistence, both in natural and artificially introduced nematode populations.

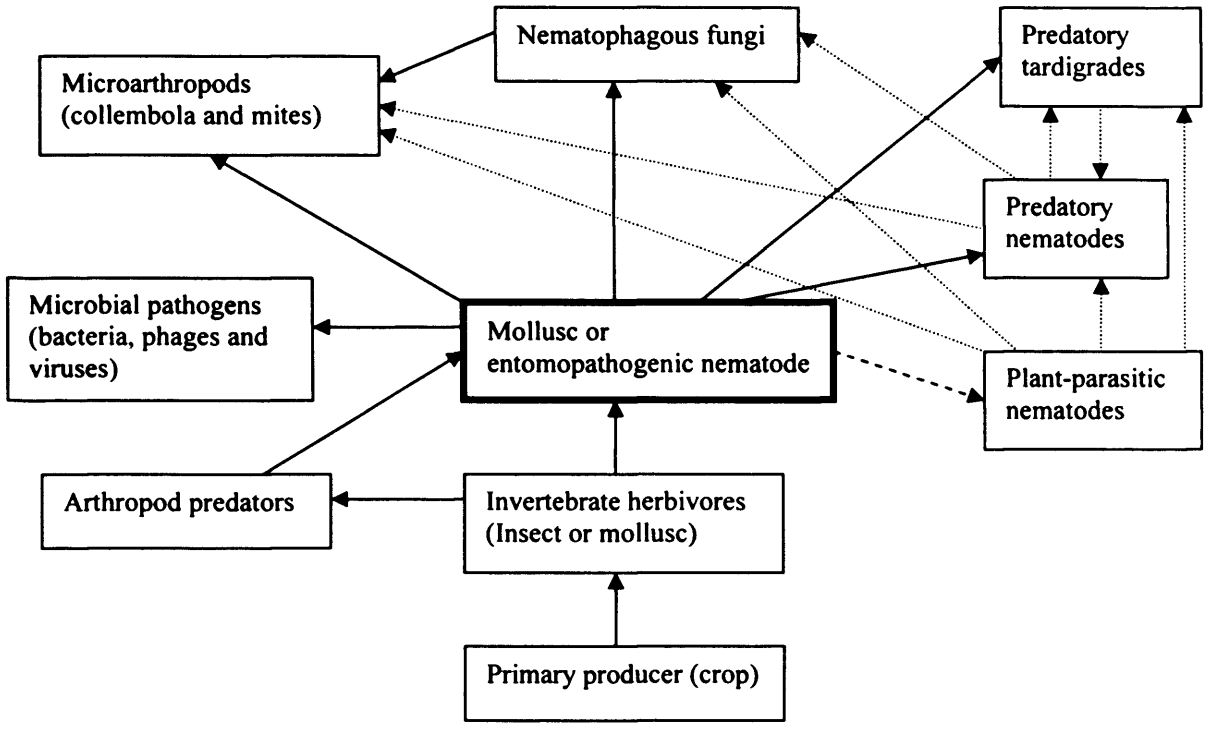


Figure 1.5.1 A simplified food-web illustrating the potential links of mollusc and entomopathogenic nematodes with other biota in the soil food-web, based on known interactions from the literature. Arrows and solid lines represent direction of energy flow. Small dashed lines (....) represent energy flow not directly involving parasitic nematodes. Large dashed line (---) represents possible suppression, although mechanism is unknown.

1.5.1. Antagonists of nematodes within the host

The ability of mollusc- and insect-parasitic nematodes to recycle within the host is of importance for their long-term persistence within the soil. Once a host is infected, it is killed within 2-3 days (Adams and Nguyen 2002) and spends a further 7-12 days (or longer) on or in the soil. After this time, the nematode IJs emerge from the host into the soil. The reduced fitness and defensive abilities of infected hosts and cadavers means that they are vulnerable to predation and scavenging, potentially killing the developing nematodes within the host. Despite the potential importance of this interaction in determining the persistence and population dynamics of parasitic nematodes, little research has been carried out in this area. Baur *et al.* (1998) conducted field experiments, indicating that scavenging ants fed on insects infected with steinernematid

nematodes, but not on hosts infected with heterorhabditid nematodes. However, even in heterorhabditid infected hosts, puncture wounds in the host integument caused desiccation and the death of the nematodes inside. Scavenging has been shown to be widespread amongst invertebrates; Sunderland *et al.* (1987) recorded 48 different species of predator that would scavenge on dead aphids, and Foltan *et al.* (2005) found that the mean length of time before the removal of *D. reticulatum* cadavers in wheat and grass pasture was between 10.5 and 11.7 h. In light of these high levels of scavenging, and natural predation pressure, it has been speculated that intraguild predation by predatory and scavenging organisms may have a significant effect on the population dynamics of parasitic nematodes (Koppenhofer and Kaya 2001).

1.5.2. Antagonists of the free-living infective nematodes in the soil

The survival of entomopathogenic nematode IJs has been found to be higher in sterilised soil than non-sterilized soil (Timper *et al.* 1991), indicating that biotic factors are important in determining persistence. A number of different groups of organisms are capable of reducing nematode numbers within the soil. Obligate bacterial parasites of nematodes have been identified in the genus *Pasteuria* (see Poinar and Jansson 1988) and the bacterium *Pasteuria penetrans* has received considerable attention for the biological control of plant parasitic nematodes (Kariuki and Dickson 2007; Dabire *et al.* 2007). An isolate of this bacterial parasite of nematodes has been recorded from *Steinernema pakistanense* (see Bajaj and Walia 2005), indicating that it may constitute a significant biotic mortality factor of parasitic nematodes in soil. Protozoan parasites have also been found infecting entomopathogenic nematodes; *Pleistopora schubergi* and *Nosema mesnili* have been shown to infect *S. carpocapsae* (see Veremtchuk and Issi 1970), and an unidentified microsporidian has been found to infect *S. glaseri* (see Poinar 1988).

Nematophagous fungi are widespread and found in soils around the world (Kaya and Koppenhofer 1996), with over 100 species capable of trapping nematodes in agricultural soils (Jatala 1986). Two basic types of nematophagous fungi are found: 1) endoparasitic fungi that attach to the host cuticle; and 2) predatory or trapping fungi, that capture nematode prey using adhesive knobs or constricting loops. Trapping fungi have been shown to reduce numbers of entomopathogenic nematodes IJs on agar substrates,

trapping 90 % of *S. feltiae* and *H. hepialus* on sand and between 40 and 60 % on soil in the laboratory (Poinar and Jansson 1988; Koppenhofer *et al.* 1996). An endoparasitic fungus, *Hirsutella rhossiliensis*, responds to nematode density (Jaffee 1992; Jaffee and Strong 2005) and can infect parasitic, entomopathogenic and free-living nematodes (Kaya and Koppenhofer 1996). However, not all entomopathogenic nematodes are equally susceptible to endoparasitic fungi, and the ability of some species to retain and shed the second stage cuticle affords a degree of protection (Koppenhofer and Kaya 2001).

Tardigrades can reach high densities within soils, up to 33,600 per m^2 (Hohberg 2006). Many species of tardigrades are predatory and have been observed feeding on plant parasitic nematodes (Hutchinson and Streu 1960; Sayre 1969). Tardigrades have been shown to reduce nematode numbers in laboratory studies (Hyvonen and Persson 1996) and it has been demonstrated that the predatory tardigrade, *Macrobiotus richtersi*, can consume 4.6 µg of nematode prey in 4 h (Hohberg and Traunsperger 2005). When this data is applied to the field, it has been calculated that a natural population of *M. richtersi* could consume 4 % of the available nematode biomass per day. Planarians are also known to be voracious nematode predators, where in artificial conditions an adult flatworm, *Dugesia gonocephala*, was able to consume a maximum of 94 adult or 197 juvenile nematodes in 3 h (Beier *et al.* 2004).

Predatory nematodes are found in the orders Mononchida, Diploglasterida, Dorylaimida and Aphelenchida, and have been used for the biological control of plant parasitic nematodes (reviewed in Khan and Kim 2007). Although no information exists on the rates of predation on entomopathogenic nematodes, it is likely that predation by other nematodes is a significant mortality factor.

Perhaps the least studied group of potential nematode antagonists are the soil-dwelling microarthropods, of which mites and collembola are the most numerous (Mankau 1980). A wide range of collembolan species have been observed feeding upon nematodes, including *Isotoma* sp. *Hypogastrura packardi*, *H. scotti*, *Sinella caeca*, *Entomobryoides dissimilis*, *Tomocerus vulgaris*, *T. flavescens*, *F. candida*, *Isotomurus palustris*, *Isotoma viridis*, and *I. cinerea*, *Onychiurus* sp., *Heteromurus nitidus*, *Protaphorura fimata* and *Proisotoma minuta* (see Brown 1954; Gilmore 1970; Epsky *et*

al. 1988; Osman *et al.*1988; Gilmore and Potter 1993; Lee and Widden 1996; Ruess *et al.* 2004, 2005a; Chamberlain *et al.* 2006a). Traditionally, collembola were thought to be exclusively fungal, detrital and plant feeders (Hopkins 1997). However, increasingly, evidence is showing that animal food constitutes an important part of collembolan diet. For example, Lee and Widden (1996) reported that the collembolan *Folsomia candida* preferentially fed on the nematode *Caenorhabditis elegans* rather than a common soil fungus, *Cladosporium cladosporioides*. Fatty acid analysis and stable carbon isotopes were used to show that the collembola *F. candida* and *Proisotoma minuta* preferred a nematode diet over fungi (Chamberlain *et al.* 2006a) and that a nematode-only diet resulted in increased fecundy and growth when compared to a fungal-only diet (Chamberlain *et al.* 2006b). Microcosm experiments have also shown that collembola are capable of suppressing nematode numbers in soil (Hyvonen and Persson 1996; Huhta *et al.* 1998). In the field, negative correlations of microarthropods and nematodes have been observed (Santos and Whitford 1981), and the presence of a non-specialist predatory mite, *Lysigamasus lapponicus*, in soil ecosystem experiments was enough to reduce the population of bacterial and fungal feeding nematodes by more than 50% (Laakso and Setala 1999).

Mites have also been shown to be predators of nematodes (Muraoka and Ishibashi 1976; Imbriani and Mankau 1983; Inserra and David 1983; Brust and House 1988; Oliveria *et al.* 2007), and microcosm experiments have shown that mites can suppress nematode numbers in the soil (Osman *et al.* 1988; Laakso and Setala 1999). Although a large number of mites are known to be nematophagous, many more species that had been labelled as fungivores may also prey on nematodes. For example, Walter (1987) found that 8 of 31 species of mycophagous arthropods collected from short-grass steppes in Nebraska were observed feeding on, and could be reared on, nematodes.

Both collembola and mites have been observed feeding on the free-living stage of entomopathogenic nematodes (Poinar 1979; Epsky *et al.* 1988). Gilmore and Potter (1993) showed that the collembola *F. candida* and *S. caeca* consumed steinernematid nematodes, and were able to reduce the effectiveness of the nematodes against hosts in the laboratory. However, the majority of these studies have either been based on laboratory feeding trials, which may not accurately reflect feeding choices in the field (Mckemey *et al.* 2003), or from associational evidence from field plots.

1.6. Determining trophic interactions

1.6.1. Previous methods

In order to investigate trophic interactions below-ground and to understand the linkages between these food-webs, it is necessary to study interactions and energy flow between the component organisms. The scale and concealed nature of below-ground ecosystems precludes methods such as direct observation, and whilst measuring the abundance of organisms may provide clues to interactions, it does not implicitly identify trophic links. One method that does not encounter these difficulties is post-mortem gut and tissue analysis. By removing the predators from an undisturbed natural system after the predation event has occurred, the diet can be determined without any of the biases associated with artificial laboratory feeding or field manipulation experiments. A number of different techniques have been used to determine the diet of invertebrates post-mortem, including visual identification, protein electrophoresis, polyclonal and monoclonal antibodies, stable isotope analysis, fatty acid analysis and DNA amplification (Symondson 2002). Visual analysis relies on the identification of undigested prey in the gut of the predator, usually exoskeleton (reviewed in Ingerson-Mahar 2002). The drawback is that this method relies on a high degree of morphological taxonomic skills, and that fluid feeders (such as spiders and many Hemipterans) are unlikely to have identifiable remains in their guts. Techniques such as stable-isotope analysis (reviewed in Hood-Nowotny and Knols 2007) and fatty acid analysis (Ruess *et al.* 2005a; Chamberlain *et al.* 2005, 2006a) rely on the body tissue of the consumer reflecting the composition of prey that it consumed. However, these methods only reflect the trophic level of the consumer and do not identify specific trophic links. Recent studies have also highlighted the high potential for variability between individuals on the same trophic level, with isotopic variation being identified between plant tissues, over time, between insect life stages, and between species, despite similar feeding habits (Daugherty and Briggs 2007).

Protein electrophoresis has been used in a wide range of ecological studies, to determine the diet of Coleoptera (Schelvis and Siepel 1988; Walrant and Loreau 1995), Hemiptera (Giller 1986) and Acari (Lister *et al.* 1987). However, the relatively low sensitivity and

the difficulty in determining mixed diets have meant this method has been superseded by later techniques. Polyclonal and monoclonal antibodies have been used extensively to study the diet of a wide range of different predators as far back as 1946 (Brooke and Proske 1946). The ability to develop antibodies with a high degree of specificity, even down to the instar level, makes this technique particularly useful. However, the high cost and time associated with developing the more specific monoclonal antibodies (Symondson 2002) has resulted in this method being used less and less for predation studies.

1.6.2. DNA techniques

1.6.2.1. Molecular detection of predation

The use of polymerase chain reaction (PCR) techniques for the detection of predation at the DNA level is a relatively recent approach, with the first successful application of this technique being used to determine predation by the sand shrimp, *Crangon affinis*, on juvenile stone flounder, *Kareius bicoloratus* (see Asahida *et al.* 1998). PCR techniques utilise the ability of short lengths of synthesised DNA, called oligonucleotides, to act as 'primers' for a DNA replication enzyme by annealing to denatured single-stranded DNA. Based on sequence composition, these oligonucleotides can be designed to anneal to a stretch of DNA unique to a species or lineage (i.e. species-specific primers), or to a wide range of species, either within a taxonomic group (i.e. group-specific primers) or as wide a range of species as possible (i.e. general primers). PCR based methods have the advantage that molecular skills and the equipment to carry out this type of research are widespread (Symondson 2002), and that once developed, species-specific primers can be cheaply acquired by researchers in different laboratories. A wide range of predator and prey DNA sequences are available from online databases, such as GenBank (http://www.ncbi.nlm.nih.gov), allowing for the design and testing of new primers.

The use of DNA-based methods can be broadly split into two approaches. The first approach is to design species-specific primers for the detection of a particular target prey species of interest. This approach has been used to detect predation by a broad array of predators on an even wider range of prey (see Table 1.1 in Appendix 1 for a

summary of studies utilising PCR based gut detection in invertebrates). Carabid beetles (Coleoptera; Carabidae) are probably the most studied invertebrate predators, probably because of their abundance in cereal crops and their generalist diet, which implies the potential to control a wide range of pest species. As a result, these beetles have been screened for a range of prey, including mosquitoes, earthworms, aphids, slugs, snails, weevils and scarab beetle larvae (Zaidi *et* al. 1999; Sheppard *et al.* 2005a; Harper *et al.* 2005, 2006; Juen and Traugott 2006).

The alternative approach for prey detection is to use group-specific or general primers to amplify a mix of DNA fragments from within the gut of the predator. These fragments can then either be separated using gel techniques such as Temperature Gradient Gel Electrophoresis (TGGE) (Harper *et al.* 2006), or cloned, sequenced and BLAST searched to identify the prey (Blankenship and Yayanos 2005). This approach has the advantage that it can be used with no prior assumptions about the diet of the predator, reducing the possibility that important prey groups are excluded from the analysis.

As the technology behind the use of PCR based detection of predation and parasitism matures, the frequency at which these studies are conducted increases (see Figure 1.8.1). As these studies move out of the laboratory into the field, a number of technical difficulties need to be addressed before results can be interpreted with any reliability.

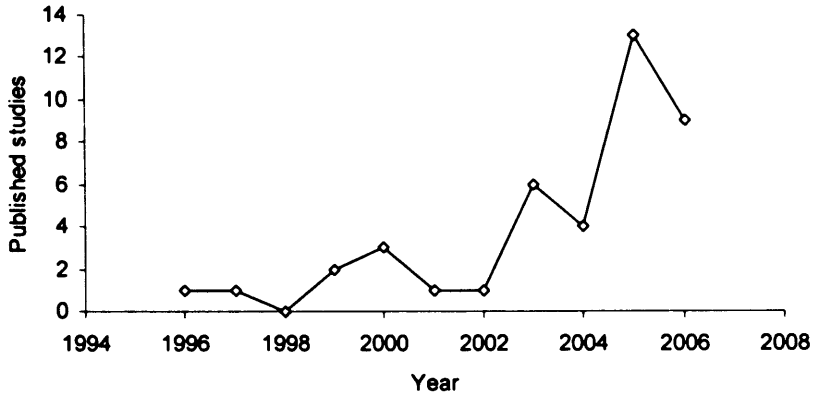


Figure 1.8.1 Number of published studies utilising PCR for the detection of predation in terrestrial invertebrate systems, against time in years.

1.6.2.2. Molecular detection of parasitism

The use of molecular methods, specifically PCR, for the detection of parasites within their hosts is not new. For example, PCR using species-specific and group-specific primers is widely used for the detection of human parasites, including parasites that cause schistosomiasis (Sandoval *et al.* 2006), leishmaniasis (Mehrabani *et al.* 2007), cryptosporidiosis (Moore *et al.* 2007), filariasis (Fischer *et al.* 2007) and malaria (Mharakurwa *et al.* 2006). Species-specific primers have been used to amplify DNA from a range of filarial nematode parasites that could then be identified to species level using restriction enzymes (Nuchprayoon *et al.* 2005). Parasites of livestock are also regularly screened, including those that infect horses (reviewed in Hodgkinson 2006) and for the detection of trypanosomes in cattle (Desquesnes and Davila 2002). For example, quantitative PCR has been used for the detection of protozoan parasites in the blood of cattle (Buling *et al.* 2007). As well as detecting parasites in blood, PCR has been used to identify parasites from faecal samples (Mochizuki *et al.* 2006; Harmon *et al.* 2006). Wimmer *et al.* (2004) used species-specific primers to detect nine different species of parasitic nematodes from six different families in the faeces of a wild, unmanaged and naturally infected population of sheep.

The detection of parasites within invertebrate hosts has received less attention, probably due to the lack of medicinal significance of most invertebrate parasites. One exception is the plasmodium parasite that causes malaria, for which a range of assays have been developed to detect their presence within their mosquito host (Moreno *et al.* 2004; Mohanty *et al.* 2007). PCR using species-specific primers and probes has been used to detect *Hematodinium*, a parasitic dinoflagellate that infects Norwegian lobsters (Small *et al.* 2006) and blue crabs (Small *et al.* 2007).

Research on terrestrial invertebrates has mainly focussed on the detection of the eggs and larvae of insect parasitoids from the orders Hymenoptera and Diptera within their insect hosts (reviewed in Greenstone 2006 and Gariepy *et al.* 2007), although PCR has also been used to detect the bacterial parasite *Wolbachia* in collembola (Czarnetzki and Tebbe 2004) and the fungal parasitic microspora, *Thelohania solenopsae*, in fire ants (Snowden *et al.* 2002). Qvarnstrom *et al.* (2007) detected the parasitic nematode

Angiostrongylus cantonensis (a common cause of human eosinophilic meningitis) in tissue and mucus secretions of a molluscan host, the semislug *Parmarion* cf. *martensi* using primers that amplified a species-specific segment of the 18S gene.

1.6.2.3. Detection of nematodes from environmental samples

PCR has been used as a diagnostic method to detect and track nematodes within a range of environmental samples. Species-specific primers have been frequently used for the detection of plant parasitic nematodes within their host plants, including for the potato parasitic nematode *Nacobbus* sp. (see Atkins *et al.* 2005), the pinewood nematode, *Bursaphelenchus xylophilus* (see Cao *et al.* 2005; Jiang *et al.* 2005), and the beet cyst nematode, *Heterodera schachtii* (see Madani *et al.* 2005).

A number of studies have used molecular methods to detect and quantify parasitic nematodes from within substrates. Species-specific primers have been developed for the detection of the root-knot nematodes *Meloidogyne arenaria*; *M. incognita*, and *M javanica* (see Qiu *et al.* 2006), and for *M. incognita* and *Pratylenchus coffeae* from within soil samples (Saeki *et al.* 2003). PCR primers and probes have also been designed for the detection of the slug parasitic nematode *P. hermaphrodita* from soil samples (MacMillan *et al.* 2006). In this instance, species-specific probes and primers based on the 18S region of ribosomal DNA were used to conduct real time PCR (RT-PCR) assays that could be used to quantify nematode numbers in the soil. A similar approach was taken by Torr *et al.* (2007) to identify and quantify the entomopathogenic nematodes *S. kraussei* and *S. affine* from soils at a number of sites in Scotland.

1.7. Parasitic nematode diversity and genetics

Research on the taxonomy of the Heterorhabditidae and Steinernematidae has received considerably more attention than for the Phasmarhabditidae, probably due to the number of species and the length of time that these have been used for biological control.

Blaxter (1998) produced a molecular framework for the Nematoda, which indicated that, despite similar life-cycles, the Heterorhabditidae and Steinernematidae are not closely related. The genus *Heterorhabditis* is more closely related to the Strongylida, a

group of vertebrate parasites, and the genus *Steinernema* is closely related to the Panagrolaimoidae and Strongyloidae, a group that contains plant parasitic, fungivorous and bacterivorous species (Adams *et al.* 2006). The Heterorhabditidae are monotypic, comprising only one genus, *Heterorhabditis* (see Poinar 1976) which contains 10 species (Adams *et al.* 2006). The Steinernematidae comprises two genera: *Steinernema* (see Reid *et al.* 1997) which includes over 40 known species (Adams *et al.* 2006) and *Neosteinernema* which comprises just one species, *Neosteinernema longicurvicauda* (see Nguyen and Smart 1994).

The genome sizes of heterorhabditid and steinernematid nematodes have been previously calculated, with an estimated 2.3×10^8 bp in *S. carpocapsae* and 3.9×10^7 bp in *H. bacteriophora* (see Lui *et al.* 2000). A number of studies have investigated the genetic diversity of these two families to identify, diagnose and delimit the species they contain. Several different approaches have been undertaken to achieve these aims, including random amplification of polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), microsatellites and sequence data.

The RAPD approach utilises a single 'randomly chosen' primer that acts as both a forward and reverse primer. When these primers bind to sites that are closer than 2 kb to each other variable fragments of DNA are amplified. Sequence differences between individuals and species will alter the characteristic banding pattern produced, allowing for species separation and identification. This has been used to assess diversity within Heterorhabditidae (Gardner *et al.* 1994; Hashmi and Gaugler 1998). Restriction fragment length polymorphism (RFLP) is a technique that works on the principle of digesting genomic DNA with a set of restriction enzymes, which will generate a unique 'barcode' of different sized DNA fragments depending on the species. RFLP has been used on the mitochondrial genome, nuclear ribosomal internal transcribed spacer (ITS) and 26S ribosomal DNA, and has been used to discriminate between *Steinernema* species (Hominick *et al.* 1997; Reid *et al.* 1997; Pamjav *et al.* 1999; Triga *et al.* 1999) and *Heterorhabditis* (see Joyce *et al.* 1994). Satellite DNA sequences, which consist of short tandemly repeated sequences, have been used less frequently for the study of nematode population genetics. Greiner *et al.* (1996a) identified two *Alu*I tandemly repeated sequences from *H. bacteriophora* and *S. glaseri*, and *HaeIII* satellite sequences

have been used for the species-specific identification of *S. carpocapsae*, *H. bacteriophora*, and *H. indicus* (see Grenier *et al.* 1996b; Abadon *et al.* 1998).

However, the most frequently used technique for describing genetic diversity amongst entomopathogenic nematodes has been to use DNA sequence data. Nuclear regions such as the ribosomal internal transcribed spacer region (ITS and 5.8S) have been used to distinguish between 10 species of *Steinernema* (see Nguyen *et al.* 2001), eight species of *Heterorhabditis* (see Adams *et al.* 1998) and to identify known and new species of *Steinernema* and *Heterorhabditis* from Arizonan soils (Stock and Gress 2006). Stock *et al.* (2001) used the 28S nuclear region to investigate the phylogenetic relationships between 21 species of *Steinernema*, and Nadler *et al.* (2006) used this region, plus mitochondrial DNA to infer relationships between 25 species of *Steinernema*. Partial 18S sequences (265 bp) have been used to infer relationships between *Steinernema* and *Heterorhabditis*. However, although these were adequate for species discrimination within *Steinernema*, it was not possible to resolve the variation within *Heterorhabditis* at species level (Lui *et al.* 1997). Mitochondrial sequences have also been used to prospect for cryptic species (Blouin 2002), assess population structure (Blouin *et al.* 1999) and infer phylogenetic relationships between species (Szalanski *et al.* 2000). Nematode mitochondrial sequences from COI, the dehydrogenase subunit 4 gene (NAD4) (Blouin 2002), COII and 16S (Szalanski *et al.* 2000) and have been used for species discrimination.

Despite the number of studies utilising molecular data to examine nematode diversity, few studies have examined the population structure and genotype distribution within species. Reid and Hominick (1992) used Restriction Fragment Length Polymorphism (RFLP) to survey 89 *Steinernema* sp. isolated from the United Kingdom, identifying five RFLP types, two of which were found in *S. feltiae*. The mitochondrial NAD4 region was used to investigate the population structure of *H. marelatus*, in which four distinct haplotypes were found and no association with geographical location was observed (Blouin *et al.* 1999). The diversity within the Heterorhabditidae has been shown to be lower than in the Steinernematidae (both in terms of genetic structure and the number of species) (Grenier *et al.* 1996; Liu *et al.* 1997) indicating that the Heterorhabditidae have diverged more recently. An alternative hypothesis is that the first generation hermaphroditic reproductive strategy in the Heterorhabditidae may

result in lower rates of genetic divergence, due to the production of genetically less diverse offspring after the infection of a host by a single nematode (Hominick *et al.* 1996), whereas the obligate first-generation sexual reproduction in *Steinernema* nematodes may generate higher levels of diversity and speciation.

The Phasmarhabditidae are monotypic, comprising of one genus *Phasmarhabditis*, which contains five species, although only two are parasites of slugs; *P. hermaphrodita* and *P. papillosa* (see Grewal *et al.* 2003). Of these, only *P. hermaphrodita* is used for biological control. This family has received less attention than the entomopathogens, with no phylogenetic studies carried out as yet. Hooper *et al.* (1999) conducted a morphological and protein profile study of *P. hermaphrodita* and *P. neopapillosa*, indicating that although highly morphologically conserved, these are indeed two different species. The rhabditid, mollusc-parasitic nematode *P. hermaphrodita* has been geographically recorded in the UK, France and Germany (Glen and Wilson 1997). It is a bacterial-feeding nematode, like the insect pathogenic species, causing speculation that it evolved from a soil-dwelling rhabditid nematode (Grewal *et al.* 2003).

1.8. Choice of gene for predation studies

The choice of target gene for the amplification of species-specific or group-specific fragments of DNA is important, as it determines, amongst other factors, the specificity, taxonomic level and sensitivity of the probes. For example, genes that are present in multiple copies in the cell have been shown to be better targets for predation studies due to the increased sensitivity of the PCR assay (Zaidi *et al.* 1999). Early attempts to detect prey DNA in predator guts utilised markers that amplified nuclear DNA. For example, Agusti *et al.* (1999) used randomly amplified polymorphic DNA derived from SCAR (Sequence Characterised Amplified Region) markers to detect the lepidopteran pest *Helicoverpa armigera* in the gut of the predator *Dicyphus armigera*. The SCAR approach has also been utilised in a number of other studies (Agusti *et al.* 2000; de Leon *et al.* 2005; Zhang *et al.* 2007a, b). Other examples of nuclear gene targets include the α-esterase gene (Zaidi *et al.* 1999) and ITS-1 (Internal Transcribed Spacer region one) (Hoogendoorn and Heimpel 2001; Morales *et al.* 2003; Ma *et al.* 2005).

Some nuclear targets are multi-copy, thus making them suitable for predation studies, and differing rates of evolution make them appropriate for primers targeting specific groups of organisms. The 18S ribosomal RNA (rRNA) is part of the ribosomal gene cluster that contains 5.8S, 18S, the 28S rRNA gene, the two internal transcribed spacers (ITS-1 and ITS-2) and an external transcribed spacer. This is a tandemly repeated region that is found in several hundred copies per cell (Beebee and Rowe 2004). The 18S rRNA region has been used for the design of group-specific primer pairs for the detection of a wide range of potential prey in marine ecosystems (Jarman *et al.* 2004), and for the amplification and separation of mixed nematode communities from the soil (Foucher *et al.* 2002). Nuclear regions have also proved to be useful markers for species discrimination in nematodes.

The majority of predation studies have utilised mitochondrial DNA (mtDNA) as a target for primer design. This is for two reasons, one being due to the fact that mitochondria are present in multiple copies per cell (Hoy 1994), thus increasing sensitivity when compared to single copy nuclear targets. For example, the mtDNA copy in the embryo of the free-living nematode *Caenorhabditis elegans* is estimated to be ~ 2.5×10^4 increasing to 7.8×10^5 in adults (Tsang and Lemire 2003). However, the mtDNA copy number in *C. elegans* is thought to be considerably lower that the estimated values in higher eukaryotic cells (Tsang and Lemire 2003). The second factor is that mitochondrial DNA, in particular Cytochrome Oxidase subunit One (COI), has been subject to a large sequencing effort worldwide due to projects such as the 'Barcode of Life' (Beardsley 2005; Ratnasingham and Herbert 2007), producing large online sequence databases that aid with primer design and testing.

Mitochondrial DNA is a circular double-stranded DNA molecule coding for protein in the mitochondrial respiratory chain (MRC) (Tsang and Lemire 2003). Nematode mitochondrial genomes are usually smaller that most other metazoan groups, varying in size from 13.6 -14.3 kb (Hu and Gasser 2006). In general, nematode mtDNA contains 12 protein-coding genes, 22 tRNA and 2 rRNA genes, and lack the *atp8* gene (see Figure 1.8.1) (Hu and Gasser 2006). Mitochondrial genes in nematodes, as with other animals, have high evolutionary rates, which have lead to their use in determining the relationships of closely related species (Szalanski *et al.* 2000), and identifying and differentiating cryptic species (Blouin 2002). For example, Agustí *et al.* (2003a) used

COI sequences to design species-specific primers to target three species of collembola in the guts of spiders in agroecosystems, and Greenstone and Shufran (2003) designed primers for the aphid *Rhopalosiphum maidis* based on COII. There are a number of mitochondrial genes that would make suitable targets for predation studies, although most studies have targeted the COI, COII, 12S and 16S regions (see Table 1. 1 in Appendix 1).

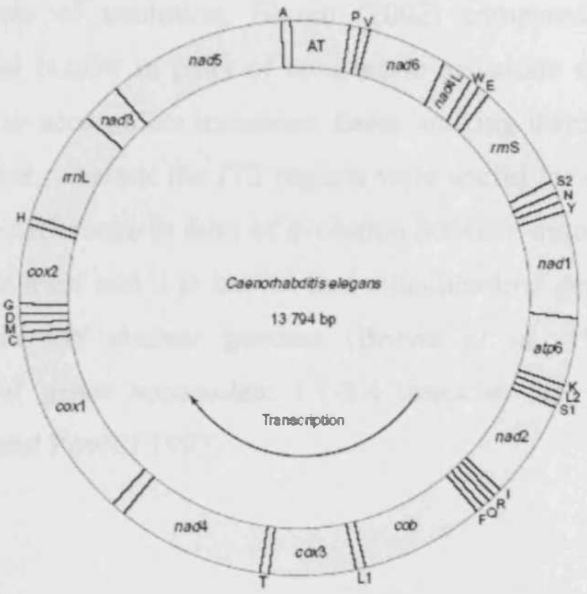


Figure 1.8.1 Mitochondrial genome structure of the free-living nematode *Caenorhabditis elegans* (From Hu and Gasser 2006).

Rates of molecular evolution within the mitochondrial regions are known to vary, where regions such as the ribosomal (rRNA) genes (12S and 16S) have been found to be slower evolving in *Drosophila* (see Ballard 2000) and salamanders (Mueller 2006), making them suitable for probes targeting a wider range of species (i.e. group-specific primers). The mitochondrial protein encoding genes such as COI and COII are comparatively faster evolving, allowing for species-specific primer design. The *ND4* gene has been compared amongst a wide range of species for nematode systematics, in which high rates of amino acid polymorphisms were found at the intra- and inter-specific levels (Blouin *et al.* 1998). As a result, *ND4* in nematodes is only suitable for comparisons of closely related species or for population genetics studies. Similar results were observed in an analysis of COI sequences of a number of species of nematode hookworm from China, Australia and Togo, where intraspecific variation ranged from

0.3-3.3 % in *Ancylostoma duodenale*, 0.5-8.6 % in *Ancylostoma caninum* and 0.3-4.3 % in *Necator americanus* (see Hu *et al.* 2002). In a comparison of the whole mitochondrial genome of two populations of the nematode *Necator americanus*, the most conserved gene was the *nad*4L gene, whereas the *nad*1 gene was least conserved at both the nucleotide and amino acid levels (Hu *et al.* 2003).

The choice between using a nuclear or mitochondrial target may be influenced by the differing rates of evolution. Blouin (2002) compared ITS-1 and ITS-2 with the mitochondrial NAD4 in pairs of congeneric nematode species. Mitochondrial regions were found to accumulate mutations faster, making them more suitable for identifying cryptic species, whereas the ITS regions were useful for distinguishing between known species. The difference in rates of evolution between mitochondrial and nuclear genes is a widespread trait, and it is known that mitochondrial genes evolve faster than most of the genes in the nuclear genome (Brown *et al.* 1982), where substitutions in mitochondrial genes accumulate 1.7-3.4 times as fast as nuclear genes on average (Moriyama and Powell 1997).

1.9 Aim, rationale and objectives of the current study

Aim:

To study the interactions between nematode biopesticides and other organisms in the soil community.

Rationale:

1. The soil ecosystem can be viewed as a 'black box' due to the concealed nature of the environment. Understanding interactions between the soil-dwelling biota has many benefits, including improved knowledge of pest control, ecosystem processes, and nutrient cycling and productivity, both above- and below-ground.
2. Due to the concealed nature of the below-ground food-web, molecular techniques need to be developed to identify trophic links between its component organisms.
3. The persistence of parasitic nematodes used for biological control in the soil after application has often been shown to be low, and improved persistence may increase pest control efficacy.
4. Interactions of parasitic nematodes used for biological control within the soil food-web (other than with the target pest) may play an important role in determining the persistence and efficacy of parasitic nematodes.

Objectives:

1. Develop molecular methods for the detection of nematode biopesticides within their hosts, within potential predators of the free-living nematode stages and within predators of the infected hosts.
2. Test these newly developed molecular tools to determine their accuracy and potential error in determining predation on nematode IJs.
3. Through laboratory-based feeding trials, investigate the potential for soil-dwelling microarthropods to limit the persistence of introduced nematode IJs.
4. Investigate the impact of the application of nematode IJs in agroecosystems and identify potential predators of the introduced nematodes to identify potential nematode antagonists.

5. Develop methods for assessing nematode diversity in the field, and use these methods for studying the impact of introducing nematode IJs on existing nematode communities.
6. Test the molecular tools for use in investigating tri-trophic interactions between potential predators of nematode-infected hosts.
7. Investigate the potential for nematode infection to alter the prey choice by invertebrate predators.

Chapter 2

General methods – Culturing of experimental organisms and the development and optimization of PCR-based detection methods

2.1. Methods

2.1.1. Culturing techniques

Carabid beetles, *Pterostichus melanarius* (Coleoptera: Carabidae), were collected in a field of winter wheat (*Triticum aestivum*) from Burdens Farm, Wenvoe, near Cardiff (map ref: Lat: 51:26:24N Lon: 3:16:29W) using pitfall traps. Beetles were stored individually in plastic containers (9 x 5 cm) with damp peat as substrate, at 16 ± 2 °C with a 16:8 h light: dark regime. They were fed upon larvae of the dipteran, *Calliphora vomitoria* (L) (Diptera: Calliphoridae) once per week. Containers were inspected twice weekly and any uneaten food remains were removed.

Grey field slugs, *Deroceras reticulatum* (Muller) (Mollusca: Pulmonata), were collected at night from the surfaces of cut lawns in Devizes, Wiltshire. Slugs were stored at 4 °C for up to 48 h before sorting into groups of 10 in plastic containers (25 x 15 cm), with a piece of moist tissue as substrate. Slugs were stored at 16 ± 2 °C for no longer than 2 weeks, and fed on a diet of cabbage and carrot. The tissue was changed every two days to prevent the build up of mucus and the spread of disease.

Mealworms, *Tenebrio molitor* (L) (Coleoptera: Tenebrionidae), were initially obtained from commercial suppliers (LivefoodUK, Somerset, UK). The larvae were placed in a large plastic container (26 x 18 cm) with a vented lid, and stored at room temperature (22 ± 2 °C). Organic wholemeal flour was used as a food source and substrate, and moisture was supplied in the form of pieces of carrot and apple every two weeks. Adult beetles were removed to new containers with fresh flour, and allowed to lay eggs. Every three months the mealworm larvae were removed by sieving the substrate, and placed in a clean container with new substrate.

Waxworms, *Galleria mellonella* (L) (Lepidoptera: Pyralidae), were obtained from commercial suppliers (LivefoodUK, Somerset, UK). The larvae were placed in plastic containers (15 x 10 cm) with vented lids and sides, and stored at room temperature (22 ± 2 °C). Egg cartons were included as substrate and the larvae were fed on commercial waxworm food (LivefoodUK, Somerset, UK), until they were ready to be used.

The collembola *Folsomia candida* (Isotomidae), *Protaphorura armata* (Onychiuridae), *Heteromurus nitidus* (Entomobryidae) and *Proisotoma minuta* (Isotomidae) were obtained from laboratory cultures at Cardiff University (original source unknown) and stored in plastic food containers (17.5 x 11.5 cm) with a moist charcoal Plaster-of–Paris base. They were fed on a diet of dried active yeast (Westmill Foods, Hertfordshire, UK), and watered once weekly. Mesostigmatid mites *Stratiolaelaps miles* (Berlese) (Mesostigmata: Laelapidae) and *Hypoaspis aculeifer* (Canestrini) (Mesostigmata: Laelapidae) were obtained from commercial suppliers (HarrodHorticultural, Lowestoft, UK) and reared in identical containers to the collembola. These were fed on a diet of grain mites, *Acarus* sp. (L) (Astigmata: Acaridae), that had been reared on dried active yeast.

Nematodes were obtained from BeckerUnderwood (Littlehampton, UK) in the form of nematode infective juveniles (IJs) in a partially desiccated state in clay aggregate. In order to reduce costs, the entomopathogenic nematodes *Heterorhabditis megidis* (Poinar, Jackson and Klein) (Rhabditida: Heterorhabditidae) and *Steinernema feltiae* (Filipjev) (Rhabditida: Steinernematidae) were occasionally reared in insect hosts for later use. Both nematode species were reared in larval *G. mellonella*. Ten larvae were placed in 10 cm plastic Petri dishes with a layer of filter paper as substrate. Nematode IJs were added at the recommended field rate of 50 per cm^2 in 2 ml of tap water. Hosts were then incubated at 20 °C for a further nine days. After this time, the hosts were arranged on a White's trap to harvest the nematodes (White, 1927). This consisted of an upturned 5 cm Petri dish standing in a 15 cm Petri dish. The smaller dish was covered with filter paper and dechlorinated tap water poured in the larger dish to a height of 5 mm. The infected hosts were placed on the filter paper, above the water line. The emerging nematodes made their way into the water, and were collected and stored at 4 °C in a glass beaker for no longer than 15 days.

2.1.2. Nematode extraction from soil samples

The number of nematodes in soil samples was measured using an adapted Baermann Funnel method (Flegg and Hooper, 1970) (see Figure 2.1.1). A 15 cm length of 4 mm diameter rubber tubing was attached to the stem of a 20 cm diameter plastic funnel. A 2-ml glass vial was attached to the end of the rubber tubing. The funnel was placed in a

clamp, and filled with dechlorinated tap water to 2 cm from the top. The soil sample, containing the nematodes, was placed on a two-ply layer of tissue, and supported on a plastic mesh at the surface of the water level. The water level was raised until the base of the soil was in contact with the water. The apparatus was left for 72 h at room temperature (22 ± 2 °C), and tapped occasionally to facilitate the nematode movement down the water column. After this time, the glass vial, containing the nematodes was carefully removed, labelled and sealed with a lid.

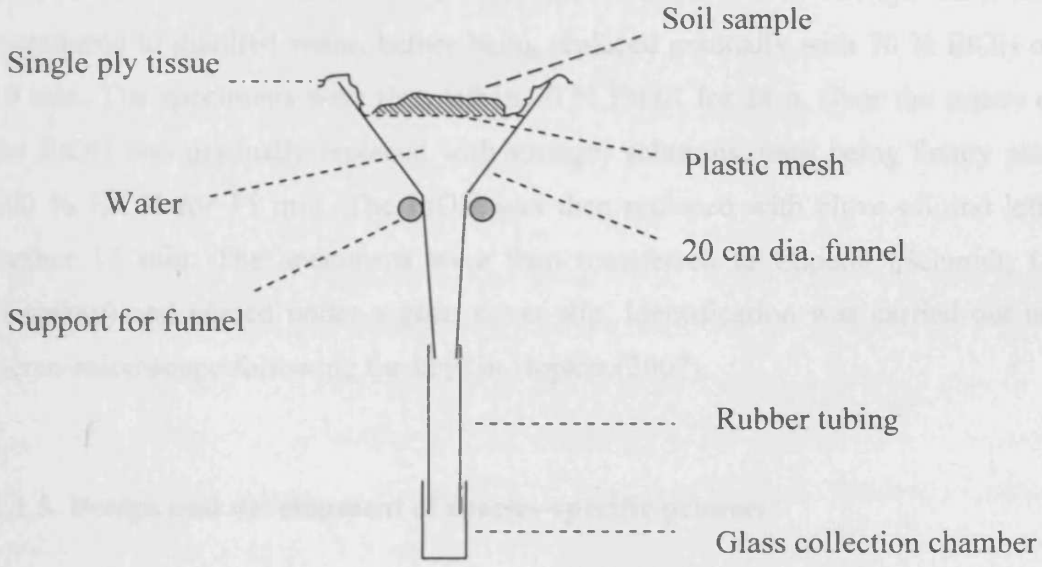


Figure 2.1.1 Baermann apparatus for the extraction of free living nematodes from soil samples.

2.1.3. Estimating numbers of nematodes

To estimate the number of nematodes in a solution, 0.1 g of nematode IJs in the supplied clay aggregate was diluted in 50 ml of tap water. This solution was mixed thoroughly, before pipetting 1.0 ml into a Sedgewick-Rafter counting cell (Graticules Limited, Tonbridge, UK). The total number of IJs in the cell was counted under a low power stereo-microscope. This was repeated three times weighing out a fresh 0.1 g of nematode-aggregate solution into 50 ml of water each time. The average number of nematodes per gram could then be calculated, and the appropriate solutions made depending on how many IJs were required. This was repeated with each fresh batch of

nematodes from the supplier, to ensure any nematode density variation between batches would not affect experimental results.

2.1.4. Identification of collembola

Samples of collembola collected from Burdens Farm, Wenvoe, near Cardiff, were preserved in 70 % ethanol (EtOH) for later identification. Collembola were prepared for identification following the method outlined in Hopkin (2007). Specimens were transferred to a 10 % solution of sodium hydroxide for 1-2 h. The specimen was then transferred to distilled water, before being replaced gradually with 70 % EtOH over 5-10 min. The specimens were then left in 70 % EtOH for 24 h. Over the course of 1 h, the EtOH was gradually replaced with stronger solutions, until being finally placed in 100 % EtOH for 15 min. The EtOH was then replaced with clove oil and left for a further 15 min. The specimens were then transferred to Euparal (Schmidt, GmbH, Germany) and placed under a glass cover slip. Identification was carried out under a stereo-microscope following the keys in Hopkin (2007).

2.1.5. Design and development of species-specific primers

2.1.5.1. Sequencing and primer design

Four species of parasitic nematode, one of molluscs, *Phasmarhabditis hermaphrodita* (Rhabditida: Rhabditidae) and three of insects, *Steinernema kraussei* (Rhabditida: Steinernematidae), *S. feltiae* and *H. megidis* (Rhabditida: Heterorhabditidae) (supplied by BeckerUnderwood, Littlehampton, UK), were used as model parasite species. DNA from 10 IJs of each species was extracted separately using the solid tissue protocol in Qiagen tissue extraction kits (Qiagen, Crawley, UK). A number of host, predator and non-target invertebrate species were also obtained, including the mealworm *T. molitor*, the waxworm *G. mellonella*, the garden chafer *Otiorhynchus sulcatus* (Fabricius) (Coleoptera: Curculionidae), the cabbage root fly *Delia radicum* (L.) (Diptera: Anthomyiidae), the turnip moth *Agrotis segetum* (see Denis and Schifferm) (Lepidoptera: Noctuidae) and the collembolan *F. candida*. All hosts, non-targets and predators were starved for at least seven days before killing at -80 °C. A small piece (<10 mg) of tissue was removed from the non-target invertebrates using a clean scalpel, and placed in a 1.5 ml centrifuge tube. DNA was extracted from whole *F. candida*

individuals. DNA extraction was conducted using the manufacturer's solid tissue extraction protocol (Qiagen, Crawley, UK).

A number of different general primers were assessed for amplification of nematode, host and predator DNA. The final primers that were used for each species were as a result of preliminary testing of the efficiency of each primer pair to amplify the target species. A 525-bp region of mtDNA from Subunit I of the Cytochrome Oxidase (COI) coding region was amplified from the four nematode species (*P. hermaphrodita*, *H. megidis*, *S. feltiae* and *S. kraussei*) and three potential hosts (*O. sulcatus*, *D. radicum* and *A. segetum*) with the general invertebrate primers *C1-J-1718* and *C1-J2191* (Simon *et al.* 1994) (Table 2.2.1) using a GeneAmp 9700 thermal cycler (ABI Perkin Elmer, Beaconsfield, UK). PCR reactions contained 2 µl template DNA, 1x PCR buffer, 2.0 mM $MgCl_2$ (both provided by Invitrogen, Paisley, UK), 0.2 mM dNTPs (Invitrogen, Paisley, UK), 0.2 units *Taq* polymerase (Invitrogen, Paisley, UK) and 0.5µM of each primer (Operon, Cologne, Germany) and were made up to 25 µl with PCR water. Thermal cycle parameters included an initial denaturation step of 2 min at 94 °C followed by 35 cycles of 94 °C for 1 min 10 sec, 45 °C for 1 min 10 sec, 72 °C for 1 min 30 sec, and a final extension stage of 72 °C for 7 min. Ultraviolet translumination was used to visualise 4 µl of PCR product following electrophoresis at 100 V for 1 h on a 1.8% (w/v) agarose gel stained with ethidium bromide in 1 x TAE buffer (1mM EDTA, 40mM Tris-Acetate). Gel images were analyzed using InGenius software (Syngene, Cambridge, UK) for PC.

A 710-bp region of COI mtDNA was amplified from *T. molitor* and *G. mellonella* using general invertebrate primers HCO and LCO (Folmer *et al.* 1994) (Table 2.2.1) using the following conditions: 2 µl template DNA, 1x PCR buffer, 3.0 mM $MgCl_2$ (both provided by Invitrogen, Paisley, UK), 0.2 mM dNTPs (Invitrogen, Paisley, UK), 0.2 units *Taq* polymerase (Invitrogen, Paisley, UK) and 0.5 µM of each primer (Operon, Cologne, Germany) and were made up to 25 µl with PCR water. Thermal cycle parameters included an initial denaturation step of 3 min at 94 °C followed by 35 cycles of 94 °C for 45 sec, 58 °C for 1 min 10 sec, 72°C for 1 min, and a final extension stage of 72°C for 10 min.

Finally, a 642-bp fragment of COI mtDNA was amplified from *F. candida* using primers *C1-J-1751* and *C1-N-2329* (Simon *et al.* 1994) (Table 2.2.1) using the following conditions: 2 µl template DNA, 1x PCR buffer, 2.0 mM $MgCl_2$ (both provided by Invitrogen, Paisley, UK), 0.2 mM dNTPs (Invitrogen, Paisley, UK), 0.2 units *Taq* polymerase (Invitrogen, Paisley, UK) and 0.5 µM of each primer (Operon, Cologne, Germany) and were made up to 25 µl with PCR water. Thermal cycle parameters included an initial denaturation step of 3 min at 94 °C followed by 35 cycles of 94 °C for 45 sec, 55 °C for 1 min 10 sec, 72°C for 1 min, and a final extension stage of 72°C for 10 min.

Products were cleaned using the GeneClean Turbo for PCR kit (Qbiogene, Cambridge, UK), following manufacturer's instructions, and 25-50 pg aliquots of purified template DNA were used in sequencing PCR with a Big Dye ™ terminator sequencing kit (Promega, Madison, WI, USA). DNA was sequenced using the relevant forward and reverse primers for each reaction, and products were resolved on an ABI 3100 automated capillary DNA analyzer (ABI Prism model 3100, Beaconsfield, UK). Sequence alignment and editing was carried out using Sequencher ™ 4.0 and secondary alignment was carried out using the ClustalW multiple alignment function of the Bioedit ™ package (Hall, 1999). Sequence data was used to design species-specific primer pairs for the four target species of nematode (*P. hermaphrodita*, *H. megidis*, *S. feltiae* and *S. kraussei*), for the two host species (*T. molitor* and *G. mellonella*) and the predator (*F. candida*).

Primers were designed to amplify fragments < 300 bp, maximizing the detection period for semi-digested DNA (Agustí *et al.* 1999; Zaidi *et al.* 1999; Hoogendoorn and Heimpel 2001; Deagle *et al.* 2006a, b). Attempts were made to select primers using the criteria that the last three bases of the 3' end of both the forward and reverse primers should lay on a site of inter-species variability, assisting with species-specific primer binding. The programs Amplicon (Jarman 2004b) and Primer3 (Rozen and Saletsky 1998) were used to identify suitable primer sites. Potential primer sites were checked for hairpins, dimers, cross-dimers, and palindromes on NetPrimer (PREMIER Biosoft International, CA, USA). A nucleotide-nucleotide BLAST search (Altschul *et al.* 1990) on the NCBI website was used to test for non-target similarities with other organisms. Primers were ordered from Operon (Cologne, Germany)

2.1.5.2 Primer testing and non-target testing

All potential primers were tested for their ability to amplify species-specific fragments of DNA in 10 µl reactions using the following conditions: 1 µl template DNA, 1x PCR buffer, 2.0 mM $MgCl_2$ (both provided by Invitrogen, Paisley, UK), 0.2 mM dNTPs (Invitrogen, Paisley, UK), 0.1 U *Taq* polymerase (Invitrogen, Paisley, UK) and 0.5 µM of each primer (Operon, Cologne, Germany). Thermal conditions included a first cycle of denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 30 sec, annealing for 45 sec, 72°C for 1 min, and a final extension stage of 72 °C for 10 min. The annealing temperature for each of the primer pairs was optimized on a gradient PCR using a BioRad DNA Engine thermal cycler (BioRad, Hercules, CA, USA), with the highest consistently successful annealing temperature selected to increase primer species-specificity.

The primers were tested against a number of non-target organisms using the optimized annealing temperatures. Primer pairs designed for use in laboratory studies were tested against all the organisms used in these studies to ensure no cross-reactivity. Primer pairs designed for use in field trials (the *P. hermaphrodita* specific primers) were tested against a wide range of non-target organisms (total 74) to ensure no cross-reactivity would occur in the highly species diverse field environment (Non-targets shown in Appendix 2, Table 2.1). The *P. hermaphrodita* primer pairs were also screened for specificity against 48 unidentified nematodes that were extracted from soil samples from the field site at Burdens Farm, Wenvoe, nr. Cardiff, to ensure no cross-reactivity would occur with non-target nematodes present at the field site. Any primer pairs that showed non-target amplification were rejected.

2.1.5.3 Primer sensitivity

The sensitivity of the primer pairs were tested in two ways: 1) by testing the efficiency of the nematode primers to amplify the DNA from a single nematode IJ; and 2) to test the efficiency of all the primers to amplify a serial dilution of target DNA. In the first experiment, ten of each of the three nematode species were extracted individually using the Qiagen DNA tissue extraction kit (Qiagen, Crawley, UK), following manufacturer's

protocols. The extracted DNA was then used in PCR reactions with the relevant species-specific primers, and visualized on an ethidium-bromide stained agarose gel as described previously. In the second experiment, the host and predator DNA extracts were quantified using a spectrophotometer. The host and predator DNA stocks were diluted to a starting concentration of 8000 pg / µl. These stock solutions were subjected to a serial dilution, halving the concentration of DNA at each stage in a total of 24 steps. This gave final concentrations from 8000 down to 0.0009 pg / µl of host and predator DNA. The nematode DNA was diluted from a starting concentration of 100 pg / µl in 16 steps to give a final dilution series of 100 down to 0.003 pg / µl. The serial dilutions were then used as templates for PCR, using the respective species-specific primers as described previously, and visualized on ethidium-bromide stained agarose gel.

2.1.5.4. Preliminary feeding trials to determine post-ingestion detection of nematode DNA in microarthropods using Qiagen DNA extraction method

Preliminary feeding trials were carried out to determine the suitability of the developed molecular methods for investigating the diet of soil dwelling microarthropods. Cultures of *F. candida* were maintained on a diet of bakers yeast (Tesco, UK). Mesostigmatid mites, *S. miles* (HarrodHorticultural, Lowestoft, UK), were used within five days of purchase. In separate feeding trials, the nematodes *P. hermaphrodita*, *S. feltiae* and *H. megidis* were re-hydrated with water and applied at 30 per cm^2 to the surface of plastic feeding arenas (125 mm dia, 70 mm depth), with a base of moistened charcoal mixed into plaster-of-Paris (1:250) and a vented lid. *F. candida* and *S. miles* were starved for a period of 48 h, placed in separate arenas and allowed to feed on the nematodes for 2 h. *F. candida* feeding trials were conducted on all three nematode species, but *S. miles* were fed on two species; *P. hermaphrodita* and *H. megidis*. After the 2 h feeding period, the predators were removed from the food source and placed in similar but clean containers and stored at 16 ± 2 °C in the dark. Ten predators were removed from these containers 1, 3, 6, 12, 24 and 48 h after the mid-point of the 2 h feeding period, placed individually in clean Eppendorf tubes and frozen at -80°C. To ensure false positives were not occurring, separate subsets (ca. 10 per species) of the predators (*F. candida* and *S. miles*) were examined under a high power stereo-microscope for signs of nematode phoresy (i.e. the attachment of the nematodes to the external surface of the

predators). Negative controls were prepared by keeping 10 of each predator species in feeding arenas lacking nematodes.

Extraction of DNA from the whole micro-arthropods, including gut contents, was carried out using the commercially available Qiagen tissue extraction kit. Screening of the extracted DNA was a two stage process. Primers *C1-J-1718* and *C1-J2191* (Simon *et al.* 1994) (Table 2.2.1) were used to amplify a 525-bp fragment of CO1 mtDNA using the same conditions and thermal cycle as described previously. Successful DNA extractions were then screened for the presence or absence of nematode DNA within the guts of the predatory micro-arthropods using *P. hermaphrodita* specific primer pair *PH-F-(284-304)* and *PH-R-(417-438)*, *H. megidis* specific primer pair *HM-F-(288-307)* and (*HM-R-(419-437)*, and *S. feltiae* specific primer pair *SF-F-(429-447)* and *SF-R-(610-629)*. PCR and thermal cycler conditions were as described in Section 2.1.5.2, using the optimum annealing temperatures in Table 2.2.3. PCR products were visualised as described previously.

2.1.6. Statistical Analysis

For the analysis of the collembola and mite feeding-trial data, Probit models, a form of Generalized Linear Model (GLM), were used to describe the change in the number of predators positive for prey DNA over time, using Minitab. To compare the calculated median detection times, the method from Payton et al. (2003), based on using 83.5 % fiducial limits as an approximation of an $\alpha = 0.05$ test was used. This allowed the statistical significance of the calculated median detection times to be compared, and differences in the values to be highlighted.

2.2. Results

2.2.1. Sequencing and primer design

A total of six general invertebrate COI primers were assessed for their suitability for use on the invertebrate material in this study (Table 2.2.1). The relative positions of these primers on the COI region of mitochondrial DNA are shown on Figure 2.2.1. COI fragments between 525-528 bp were successfully amplified and sequenced from 10 individuals each of *Heterorhabditis megidis, Phasmarhabditis hermaphrodita, Steinernema feltiae* and *Steinernema kraussei* (Genebank accession numbers DQ285542, DQ285543, DQ285544 and DQ285545, respectively). No intra-specific variation was found, allowing a single consensus sequence for each species to be used. Fragments between 684-695 bp were amplified for 5 individuals of *Galleria mellonella* and *Tenebrio molitor*. No intra-specific variation was found in *G. mellonella* (see Appendix 2, Figure 2.6), but three haplotypes were found in *T. molitor* (Appendix 2, Figures 2.7, 2.8, and 2.9). A 643 bp fragment was sequenced for *Folsomia candida* with no intra-specific variation (Appendix 2, Figure 2.10). 527-529 bp sequences were obtained for *Agrotis segetum*, *Otiorhynchus sulcatus* and *Delia radicum* with no intra-specific variation (Appendix 2, Figures 2.11, 2.12 and 2.13, respectively).

Primers were successfully designed for all of the target nematodes (Table 2.2.2) except *S. kraussei*, where no suitable species-specific primer sites could be identified. Because of this, *S. kraussei* was excluded from the rest of the study as a target nematode. Primers were then paired up in a number of different combinations (Table 2.2.3), ensuring fragment sizes stayed below 400 bp in length. Alignments of *P. hermaphrodita*-specific primer-pair *PH-F-(284-304)* and *Ph-R-(479501)* is shown in Table 2.2.4, and *S. feltiae*-specific primer-pair *SF-F-(429-447)* and *SF-R-(610-629)* in Table 2.2.5, showing the specificity of these primers against a number of non-target predators and hosts.

For the two model hosts (*G. mellonella* and *T. molitor*) and one model predator (*F. candida*) species-specific primers were designed for use in feeding trials (Chapter 3 and 6). A number of forward and reverse primers were designed for each species (Table 2.2.6) and potential primer pairings examined (Table 2.2.7).

Table 2.2.1 General COI invertebrate primer pairs assessed and used in the current study, with information about the primer sequence, GC content, melting temperature of the primers and the size of the amplified fragment.

Primer name	Source	Primer sequence	GC (%)	Melting temp (°C)	Fragment size (bp)
C1-J-1718	Simon *et al.* (1994)	5'-GGTGGTTTTGGTAACTGATTTTACC-3'	40.0	60.69	525
C1-J-1751	"	5'-GGAGCACCTGATATAAGATTTCC-3'	47.48	57.03	
C1-N-2191	"	5'-GCTGGTAAAATCAAAATATATACTTC-3'	26.92	54.46	710
C1-N-2329		5'-ACTGTATATATGATGAGCTCA-3'	33.33	44.5	
LCO	Folmer *et al.* (1994)	5'-GGTCAACAAATCATAAAGATATTGG-3'	32.0	57.44	642
HCO	"	5'-TAAACTTCAGGGTGACCAAAAAATCA-3'	34.62	63.57	

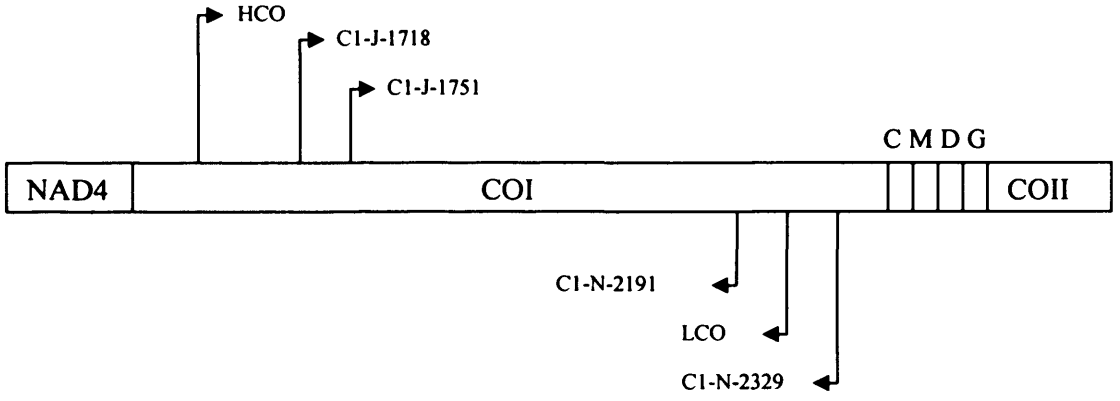


Figure 2.2.1 Relative positions of COI general invertebrate primers used in the current study. Arrows represent position and direction of primers. The abbreviations used are: COI and COII (cytochrome oxidase I and II genes), NAD4 (dehydrogenase 1), C M D and G (tRNA genes).

Table 2.2.2 Species-specific primers designed to amplify DNA from the nematodes *Steinernema feltiae*, *Steinernema kraussei*, *Heterorhabditis megidis* and *Phasmarhabditis hermaphrodita*, including information about the primer sequence, GC content, melting temperature of the primers and the rating of the primer based on NetPrimer software (PREMIER Biosoft International, CA, USA).

Primer name	Primer sequence (5' to 3')	GC(%)	Melting temp (°C)	bp	Rating (%)
SF-F-(326-346)	GTTTTTGGTTATTACCCACTG	38.1	52.18	21	86
SF-F-(429-447)	AGGCCATCCTGGAAACAGG	57.89	59.5	19	78
SF-R-(427-446)	CTGTTTCCAGGATGGCCTAA	50.0	57.66	20	79
SF-R-(610-629)	GCTAAAACCGGTAAAGAAAG	40.0	52.0	20	77
SF-R-(632-656)	TCTGTAAGAAGTATAGTAATTGCCC	36.0	54.95	25	90
SK-F-(433-451)	CACCCTGGTAATAGTGTTG	47.37	47.88	19	92
SK-R-(610-628)	CCAAGACAGGTAGTGATAG	47.37	44.03	19	100
HM-F-(288-307)	GGCACCTGATATGAGTTTTC	45.0	51.95	20	92
HM-R-(419-437)	GGATGACCTAAAGTTCTCA	42.11	46.4	19	91
Ph-F-(271-291)	GTTCCTCTTATGTTGGGTGCC	52.38	58.65	21	100
PH-F-(284-304)	TGGGTGCCCCTGATATAAGAT	47.62	58.66	21	91
PH-R-(411-431)	CCAAGGGTACTTAATGGTGGG	52.38	58.77	21	88
PH-R-(417-438)	CGGATGACCAAGGGTACTTAAT	45.45	57.88	22	89
PH-R-(479-501)	ACCCAAAATAGACCTTAAACCGG	43.48	61.03	23	80
PH-R-(616-636)	AGCACCTGCTAAAACAGGAAG	47.62	56.77	21	79

Table 2.2.3 Nematode-specific primer pairs for *Steinernema feltiae*, *Steinernema kraussei*, *Heterorhabditis megidis* and *Phasmarhabditis hermaphrodita* tested in this study with information about the amplified fragment length and the optimum annealing temperature. Shaded primer pairs indicate primers that were selected after testing.

Forward primer	Reverse Primer	Fragment length (bp)	Optimum annealing temperature (°C)
SF-F-(326-346)	SF-R-(427-446)	120	58
SF-F-(326-346)	SF-R-(610-629)	303	61
SF-F-(326-346)	SF-R-(632-656)	330	58
SF-F-(429-447)	**SF-R-(610-629)**	**200**	**62**
SF-F-(429-447	SF-R-(632-656)	227	60
SK-F-(433-451)	SK-R-(610-628)	195	63
Ph-F-(271-291)	PH-R-(417-438)	167	62
Ph-F-(271-291)	PH-R-(411-431)	160	58
Ph-F-(271-291)	PH-R-(479-501)	230	58
Ph-F-(271-291)	PH-R-(616-636)	365	60
PH-F-(284-304)	PH-R-(411-431)	147	60
PH-F-(284-304)	**PH-R-(417-438)**	**154**	**62**
PH-F-(284-304)	**PH-R-(479-501)**	**217**	**62**
PH-F-(284-304)	PH-R-(616-636)	352	57
HM-F-(288-307)	HM-R-(419-437)	149	60

Table 2.2.4 Alignment of *Phasmarhabditis hermaphrodita* specific primers PH-F-(284-304) and Ph-R-(479501) with a number of non-target predators and potential host species.

Species	Forward primer sequence (5'-3') PH-F-(284-304)	Reverse primer (Reverse complement) (5' - 3') PH-R-(479-501)
	TGGGTGCCCCTGATATAAGAT	CCGGTTTAAGGTCTATTTTGGGT
Phasmarhabditis hermaphrodita	--------------------	----------------------
Heterorhabditis megidis	----G--A--------G--T-	-T--------T--------A--T
Steinernema carpocapsae	----G--T----------T-	-A--AA-T--T--A-----A--G
Steinernema kraussei	-T-----T--A--------T-	-A---G-T--C------C-A--C
Steinernema feltiae	-T-----T--G---------	-T---G----C----------G
Caenorhabditis elegans	-A--A--A--C---------	-A--GT----A--------A--T
Folsomia candida	-T--G--T---------GCA-	-A---GCT--A--A-----A--G
Isotomurus sp.	-T--A--T--A------GCT-	-----GCGTCA--A-----A--T
Isotoma anglicana	-T--A--G--G-----GGCC-	-A--AGCGTCT--------A--G
Otiorhynchus sulcatus	T--G-----A-----GGCT-	-A--GG-TTCA-----CC-A--T
Agrotis ipsilon	-A--A--T--A------GCT-	-T--AA-TTCC------C-A--A
Delia radicum	-A-----T--A------GC--	-A--AA-TTCT--A-----A--A
Melolontha melolontha	-C--C----------GC--	----AA-CTCT--C---C-A--A
Stratiolaelaps lamington	-TTC-T-T---------GCC-	-A---GCCTCA--------AA-T
Deroceras reticulatum	-T--A--T--G--------T-	-A--GA-GTCT--------A--T
Arion hortensis	-A--G--T--G--C-----G-	-G--AA-GTCT--G--------T

Table 2.2.5 Alignment of *Steinernema feltiae* specific primers SF-F-(429-447) and SF-R-(610-629) with a number of non-target predators and potential host species.

Species	Forward primer (5'-3') SF-F-(429-447)	Reverse primer (Reverse complement) (5' - 3') SF-R-(610-629)
	AGGCCATCCTGGAAACAGG	CTTTCTTTACCGGTTTTAGC
Steinernema feltiae	-------------------	--------A----------
Phasmarhabditis hermaphrodita	T--T-----G--T-GA--T	T-A--AC----T--------
Heterorhabditis megidis	---T-----G----GA---	T-G--A--A--T--------
Steinernema carpocapsae	---T--C--C--T-GA--T	T-A-----A--T--------
Steinernema kraussei	---G--C-----T--T--T	--A--AC-A--T--C--G--
Caenorhabditis elegans	G--G--C--C----GT--A	T-A---C-A----------
Folsomia candida	C-CT---G-A---GCATCA	T-A--GC-A--T--------
Isotomurus sp.	--CA---G-A---GCGTCT	--A--AC----A--------
Isotoma anglicana	C-CG---G-----GCATCT	--A--AC----T--GC----
Otiorhynchus sulcatus	T-CA---GAA--TGC-TC-	-----AC----A--A-----
Agrotis ipsilon	T-CT---GGA----GATCA	T-A-----A--A--------
Delia radicum	C-CT---GGC---GCTTCT	--A-----A--A--------
Melolontha melolontha	T-C----AGA---GC-TCA	-----C--A--A--------
Stratiolaelaps lamington	TTCA---AGA--TGCTGC-	T-A-----A--A--------
Deroceras reticulatum	---T---G----GGCATCT	T-A-----A--T--------
Arion hortensis	--CT---AGG--GGCATCT	--C--A--G-----C-----

Table 2.2.6 Host and predator specific primers designed to amplify DNA from *Folsomia candida*, *Tenebrio molitor* and *Galleria mellonella*. Including information about the primer sequence, GC content, melting temperature of the primers and the rating of the primer based on NetPrimer software (PREMIER Biosoft International, CA, USA).

Primer name	Primer sequence (5' to 3')	GC(%)	Melting temp (°C)	bp	Rating (%)
FC-F-(406-427)	GTTTACCCCCCACTGTCAAGAG	54.55	60.2	22	100.0
FC-F-(440-462)	CAGGGGCTTCCGTAGATTTATCA	47.83	62.54	23	92.0
FC-F-(538-558)	CGAGTCGAAGGAATAACATGA	42.86	55.43	21	87.0
FC-R-(540-562)	GGTCTCATGTTATTCCTTCGACT	43.48	57.11	23	86.0
FC-R-(666-689)	GGATCAAAGAAGGAGGTATTTAGG	41.67	58.1	24	90.0
TM-F-(524-546)	CAGTAATCAACATACGACCACAG	43.48	55.25	23	100.0
TM-F-(332-354)	GATTGCTACCCCCTTCACTAAGA	47.83	59.66	23	91.0
TM-F-(406-428)	GTTTATCCACCCTTATCCTCTAA	39.13	54.75	23	92.0
TM-R-(526-548)	CCCTGTGGTCGTATGTTGATTAC	47.83	58.87	23	94.0
TM-R-(666-689)	GGGTCAAAGAAGGATGTATTAATG	37.5	57.24	24	84.0
GM-F-(295-315)	GACATAGCTTTCCCCCGACTT	52.38	60.01	21	88.0
GM-F-(422-447)	CTTCTAATATTGCCCATAGAGGTAGA	38.46	58.43	26	85.0
GM-R-(469-491)	GAAATTCCAGCTAAATGGAGAGA	39.13	57.67	23	84.0
GM-R-(672-692)	GCAGGGTCAAAAAATGATGTG	42.86	58.02	21	100.0

Table 2.2.7 Host and predator specific primer pairs designed to amplify DNA from *Folsomia candida*, *Tenebrio molitor* and *Galleria mellonella*. Also included, information about the amplified fragment length and the optimum annealing temperature. Shaded primer pairs indicate primers that were selected after testing.

Forward primer	Reverse Primer	Fragment length (bp)	Optimum annealing temperature (°C)
FC-F-(406-427)	**FC-R-(540-562)**	156	**60**
FC-F-(406-427)	FC-R-(666-689)	283	62
FC-F-(440-462)	FC-R-(540-562)	122	61
FC-F-(440-462)	FC-R-(666-689)	249	60
FC-F-(538-558)	FC-R-(666-689)	151	62
TM-F-(524-546)	TM-R-(666-689)	165	59
TM-F-(332-354)	**TM-R-(526-548)**	216	**60**
TM-F-(406-428)	TM-R-(526-548)	142	60
TM-F-(406-428)	TM-R-(666-689)	283	62
GM-F-(295-315)	GM-R-(469-491)	196	66
GM-F-(295-315)	GM-R-(672-692)	397	64
GM-F-(422-447)	GM-R-(672-692)	270	63

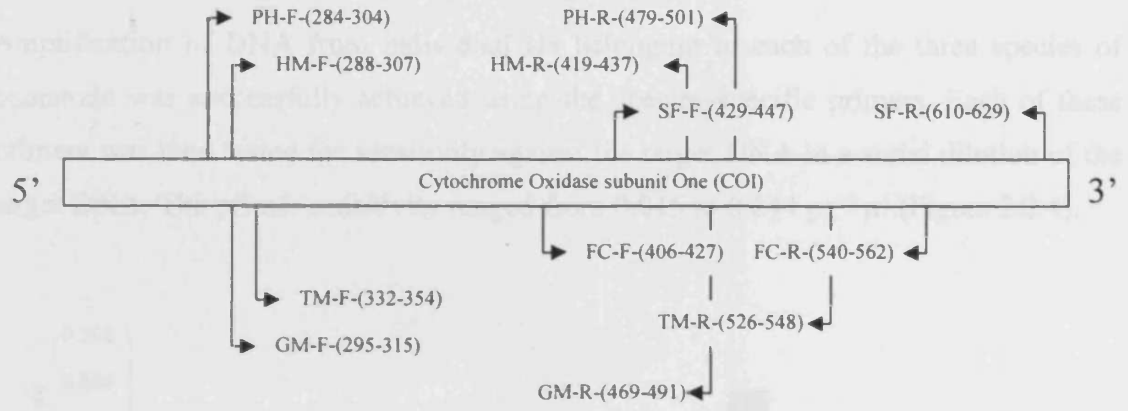


Figure 2.2.2 Position of species-specific primers on Cytochrome Oxidase subunit one (COI), for the nematodes *Phasmarhabditis hermaphrodita*, *Heterorhabditis megidis* and *Steinernema feltiae*, and for the insect hosts *Tenebrio molitor* and *Galleria mellonella*, and the collembolan *Folsomia candida*.

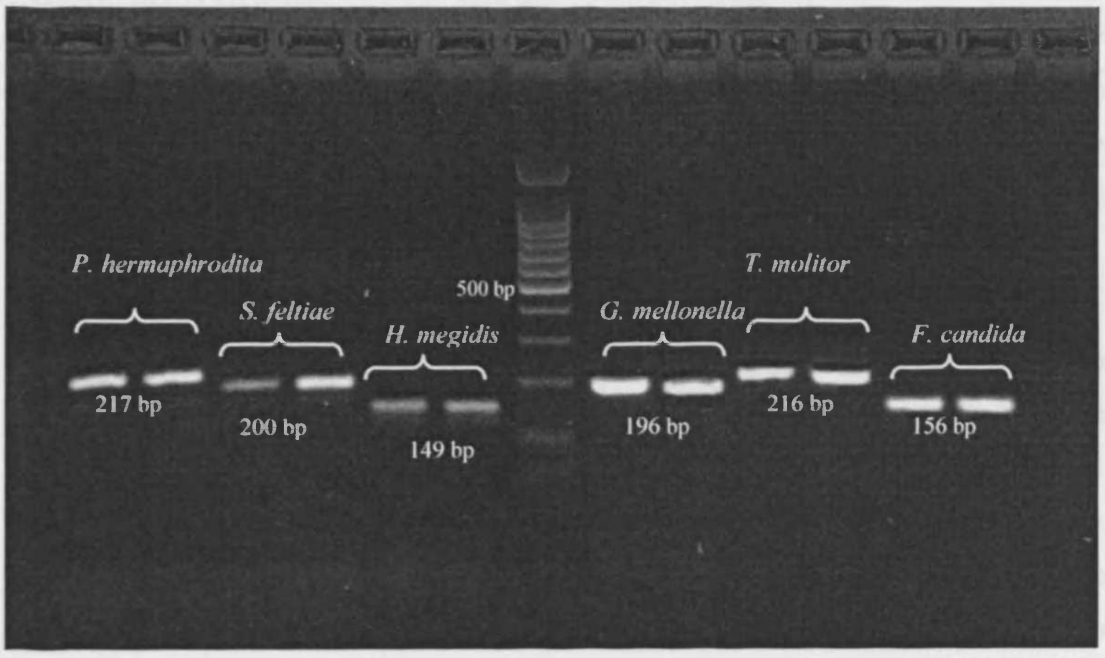


Figure 2.2.3 Ethidium-bromide stained agarose gel showing size of amplified products using species specific primers for the nematodes *Phasmarhabditis hermaphrodita*, *Heterorhabditis megidis* and *Steinernema feltiae*, and for the insect hosts *Tenebrio molitor* and *Galleria mellonella*, and the collembolan *Folsomia candida*.

2.2.2. Primer testing and non-target testing

Amplification of DNA from individual IJs belonging to each of the three species of nematode was successfully achieved using the species-specific primers. Each of these primers was then tested for sensitivity against the target DNA in a serial dilution of the target DNA. The primer sensitivity ranged from 0.015 to 0.244 pg / µl (Figure 2.2.4).

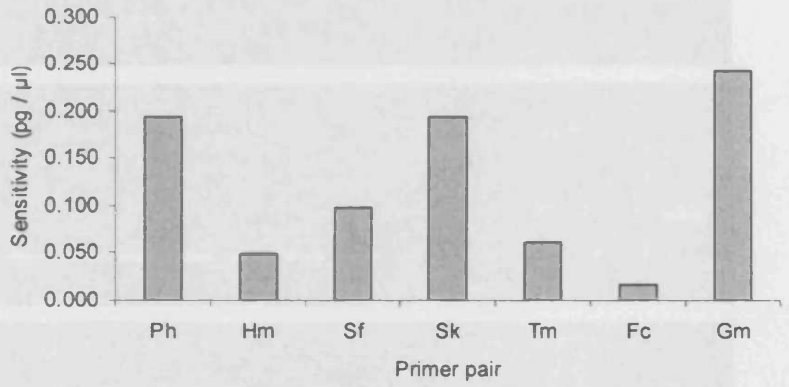


Figure 2.2.4 Sensitivity of the primer pairs used in this study calculated from serial dilutions of the template DNA. Sensitivity is shown in (pg / µl) of template DNA. Ph = *Phasmarhabditis hermaphrodita*. Hm = *Heterorhabditis megidis*. SF = *Steinernema feltiae*. SK = *Steinernema kraussei*. TM = *Tenebrio molitor*. FC = *Folsomia candida*. GM = *Galleria mellonella*.

Each of the primer pairs were tested for optimum annealing temperatures on a gradient PCR. Ethidium-bromide stained agarose gels showing the PCR products from these optimisation PCRs are shown in Figure 2.2.5. Primers were then tested against a number of non-target organisms to ensure species-specificity (Table 2.1 in Appendix 2 shows non-target species tested with *P. hermaphrodita* specific primers). The final primer pair for each of the target species are shown, highlighted, in Tables 2.2.3 and 2.2.7). None of these primers showed any non-target amplification with any of the organisms used in this study (Figure 2.1 in Appendix 2 shows the results of non-target testing for primers *PH-F-(284-304)* and *Ph-R-(479501)* on an ethidium-bromide stained gel). These were the primer pairs that were used for the rest of this investigation.

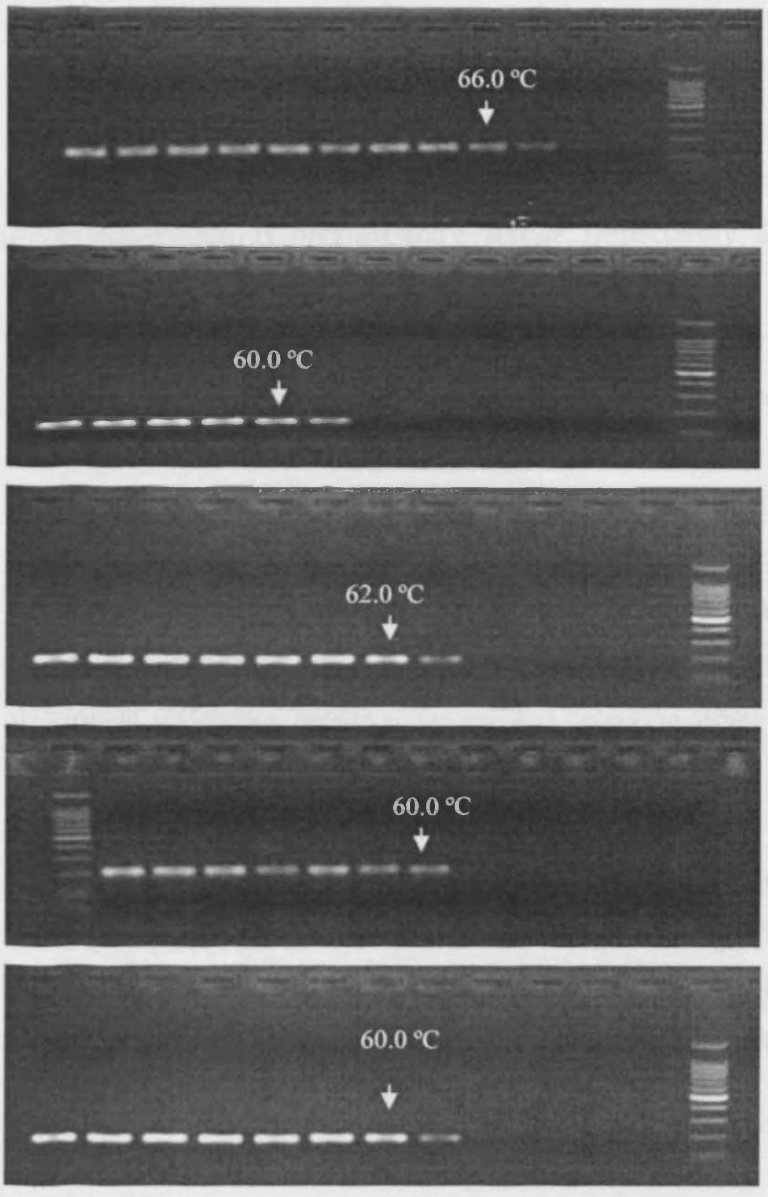


Figure 2.2.5 (A-E) Ethidium-bromide stained agarose-gels showing PCR products from gradient PCRs for the amplification of DNA from A) *Galleria mellonella* using primers *GM-F-(295-315)* and *GM-R-(469-491)*; B) *Heterorhabditis megidis* using primers *HM-F-(288-307)* and *HM-R-(419-437)*; C) *Steinernema feltiae* using primers *SF-R-(610-629)* and *SF-F-(429-447)*; D) *Tenebrio molitor* using primers *TM-R-(526-548)* and *TM-F-(332-354)*; and E) *Folsomia candida* using primers *FC-R-(540-562)* and *FC-F-(406-427)*. Arrows and temperatures indicate chosen optimum annealing temperature for subsequent PCR.

2.2.3. Post-ingestion detection of nematode DNA

In separate feeding trials, the collembolan *F. candida* was screened for the presence of target nematode DNA within its gut after feeding *ad libitum* for 2 h on nematode IJs of *P. hermaphrodita*, *H. megidis* and *S. feltiae*, respectively. DNA extraction success using the Qiagen kit was confirmed to be 100 % after screening with the general invertebrate primers *C1-J-1718* and *C1-J2191*. Screening DNA extracts with nematode-specific primers showed nematode DNA could be detected for up to 24 h after feeding by *F. candida* on each of the three nematode species (Figure 2.2.6). Fitted Probit models described the data well at the 95% level for the *H. megidis* and *S. feltiae* feeding trials (Table 2.2.8). The model fit was not as good for the *P. hermaphrodita* feeding trial, although it described the data adequately. Median detection times ranged from 10.48 h to 14.76 h. (Table 2.2.9). Overlapping 83.5% fiducial limits indicated there was no significant difference between the median detection times of the three feeding trials at the 95% level.

Detection of *H. megidis* and *P. hermaphrodita* DNA within the gut of the mite *S. miles* was possible for up to 12 h after ingestion (Figure 2.2.7). Fitted Probit models described the data well at the 95 % level for both feeding trials (Table 2.2.8). Overlapping 83.5% fiducial limits indicated no significant difference at the 95% level (Table 2.2.9).

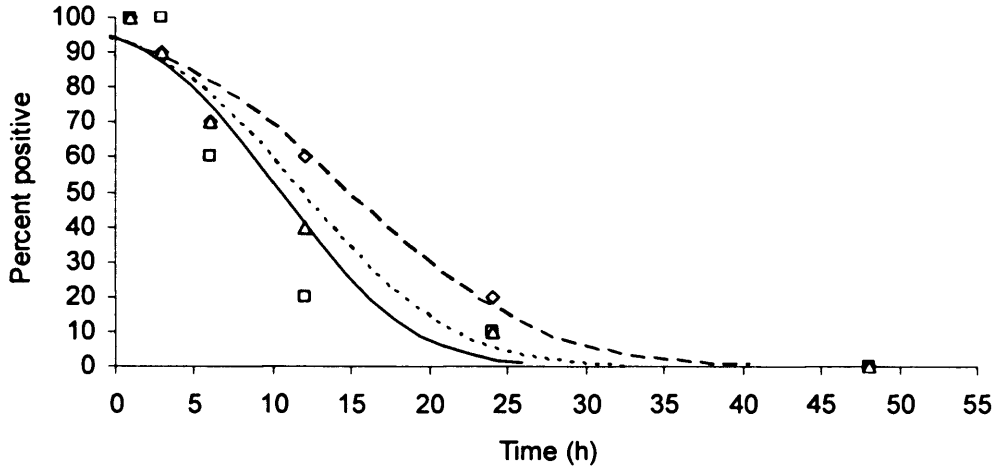


Figure 2.2.6 Post-ingestion detection of DNA from the nematodes *Phasmarhabditis hermaphrodita* (□ ——), *Heterorhabditis megidis* (◊ — —), and *Steinernema feltiae* (Δ ----) in the gut of *Folsomia candida* using primers Ph-F-(271-291) and PH-R-(417-438) against time since feeding. Lines represent fitted Probit models.

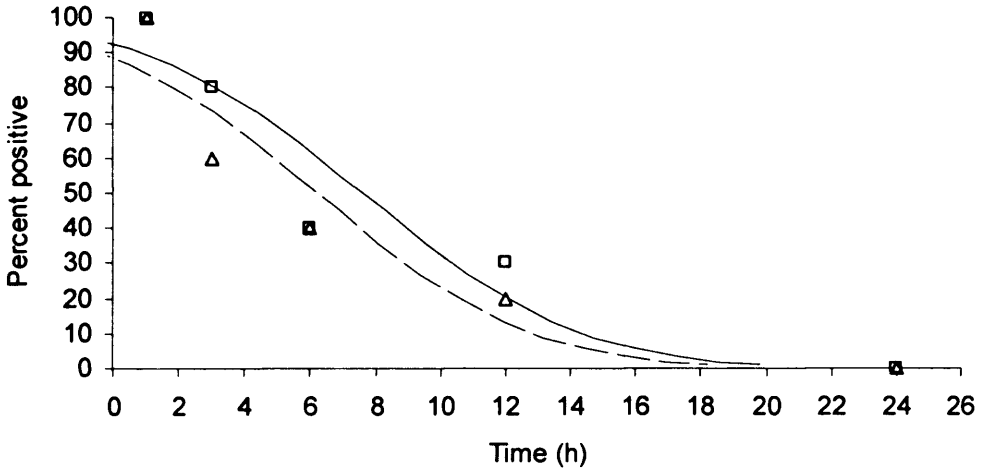


Figure 2.2.7 Post-ingestion detection of DNA from the nematodes *Phasmarhabditis hermaphrodita* (□ ——) and *Heterorhabditis megidis* (Δ — —) in the gut of *Stratiolaelaps miles* using primers Ph-F-(271-291) and PH-R-(417-438) against time since feeding. Lines represent fitted Probit models.

Table 2.2.8 Goodness of fit tests of the Probit models for probability of detecting DNA from the nematodes *Phasmarhabditis hermaphrodita*, *Heterorhabditis megidis*, and *Steinernema feltiae* in the gut of *Folsomia candida* and *Stratiolaelaps miles*.

Predator	Prey	Statistic	Chi-Square	df	P-Value
F. candida	*P. hermaphrodita*	Pearson	8.464	4	0.076
		Deviance	9.072	4	0.059
	H. megidis	Pearson	1.867	4	0.76
		Deviance	2.510	4	0.643
	S. feltiae	Pearson	1.997	4	0.736
		Deviance	2.643	4	0.619
S. miles	*P. hermaphrodita*	Pearson	3.829	3	0.280
		Deviance	4.749	3	0.191
	H. megidis	Pearson	3.721	3	0.293
		Deviance	5.150	3	0.161

Table 2.2.9 Overlap of 83.5% confidence intervals for the mean for detection of nematode DNA from *Phasmarhabditis hermaphrodita*, *Heterorhabditis megidis*, and *Steinernema feltiae* in the gut of *Folsomia candida* and *Stratiolaelaps miles*, calculated from the fitted Probit model.

Predator	Prey	Parameter	Estimate (hours)	Standard Error	Lower 83.5% CI	Upper 83.5 % CI
F. candida	*P. hermaphrodita*	Mean	10.48	1.58	8.291	12.670
	H. megidis	Mean	14.76	2.32	11.552	17.983
	S. feltiae	Mean	11.87	1.80	9.370	14.368
S. miles	*P. hermaphrodita*	Mean	7.606	1.25525	5.863	9.349
	H. megidis	Mean	6.21849	1.1648	4.601	7.836

2.3. Discussion

Probes for COI PCR were designed for specificity towards three species of insect and mollusc parasitic nematode (*Phasmarhabditis hermaphrodita*, *Heterorhabditis megidis* and *Steinernema feltiae*), two insect hosts (*Tenebrio molitor* and *Galleria mellonella*) and one potential micro-arthropod predator (*Folsomia candida*). Although a number of different general primers were used for the amplification and sequencing of the nematode, host and predator targets, there was sufficient overlap between the comparative positions of the sequences to design species-specific primers based on the intra-species variability between species. The design of multiple forward and reverse primers for each of the target species allowed several primer pairs to be tested for each species, so that the optimum pair could be selected for screening. This was important, as high theoretical primer rating and specificity did not always translate into primers that performed acceptably. Primer pairs were tested for the ability to amplify the target DNA, optimum annealing temperature, and specificity and sensitivity towards the target.

Unlike previous molecular methods used for identifying prey remains in the guts of predators, such as monoclonal antibodies, diagnostic PCR is not quantifiable (Symondson 2002). Therefore, it is important to characterise and optimise the PCR system to ensure no false positives or negatives occur during screening. The PCR reaction must consistently amplify the target DNA to ensure no false positives occur. It is expected that the quantity of DNA in the gut of a predator will decline with time, possibly due to digestion and movement of the stomach contents through the digestive system. It is also likely that the prey DNA will be diluted within large quantities of the predator DNA (Deagle *et al.* 2006), or if the predator is a generalist, DNA from alternative prey (Harper *et al.* 2006). As a result, it is desirable to use a PCR reaction with a high sensitivity towards the target. High PCR sensitivity is also advantageous to help overcome potential PCR inhibition that may originate from the prey or predator tissues. PCR inhibition may also originate from the environment, as soil is known to contain substances that inhibit PCR (Dauch *et al.* 2006; Sutlovic *et al.* 2005), a problem that is particularly apparent when screening soil-dwelling organisms (Juen and Traugott 2006). The sensitivity of the primers used in this study varied from 0.015 to 0.244 pg /

µl. These results are comparable to those of Juen and Traugott (2005) whose primers were designed to detect parasitoids within Lepidopteran pests.

Primers were designed to amplify fragments < 300 bp, due to reports that the length of the amplified target DNA influences the detection period. There have been several different studies on this subject. Chen *et al.* (2000) found no difference in the amplification of fragments of 198, 246 and 339 bp in one predator (*Hippodamia convergens*), but found the larger fragment to have a significantly lower detection half-life in the gut of another predator (*Hippodamia plorabunda*). Similarly, Juen and Traugott (2005) found no difference in the amplification of fragments between 175 and 585 bp up to 24 h post feeding, although at 32 h the 585 bp fragment was significantly less likely to be detected. More recently, in an investigation into the detection of scarab beetle larvae in the gut of a carabid predator, no difference was found in the detection periods utilising 127 bp and 463 bp fragments (Juen and Traugott, 2006). Support for fragment length influencing detection periods comes from studies carried out by Agusti *et al.* (1999, 2000), Hoogendoorn and Heimpel (2001), Zaidi *et al.* (1999) and de Leon *et al.* (2006), all of which found that smaller amplified fragments have longer detection periods than larger fragments within the gut of invertebrate predators. In this study there was no significant difference between the median detection times of fragments ranging from 149 bp to 217 bp, although any potential size related effects may have been obscured by the fact that these primers amplified DNA from different nematode species, and were used to screen different feeding trials.

Field environments are highly diverse, both in terms of the number of species and genetic diversity. Because of this it is important to design primer pairs that have a high specificity to the target, and to test them against a wide range of common non-target organisms found at the study site. In this investigation, only primer pair *Ph-F-(271-291)* and *PH-R-(479-501)* was used to screen field caught predators (Chapter 4). This primer pair was tested against 74 species of non-target invertebrate and a total of 48 unidentified nematodes collected from the field site. Although this represents a small fraction of the total diversity found in the field, the fact that no cross-amplification occurred allows the primers to be used with confidence.

In the laboratory feeding trials it was possible to detect DNA from three different species of nematode within the gut of *F. candida* for up to 24 h post feeding, with a mean detection half life of 12.37 h. Whilst this is less than some previous studies with macroarthropod predators (e.g. Zaidi *et al.* 1999; Agustí *et al.* 2003a; Sheppard *et al.* 2004), the detection period was sufficient to determine whether predation is occurring within ecologically relevant time periods (e.g. day / night), but not so long that it obscured temporal feeding patterns that might occur in the field (Holland *et al.* 1999). The decay rates of DNA in the guts of the predators did not differ significantly between the three species of nematode. The detection period for nematode DNA within the guts of the predatory soil mite, *S. miles*, was 45% less than the mean for collembola. This may be caused by a number of factors, including voracity, metabolic rates and body size. Adult *S. miles* are approximately 810 µm long (Jess and Bingham, 2004), whereas *F. candida* are up to 3000 µm in length (Fountain and Hopkin, 2004). The mites can probably consume fewer nematodes per individual than are consumed by collembola in the time that was given for them to feed. Although less, the detection half-life in this species of mite is still sufficient to determine temporal feeding patterns in the field. The only comparable study was by Cuthbertson *et al.* (2003), investigating predation by the predatory mite *Anystis baccarum* on the apple grass aphid, *Rhopalosiphum insertum*. Although detection periods of up to 48 h were achieved, it is hard to compare these results to the present study due to the lack of replication in the *A. baccarum* feeding trial.

The gut of the collembolan *F. candida* is a cylindrical structure divided into three parts, a cuticle lined foregut and hindgut, and a midgut derived from endodermal cells (Fountain and Hopkin 2005). The gut passage time of *F. candida* has previously been determined by Thimm *et al.* (1998) by feeding starved adults with brewers yeast coloured with charcoal. They found the gut retention time to be approximately 35 min. This visual analysis of gut retention time is far shorter than the detection times experienced in this study. However, Wolters (1985) found that the gut retention time in the collembolan *Tomocerus flavescens*, when starved after feeding (as occurred in the feeding trials in this study), increased to approximately 48 h, and at lower temperatures *T. flavescens* can increase its gut retention times to more than two weeks (Aitchison 1983).

The results obtained from these feeding trials have shown that these molecular methods are suitable for reliably detecting predation by microarthropods, and that these detection periods are long enough to determine feeding patterns in the field.

Chapter 3

Molecular detection of predation by soil-dwelling microarthropods on nematodes: Quantification of detection errors.

Abstract

Molecular detection of predation using species-specific probes for PCR has successfully been applied to detect the diet of a number of different macroarthropod species. So far, however, there have been few examples of these techniques being applied for the detection of predation by microarthropods, especially in below-ground soil food-webs. Species-specific probes for the mollusc and entomopathogenic nematodes *Heterorhabditis megidis*, *Steinernema feltiae* and *Phasmarhabditis hermaphrodita* were used as a model system for the detection of predation by the collembolan *Folsomia candida*. A number of potential sources of prey detection error were investigated, including those that cause false positives (infection of predator, phoresy and scavenging) and changes in detection times (predator size and ambient temperature). Both infection and phoresy were not significant sources of error. However, due to the persistence of nematode DNA after death (T_{50} = 96 h), scavenging was identified as a significant source of error in this system. Both temperature and the body size of the model collembolan predator were found to affect prey detection times, with increasing temperature and body size resulting in decreased median detection periods. Feeding-trials were used to quantify the effect of predation by *F. candida* on the persistence and efficacy of the entomopathogenic nematode *H. megidis*. Predation was found to cause significant reductions in nematode numbers on plaster-of-Paris (46 % in 10 h) and soil (49 % in 120 h). Importantly, this translated into a reduction in the number of nematode infected hosts.

3.1 Introduction

Little is known about the factors that affect nematode abundance and community composition or about the relative contributions of bottom-up (food resources) and top-down (especially predation) processes on nematode population dynamics. A major factor inhibiting such research is that soils are relatively impervious to direct observation, forcing research in this area to concentrate upon observations under artificial conditions in the laboratory, or to rely on associational evidence in manipulated plots. Species diversity can be measured by extracting nematode DNA from substrates (Foucher *et al.* 2004) but this reveals little about population density or trophic interactions.

The role of top-down processes on influencing nematode population dynamics has received relatively little attention, mainly due to the difficulties in identifying the organisms that are potential nematode predators. Despite this, there is some evidence that nematophagy may be widespread, with potential predators of nematodes including turbellarians, other nematodes, tardigrades, oligochaetes, mites, collembola and insects (Muraoka and Ishibashi 1976; Jatala 1986; Sayre and Walter 1991; Kaya and Koppenhofer 1996). The collembola are a widespread and diverse group of arthropods, with densities of up to 100 000 individuals per m^2 recorded in agricultural fields (Koehler 1999). The feeding habits of collembola have been investigated using a number of different methods, including visual gut analysis, stable isotopes, fatty acid analysis, digestive enzymes and laboratory-based choice tests. Visual identification of the gut contents of collembola has been conducted in a variety of habitats, including a coastal temperate forest (Addison *et al.* 2003), temperate chestnut woodland (Anderson and Healy 1972), beech forests (Ponge 2000), moss (Varga *et al.* 2002) fungal fruiting bodies (Greenslade *et al.* 2002) and polluted habitats including tar (Gillet and Ponge 2005) and metal polluted sites (Gillet and Ponge 2003). The majority of studies based on visual identification of gut contents have found fungal hyphae to be the dominant food item (Greenslade *et al.* 2002; Addison *et al.* 2003), along with fungal spores (Anderson and Healey 1972), detritus (Varga *et al.* 2002), micro-algae and pollen grains (Ponge 2000). Visual identification of gut contents relies on the identification of food items that have resisted digestion, and may therefore lead to an overestimation of the indigestible portion of the diet. Evidence of this can be found in a survey of the diet of

collembola in a beech forest, in which no individuals of the collembolan *Freesia truncata* were found to contain identifiable food remains (Ponge 2000). This genus is known to mainly eat micro-fauna, eggs and the moults of small animals (Singh 1969), indicating that predation may be underestimated in visual gut analysis. A high proportion of collembola collected from the field do not have any visible gut contents (Anderson and Healy 1972; Greenslade *et al.* 2002), and this may be due to food being transparent, liquid or quickly digested.

Stable isotopes of carbon and nitrogen, and the analysis of fatty acids, have been used to identify feeding preferences both in the laboratory and the field (Chamberlain *et al.* 2005; Ruess *et al.* 2005; Haubert *et al.* 2006). In the laboratory, fatty acid analysis has been used to show that the collembola *Folsomia candida* and *Proisotoma minuta* preferentially fed on the nematode *Panagrellus redivivus* over the soil fungus *Cladosporium cladosporioides* (see Chamberlain *et al.* 2006), and that fungal, bacterial and nematode diets are readily consumed (Ruess 2004; Haubert *et al.* 2005, 2006). Application of nitrogen stable isotope ratios to field-collected collembola have shown that collembolan species form a continuum, from phycophages/herbivores to primary and secondary decomposers (Chahartaghi *et al.* 2005), indicating that dietary specialization must be occurring. One of the advantages of fatty acid analysis and C/N ratios is that food sources are identified based on the composition of body tissues, not the gut contents. This allows for the extraction of collembola from soil samples via a temperature gradient, a process that is often time consuming, and could result in a change in the composition of the gut contents. A disadvantage is that these techniques can only group individuals into general trophic levels based on the trophic shift of stable isotopes (Scheu 2000) or the composition of fatty acids found in the body tissues (Chahartaghi *et al.* 2005). As a result it is only possible to make broad generalizations about trophic links, and it is not possible to identify species-specific links in the food chain.

Information from laboratory-based choice tests have shown clear evidence for feeding preference between collembolan species, including the choice of nematodes over fungi (Lee and Widden 1997) and a preference for particular species and groups of fungi over others (Anderson and Healy 1972; Varga *et al.* 2002; Jorgensen *et al.* 2003). However,

laboratory-based food preference tests do not generally correspond with gut contents analyses of field specimens (Werner and Dindal 1987).

DNA-based gut analysis has been successfully applied to many different macroarthropods, including carabid beetles (Harper *et al.* 2005; Juen and Traugott 2005), linyphiid spiders (Agustí *et al.* 2003) and predatory bugs (Ma *et al.* 2005). However, microarthropods have rarely been the subject of investigation: Cuthbertson *et al.* (2003) screened the predatory mite *Anystis baccarum* for predation on the apple grass aphid *Rhopalosiphum insertum,* and Jorgensen *et al.* (2005) screened the collembolan *Protaphorura armata* for ingested fungal species. Despite these studies, little is known about the factors that influence detection periods and the accuracy of PCR-based gut contents screening. Before PCR-based techniques can be applied to screen microarthropods for their gut contents from the field, they must be fully tested and any potential errors quantified.

A number of different factors may influence the detection of DNA in the guts of predators, causing false positives or negatives, or altering detection period. As this form of diet detection is essentially diagnostic, with a positive indicating predation and the absence of a positive indicating none, the need to have a low level of error and the elimination of false positives or negatives is obvious. Factors that may cause false positives in the current study system include external contamination of target prey DNA through phoresy by prey nematodes and scavenging on nematode cadavers. The role of scavenging in causing false PCR positives has been assessed for carabid beetles feeding on slugs and aphids (Foltan *et al.* 2005) and on scarab beetle larvae (Juen and Traugott 2005). The potential error caused by secondary predation (in which a predator consumed another predator which has in turn consumed the target prey) has also been assessed for carabid beetles, feeding on aphid prey (Sheppard *et al.* 2005). However, the magnitude of these errors depends on a number of factors, and may be different for each predator-prey system that is tested.

Accurate determination of detection times, usually measured through median detection times (the time for 50 % of the individuals to test positive after consuming the prey item), is important if comparisons about the impact of predation by more than one species of predator are to be done. If this is not conducted, the impact of predators with

longer prey detection times, due to slower rates of digestion, will be overestimated when compared to predators with short detection times. A number of factors may influence detection times in the guts of invertebrate predators, including: ambient temperature, size of the predator, locomotory activity, amount of prey consumed and the presence of alternative prey in the gut.

This chapter describes a study to investigate the potential for DNA-based detection methods to determine the diet of soil-dwelling microarthropods, with a particular focus on the consumption of the infective juveniles (IJs) of commercially available insect and mollusc parasitic nematodes. Model microarthropod predators were used for this study, including the collembolan *Folsomia candida* (Collembola: Isotomidae) and the mesostigmatid mite *Stratiolaelaps miles* (Mesostigmata: Laelapidae). As model prey, three parasitic nematode species were used: one mollusc parasite, *Phasmarhabditis hermaphrodita* (Rhabditida: Rhabditidae), and two insects parasites, *Steinernema feltiae* (Rhabditida: Steinernematidae) and *Heterorhabditis megidis* (Rhabditida: Heterorhabditidae). Species-specific primers (for development, see Chapter 2, Section 2.1.5.1) were used to investigate predation by microarthropods on these nematode species, investigate the factors that influence median detection periods, and to explore the potential sources of error involved in DNA-based gut screening. However, as the PCR-based techniques used in this study cannot quantify rates of predation, the influence of collembolan predation on the persistence and efficacy of mollusc- and insect- parasitic nematodes was investigated in a number of laboratory-based experiments. Development of these techniques will allow a better understanding of the dietary choices of soil-dwelling microarthropods, which in turn will provide a clearer picture of the potential impact of top-down control by nematophagous microarthropods upon nematode populations.

3.2 Materials and Methods

3.2.1 Non-target effect of parasitic nematodes on microarthropods

To test if entomopathogenic and mollusc-parasitic nematodes in this study have any negative effects (including the ability to infect and reproduce) on soil-dwelling microarthropods, the following experiment was carried out. The collembolan *Folsomia candida* and the mesostigmatid mite *Stratiolaelaps miles* were used as model organisms. Ten adult *F. candida* (defined as those that would not pass through a 250 μm mesh) and *S. miles* were placed in bioassay arenas consisting of plastic containers (85 mm dia., 45 mm depth) with a moistened activated charcoal: plaster-of-Paris base (1:250). Food was provided *ad libitum* in the form of dried active yeast (Allinson, UK) for the collembola, and grain mites *Acarus* sp. (Astigmata: Acaridae) for the predatory mites. In separate bioassays, the collembola and mites were exposed to the commercially recommended field dose of *Phasmarhabditis hermaphrodita* (30 per cm^2), *Heterorhabditis megidis* (50 per cm^2) and *Steinernema feltiae* (50 per cm^2). Controls consisted of identical bioassay arenas, with no nematodes added. Four replicates of each treatment were carried out. The bioassay arenas were maintained at 16 ± 1 °C in the dark. Collembola and mites were examined on a daily basis for a total of 15 days and the number of dead individuals was recorded. Any dead microarthropods were examined under a stereo microscope for signs of nematode infection.

3.2.2 Phoresy by parasitic nematodes on microarthropods

To investigate the potential for phoresy (attachment to the outside of other organisms for transport) by parasitic nematodes on soil-dwelling microarthropods, live IJs of *P. hermaphrodita*, *H. megidis*, or *S. feltiae* were added separately to bioassay arenas as described previously (Section 3.2.1). The nematodes were applied in 2 ml of water at the commercially recommended field rate (*P. hermaphrodita* 30 per cm^2, *H. megidis* and *S. feltiae* 50 per cm^2). To each of these containers, twenty adult *F. candida* were added. The containers were left for 2 h in the dark at 16 ± 1 °C). After this time, the collembola were carefully removed using a pooter and transferred to an identical container with no nematodes. Ten model invertebrate hosts, *Deroceras reticulatum* (100-150 mg), *Tenebrio molitor* (80-120 mg) and *Galleria mellonella* (100-120 mg), were added to containers with collembola that had been exposed to *P. hermaphrodita*,

H. megidis and *S. feltiae* respectively. Five replicates were carried out for each treatment. The collembola were left with the hosts and stored at 20 ± 2 °C in the dark for a further 72 h, after which point the mortality of the hosts and signs of infection was recorded.

To test whether nematode phoresy or contamination by nematode DNA was a possible source of error when using molecular methods to screen predatory microarthropods, *F. candida* were placed in feeding arenas and exposed to the three nematode parasites as described previously. To investigate whether potential false PCR-positives could be due to active attempts by live nematodes to attach to the collembola (phoresy), or due to contamination by nematode DNA, the previous experimental setup was replicated, but using either live or pre-killed nematode IJs (frozen at -80 °C for 24 h and then defrosted at room temperature for 1 h). The containers were left for 2 h in the dark at 16 ± 1 °C. After this time, the collembola were carefully removed using a pooter. Eight collembola from each treatment were killed by freezing at -80 °C for 15 min. These were examined under a high power stereo microscope for any signs of nematode attachment. A further sixteen *F. candida* from each treatment were placed individually in 1.5 ml centrifuge tubes with 500 µl of DNA-free water (Sigma, UK). The tubes were vortexed at high speed for approximately 30 s to detach any nematodes or nematode fragments from the surface of the collembolan cuticle. The collembola were then removed using a pair of sterile tweezers and discarded. The tube was centrifuged for 10 min at 13,000 rpm to pellet any nematode fragments present in the water. The excess water was carefully removed using a sterile pipette, leaving approximately 25 µl and the samples were stored at -80 °C. The samples were screened for the presence of *P. hermaphrodita* DNA (see section 3.2.8).

3.2.3 Detecting predation by microarthropods in the laboratory

To investigate how widespread nematophagy was amongst collembola and mites in laboratory feeding-trials, 13 different species of microarthropod were obtained, including the collembola: *F. candida*, *Protaphorura armata* (Collembola: Onychiuridae), *Heteromurus nitidus* (Collembola: Entomobryidae), *Isotomurus palustris* (Collembola: Isotomidae), *Isotoma viridis* (Isotomidae), *Orchesella villosa* (Entomobryidae), *Orchesella cincta* (Entomobryidae), *Entomobrya nivalis*

(Entomobryidae), *Proisotoma minuta* (Entomobryidae), *Lepidocyrtus cyaneus* (Entomobryidae), *Tomocerus longicornis* (Collembola: Tomoceridae), and the mites *S. miles* and *Hypoaspis aculeifer* (Mesostigmata: Laelapidae). The collembola *F. candida*, *H. nitidus*, *P. minuta* and *P. armata* were obtained from established laboratory cultures at Cardiff University (see Chapter 2, Section 2.1.1). The remaining collembolan species were either collected from arable fields at Wenvoe, near Cardiff, or from gardens in Cardiff. The mites were bought from commercial suppliers (HarrordHorticultural, Lowestoft, UK) and used within two days of purchase. A minimum of 20 individuals from each species were collected and stored in plastic containers with a moistened charcoal: plaster-of-Paris base (1:250), and starved for at least 72 h before the start of the experiment. *Phasmarhabditis hermaphrodita* IJs were added to each container at a rate of 30 per cm^2 in 1 ml of water. The microarthropods were allowed to feed for 2 h in the dark at 16 ± 1 °C. Sixteen individuals of each species were removed using a pooter and stored at -80 °C. An effort was made to select individuals that had visible gut contents, although this was not always possible with the larger or more heavily pigmented species. After DNA extraction, the samples were screened for extraction success using general primers, and samples that had amplifiable DNA were then screened for the presence of *P. hermaphrodita* (see section 3.2.8).

3.2.4 The effect of collembolan size class on the post-ingestion detection period of nematode DNA

Within a species of microarthropod, the age and/or size of individuals may have a significant influence on diet choice, digestion ability and gut retention time. To investigate the influence of the size of the collembolan *F. candida* on post-ingestion, prey detection times, individuals were starved for 72 h, and divided by sieving into five different size groups, according to the nominal aperture that they would fit through (+ 400 µm, 400-250 µm, 250-200 µm, 200-140 µm and 140-125 µm). At least 70 individuals were collected at each size class, and placed in clean bioassay arenas, as described previously. The collembola were fed live *P. hermaphrodita* IJs at a rate of 30 per cm^2 in 1 ml of tap water. The collembola were allowed to feed for 2 h, before being removed to clean containers and stored in the dark at 16 ± 1 °C. Eight collembola from each size class were carefully removed using a pooter 1 h, 3 h, 6 h, 12 h, 24 h and 36 h after the mid-point of the feeding period, placed individually in 1.5 ml centrifuge tubes,

and stored at -80 °C. The samples were screened for extraction success using *F. candida* specific primers and all samples with amplifiable DNA were then screened for *P. hermaphrodita* DNA (see section 3.2.8).

3.2.5 Effect of ambient temperature on the detection of nematode DNA in the gut of the collembolan *Folsomia candida*

Ambient temperature may have a significant effect on the detection times of prey DNA within predatory microarthropods. To investigate this, feeding trials were carried out involving adult *F. candida* (defined as those that would not pass through a 250 μm mesh), placed in clean bioassay arenas, as described previously. The collembola were starved for a period of 72 h before the feeding trial. *P. hermaphrodita* IJs were applied to the surface of each bioassay arena in 1 ml of water at a rate of 30 cm^2. The collembola were allowed to feed for a period of 2 h at 16 ± 1 °C in the dark, after which the collembola were removed using a pooter, divided and placed in six clean containers. The containers were stored at the following constant temperatures; 4, 8, 12, 16, 20, and 24 ± 1 °C. Eight individual *F. candida* were removed at times 1, 3, 6, 12, 24, 36, 48 and 72 h from each of the containers after the mid point of the initial feeding period (see Figure 3.2.1). These were placed individually in clean 1.5 ml micro-centrifuge tubes and immediately frozen at -80 °C to preserve the samples. Negative control consisted of 20 starved *F. candida*. Individuals of *F. candida* were placed in 1.5 ml centrifuge tubes and stored at -80 °C. The samples were screened for extraction success using *F. candida* specific primers, and all samples with amplifiable DNA were then screened for *P. hermaphrodita* DNA (see section 3.2.8).

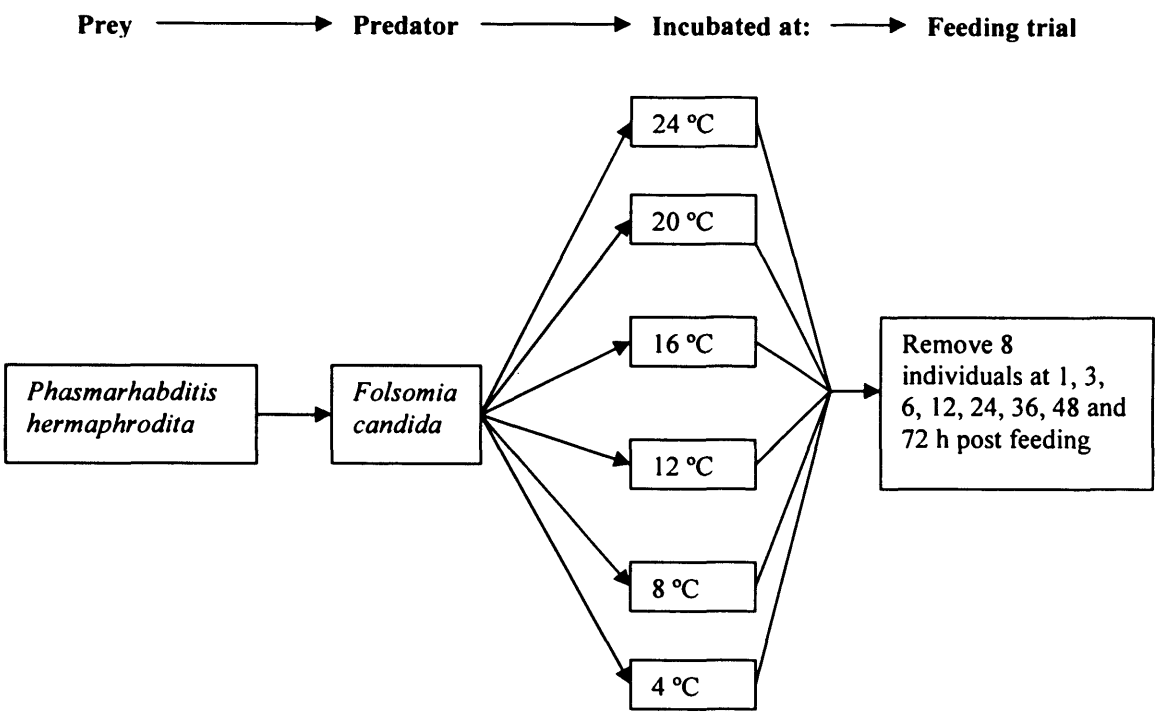


Figure 3.2.1 Feeding trial methodology to investigate the effects of temperature on the detection of nematode DNA in the gut of the collembolan *Folsomia candida* at six different temperatures.

3.2.6 Influence of decay on the detection of DNA from dead nematodes

DNA-based detection methods are not able to distinguish between predation and scavenging of dead animal material (Juan and Traugott 2005; Foltan *et al.* 2005). To investigate the potential for error caused by the presence of decaying nematode cadavers, the effect of decay on molecular detection was examined on two different substrates. The first substrate was 100 g of soil from Wenvoe, nr. Cardiff, stored in plastic containers (85 mm diameter, 45 mm depth). The soil had been partially sterilized and defaunated by heating at 60 °C for 48 h before the start of the experiment, and allowed to cool before being re-hydrated to a level of 15 % w/v with distilled water. The second substrate was 100 ml of sterile DNA-free water (Sigma, UK) stored in a glass, screw-top bottle. *Phasmarhabditis hermaphrodita* IJs were diluted with tap water to a concentration of approximately 850 nematodes per ml. The nematodes were killed by storing at -80 °C for 10 days. The nematode suspension was allowed to defrost at room temperature (22 ± 2 °C) for 1 h before the start of the experiment. Two millilitres of this solution were then applied evenly to the soil substrate and the distilled water using a

Pasteur pipette. The containers were stored in the dark at 16 ± 1 °C. Approximately 1 g of soil or 1 ml of water was removed at 0, 24, 48, 96, 144, 192, 240 and 288 h after the initial nematode application. Three replicates of each treatment were carried out. To investigate the effect of decay on DNA detectability, the samples were separately diluted in 10 ml of DNA free water and placed in shallow Petri dishes. Eight nematode IJs were removed from the substrate-water solution, using a sterile pair of fine tweezers. Nematodes were placed individually in 20 µL PBS buffer in clean 1.5 ml centrifuge tubes and stored at -80 °C. All samples were then screened for the presence of *P. hermaphrodita* DNA (see section 3.2.8).

3.2.7 DNA extraction

DNA extractions for both the collembola and mites were carried out using a modified version of the PureGene Tissue extraction kit (Gentra, Minnesota, USA) as follows: 300 µl of cell lysis solution and 1.5 µl of Proteinase K solution were added to the whole, intact collembola or mites in 1.5 ml centrifuge tubes. The tissue was then broken up using a sterile plastic pestle, before incubating at 55 °C for 3 h. RNase solution (1.5 µl) was added to each sample, followed by incubation for 30 min at 37 °C. The samples were cooled to 4-10 °C on ice for 3 min, before adding 100 µl of protein precipitation solution and vortexing at high speed for 20 s. The samples were then centrifuged at 12,000 rpm for 5 min, and the supernatant was poured off into a clean 1.5 ml centrifuge tube containing 300 µl of 100% isopropanol and 0.5 µl of glycogen to act as a DNA carrier. The tube containing the precipitated protein was discarded. The sample was mixed gently by inverting 50 times, before centrifuging at 12,000 rpm for 5 min. The supernatant was poured off, and the tube air dried for 2 min on clean absorbent paper. To clean the DNA pellet, 300 µl of 70 % ethanol was added and inverted gently three times. The samples were centrifuged a final time at 12,000 rpm for 2 min, after which the ethanol was discarded and the tubes air dried for 5-10 min. The DNA was rehydrated using 50 µl of DNA hydration solution, and incubated at 65 °C for no more than 1 h. The extracted DNA samples were stored at -20 °C until needed for screening.

3.2.8 Screening with species-specific primers

Screening of all feeding trials was a two stage process. The first stage involved screening the DNA extract with either general primers or primers specific to the predator organism, depending on the feeding trial. This process confirmed the success or failure of the DNA extraction. Any samples that did not amplify using the general or predator-specific primers were tested one more time. If no band was observed for a second time, the sample was discarded from the analysis.

General invertebrate primers *C1-J-1718* and *C1-J2191* (Simon *et al.* 1994) (Chapter 2, Section 2.2.1) that amplified a 525-bp region of mtDNA from Subunit I of the Cytochrome Oxidase (*COI*) gene were used to screen experiment 3.2.3. All PCR reactions were done using a GeneAmp 9700 thermal cycler (ABI Perkin Elmer, Beaconsfield, UK). PCR reactions contained 2 µl template DNA, 1x PCR buffer, 2.0 mM $MgCl_2$ (both provided by Invitrogen, Paisley, UK), 0.2 mM dNTPs (Boehringer Mannheim, Indianapolis, USA), 0.2 units *Taq* polymerase (Invitrogen, Paisley, UK) and 0.5 µM of each primer and were made up to 25 µl with PCR water. Thermal cycle parameters included an initial denaturation step of 2 min at 94°C followed by 35 cycles of 94°C for 1 min 10 sec, 45°C for 1 min 10 sec, 72°C for 1 min 30 sec and a final extension stage of 72°C for 7 min. Five µl of PCR product from each reaction were visualized with ultraviolet translumination following electrophoresis at 100 V for 1 h on a 1.8% (w/v) agarose gel in 1 x TAE buffer (1mM EDTA, 40mM Tris-Acetate).

Experiments 3.2.2, 3.2.4 and 3.2.5 were screened with *F. candida* specific primers *FC-F-(406-427)* and *FC-R-(540-562)* (Chapter 2, Section 2.2.1) that amplified a 156 bp fragment on a GeneAmp 9700 thermal cycler (ABI Perkin Elmer, Beaconsfield, UK) using the following optimized conditions: 1 µl template DNA, 1x PCR buffer, 2.0 mM $MgCl_2$ (both provided by Invitrogen, Paisley, UK), 0.2 mM dNTPs (Invitrogen, Paisley, UK), 0.1 U *Taq* polymerase (Invitrogen, Paisley, UK) and 0.5 µM of each primer (Operon, Cologne, Germany). Thermal conditions included a first cycle of denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 30 sec, annealing at 62 °C for 45 sec, 72°C for 1 min, and a final extension stage of 72 °C for 10 min. Products were visualized as described previously.

The second stage of the screening process involved screening for the presence of prey DNA belonging to *P. hermaphrodita*, using primers *Ph-F-(271-291)* and *Ph-R-(479-501)* (Chapter 2, Section 2.2.1) (Experiments 3.2.2, 3.2.3, 3.2.4, 3.2.5 and 3.2.6) amplifying a 217 bp fragment, on a GeneAmp 9700 thermal cycler (ABI Perkin Elmer, Beaconsfield, UK) using the following optimized conditions: 1 µl template DNA, 1x PCR buffer, 2.0 mM $MgCl_2$ (both provided by Invitrogen, Paisley, UK), 0.2 mM dNTPs (Invitrogen, Paisley, UK), 0.1 U *Taq* polymerase (Invitrogen, Paisley, UK) and 0.5 µM of each primer (Operon, Cologne, Germany). Thermal conditions included a first cycle of denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 30 sec, annealing at 62 °C for 45 sec, 72°C for 1 min, and a final extension stage of 72 °C for 10 min. Products were visualized as described previously.

3.2.9 Effects of predation by collembola on nematode numbers

The collembolan *F. candida* was used to test the ability of predatory soil-dwelling microarthropods to reduce the numbers of the insect pathogenic nematode, *H. megidis*. Approximately 100 IJs (± 5) were added to 2 ml of dechlorinated tap water, and applied to feeding arenas consisting of a plastic container (85 mm dia., 45 mm depth) with a moistened charcoal: plaster-of-Paris base (1:250). Adult *F. candida* were starved for 48 h and added to the arenas at five different densities: 5, 10, 25, 50 and 100 predators per arena. Each treatment was repeated five times and two controls were included: 1) collembola and no nematodes; and, 2) nematodes and no collembola. The containers were maintained at 16 ± 1 °C (16:8 L:D cycle) for 48 h to allow feeding to occur. After this time, nematode abundance in each container was determined by washing the plaster-of-Paris base with 5 ml of water into a clean Petri-dish, and counting the nematodes using a Sedgewick rafter counting cell (Graticules Ltd, Tonbridge, UK) under a low power stereo-microscope (Olympus, UK).

In a second experiment, the rate of *F. candida* predation upon *H. megidis* IJs was measured. The nematode IJs were added to feeding arenas (85 mm dia., 45 mm depth) with a moistened charcoal: plaster-of-Paris base (1:250), at a density of 100 per container. Ten starved adult collembola or mites were added to each container and maintained at 16 ± 1 °C in the dark. Five containers were sampled at 0, 2, 4, 6, 8 and 10 h after the start of the experiment. The plaster-of-Paris bases were washed in 5 ml of

water and the number of nematodes remaining was estimated as above. Five control containers, with nematodes but no collembola, were also sampled at each time interval.

In a third experiment, to investigate predation on a soil substrate, IJs of the nematode *H. megidis* were added at a rate of 50 per cm^2 in 2 ml of water using a Pasteur pipette, to plastic containers (85 mm diameter, 45 mm depth) containing 100 g of soil from Wenvoe, nr Cardiff. The soil had been previously defaunated at 60 °C for 48 h, before being rehydrated to ca.15 % w/v with tap water. Twenty adult *F. candida* were added to each container. Controls consisted of containers with nematodes but no collembola, and containers with just soil and no additional nematodes or collembola. The containers were stored at 16 ± 1 °C in the dark. Five containers from the treatment and each of the controls (total 15) were removed 0, 24, 48, 72, 96 and 120 h after the start of the experiment. Collembola were removed using a pooter, and the number of visible nematodes on the soil surface was counted under a stereo microscope, and expressed as number of individuals per cm^2. The soil was then mixed thoroughly, and fifty grams of soil from each container was placed into a Baermann funnel apparatus (see Chapter 2, Section 2.1.2) for nematode extraction. Samples were left for 72 h in the Baermann funnel at 22 ± 2 °C, before removing the water and counting the number of nematodes under a low power microscope in a Sedgewick rafter counting cell (Graticules Ltd, Tonbridge, UK).

To investigate the potential for soil-dwelling microarthropods to reduce the efficacy of nematode biocontrol agents, twenty adult *F. candida* were placed in plastic containers (85 mm dia., 45 mm depth) with either a moistened charcoal: plaster-of-Paris base (1:250) or 100 g of defaunated, re-moistened (15 % w/v) soil from Burdens Farm, Wenvoe. *Heterorhabditis megidis* IJs were applied at a rate of 50 per cm^2 to the substrate surface in 2 ml of water. The containers were stored at 16 ± 1 °C in the dark. After 0, 1, 3, 5, 7, 9, 11, 13 and 15 days, the containers were sampled, and all the collembola removed from each container using a pooter. Ten mealworms (*T. molitor*) weighing 40-70 mg were placed in both the soil and the plaster-of-Paris arenas. The containers were then stored at 20 ± 1 °C in the dark for a further 72 h, after which point the mortality of the mealworms was recorded. Controls consisted of containers in which nematodes were added, with no collembola, and containers with no collembola and no nematodes. Four replicates of each treatment were sampled at each time point.

3.2.10. Statistical Analysis

For the analysis of the collembola feeding-trial data (Sections 3.3.4 and 3.3.5), Probit models, a form of Generalized Linear Model (GLM), were used to describe the change in the number of predators positive for prey DNA over time. To compare the calculated median detection times, the method from Payton *et al.* (2003), based on using 83.5 % fiducial limits as an approximation of an $\alpha = 0.05$ test was used. This allowed the statistical significance of the calculated median detection times to be compared and differences in the values to be highlighted.

Changes in the numbers of nematodes in both soil and plaster-of-Paris feeding trials were analysed using linear regression on MINITAB 14. Analysis of Covariance (ANCOVA) was used to compare regression lines for significant differences in the slope and intercept. Significant differences in the mean number of nematodes at different treatments were tested for using the non-parametric Kruskal-Wallis test. Pairs of means were compared using the Two-tailed Mann-Whitney test. All tests were conducted using MINITAB 14.

3.3 Results

3.3.1 Non-target effect of parasitic nematodes on microarthropods

Four species of mollusc and insect parasitic nematode were tested for their potential to infect and kill the collembolan *Folsomia candida* and the mite *Stratiolaelaps miles*. No mortality or infection was observed in either the collembola or the mites when exposed to any of the four species of nematode.

3.3.2 Phoresy by parasitic nematodes on microarthropods

The collembolan *F. candida* was examined for the potential to act as a phoretic host for mollusc and entomopathogenic nematodes. No mortality was observed in any of the insect and mollusc hosts exposed to collembola that had in turn been exposed to their respective parasitic nematodes, indicating that phoresy by live nematodes was not occurring.

To test whether contamination by nematode DNA was a source of error when screening whole collembola for their gut contents, six feeding trials were carried out using three nematode species and two treatments: live nematode IJs and dead nematode IJs. No PCR positives were recorded using the nematode species-specific primers in any of the treatments, indicating that nematode attachment either through phoresy or contamination on the outside of the collembola was not a significant source of error in this system.

3.3.3 Detecting predation by microarthropods in the laboratory

Thirteen different microarthropods were screened to test for predation on the nematode *Phasmarhabditis hermaphrodita* in laboratory based feeding trials. Two species, *Entomobrya nivalis* and *Lepidocyrtus cyaneus* did not test positive for predation on the nematode IJs. All other species tested were $\geq$ 75% positive, apart from *Orchesella villosa* individuals that tested 62.5% positive (Figure 3.3.1).

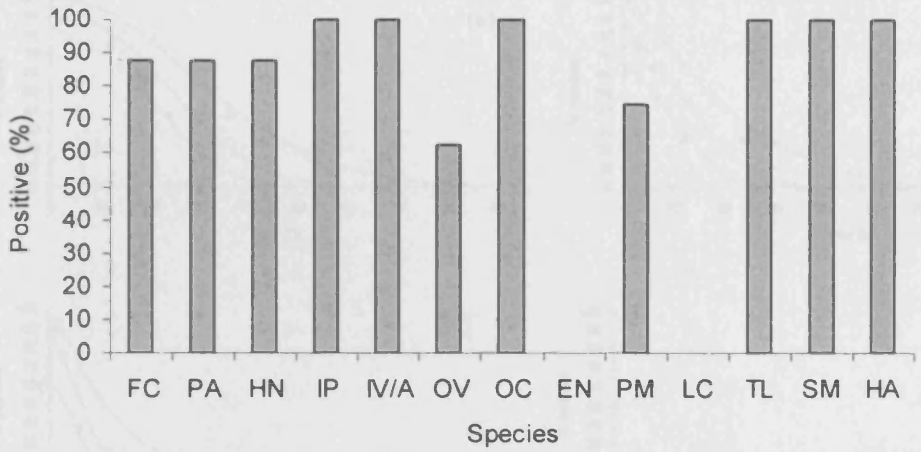
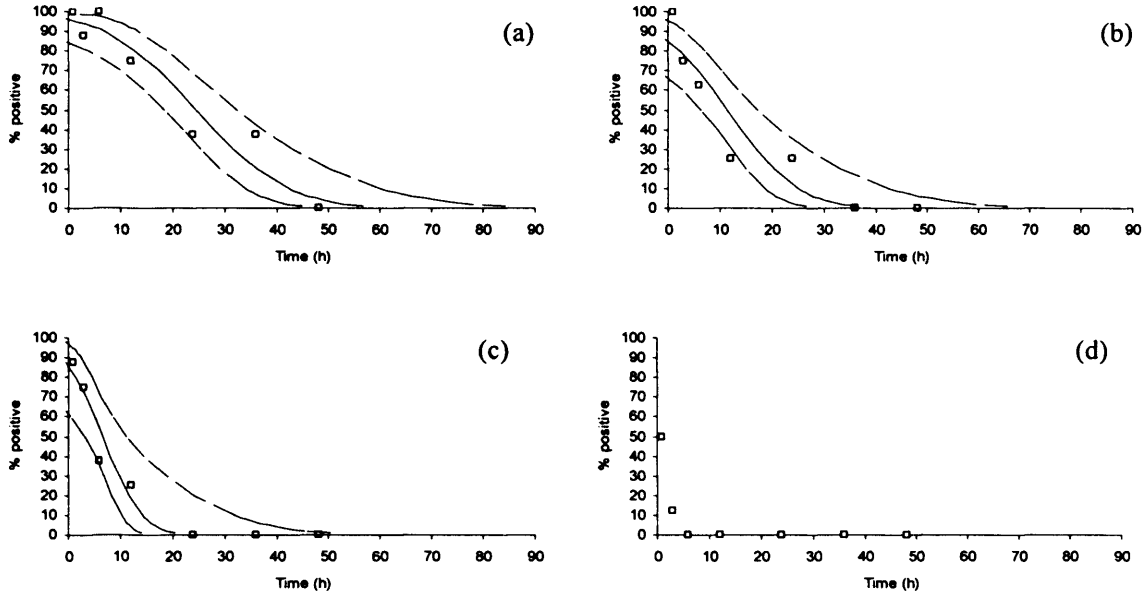


Figure 3.3.1 Percentage of individuals testing positive for predation on the nematode *Phasmarhabditis hermaphrodita* in 13 species of microarthropod. Figure symbols represent the collembola FC *Folsomia candida*; PA *Protaphorura armata*; HN *Heteromurus nitidus*; IP *Isotomurus palustris*; IV/A *Isotoma viridis/anglicana*; OV *Orchesella villosa*; OC *Orchesella cincta*; EN *Entomobrya nivalis*; PM *Proisotoma minuta*; LC *Lepidocyrtus cyaneus*; TL *Tomocerus longicornis*; and the mites SM *Stratiolaelaps miles*; HA *Hypoaspis aculeifer*.

3.3.4 The effect of collembolan size class on the post-ingestion detection period of nematode DNA

Probit models were used to describe the fit of data representing the detection of *P. hermaphrodita* DNA in the gut of *F. candida* over time for three different size classes of collembola (Figures 3.3.2 a, b, c and d). Feeding trials carried out with the '200-140 μm' size class did not contain enough positives to fit the Probit model (Figure 3.3.2 d), and the feeding trial carried out using the '140-125 μm' size class resulted in no positives at any time points (graph not shown). The Probit model described the data well for the three feeding trials that could be analyzed: + 400 μm, 400-250 μm and 250-200 μm (Table 3.3.1). In all models, time had a significant impact on the probability of detecting *P. hermaphrodita* DNA (+ 400 μm, 400-250 μm $P < 0.001$, 250-200 μm $P < 0.05$).



Figures 3.3.2 (a) (b) (c) (d) Detectability of nematode DNA (*Phasmarhabditis hermaphrodita*) in the gut of the predator (*Folsomia candida*) belonging to four different size classes, (a) + 400 μm, (b) 400-250 μm, (c) 250-200 μm and (d) 200-140 μm using primers *Ph-F-(271-291) and PH-R-(479-501).* Solid lines represent fitted Probit models with dashed lines representing 95% fiducial limits. Points represent the percentage of individuals positive for *P. hermaphrodita* DNA at each time point.

There was a significant difference between the median detection times for the '+ 400 μm' size class feeding trial compared to the '400-250 μm' and '250-200 μm' size class feeding trials, with the larger collembola having longer detection times (Figure 3.3.3). The 83.5% CI for the '400-250 μm' and '250-200 μm' size class feeding trials overlapped, indicating no significant difference at the 95% level.

Table 3.3.1 Goodness of fit tests for Probit models describing the detection of *Phasmarhabditis hermaphrodita* in the gut of five different size classes of *Folsomia candida*.

Size Class	Goodness of fit	Chi-Sq	df	P-Value
+ 400 µm	Pearson	4.461	5	0.485
	Deviance	5.546	5	0.353
400-250 µm	Pearson	4.831	5	0.437
	Deviance	6.071	5	0.299
250-200 µm	Pearson	1.282	5	0.937
	Deviance	1.294	5	0.936
200-140 µm	Pearson	N/A	N/A	N/A
	Deviance			
140-125 µm	Pearson	N/A	N/A	N/A
	Deviance			

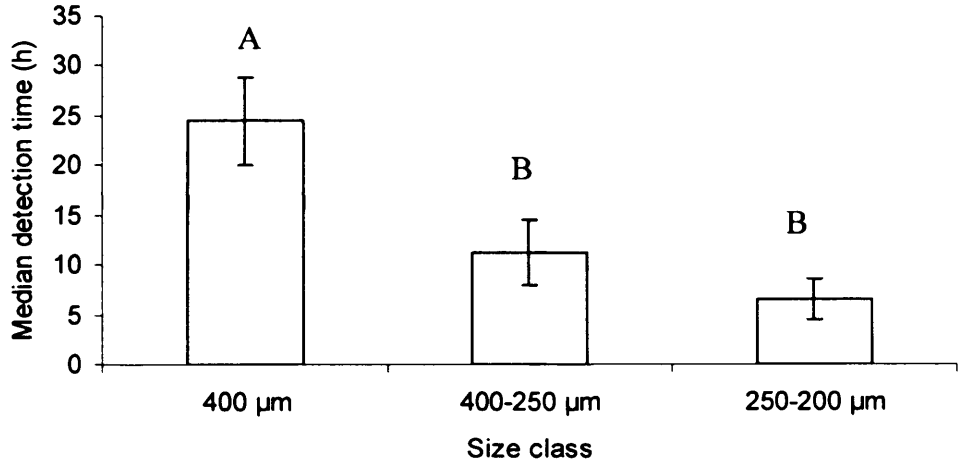
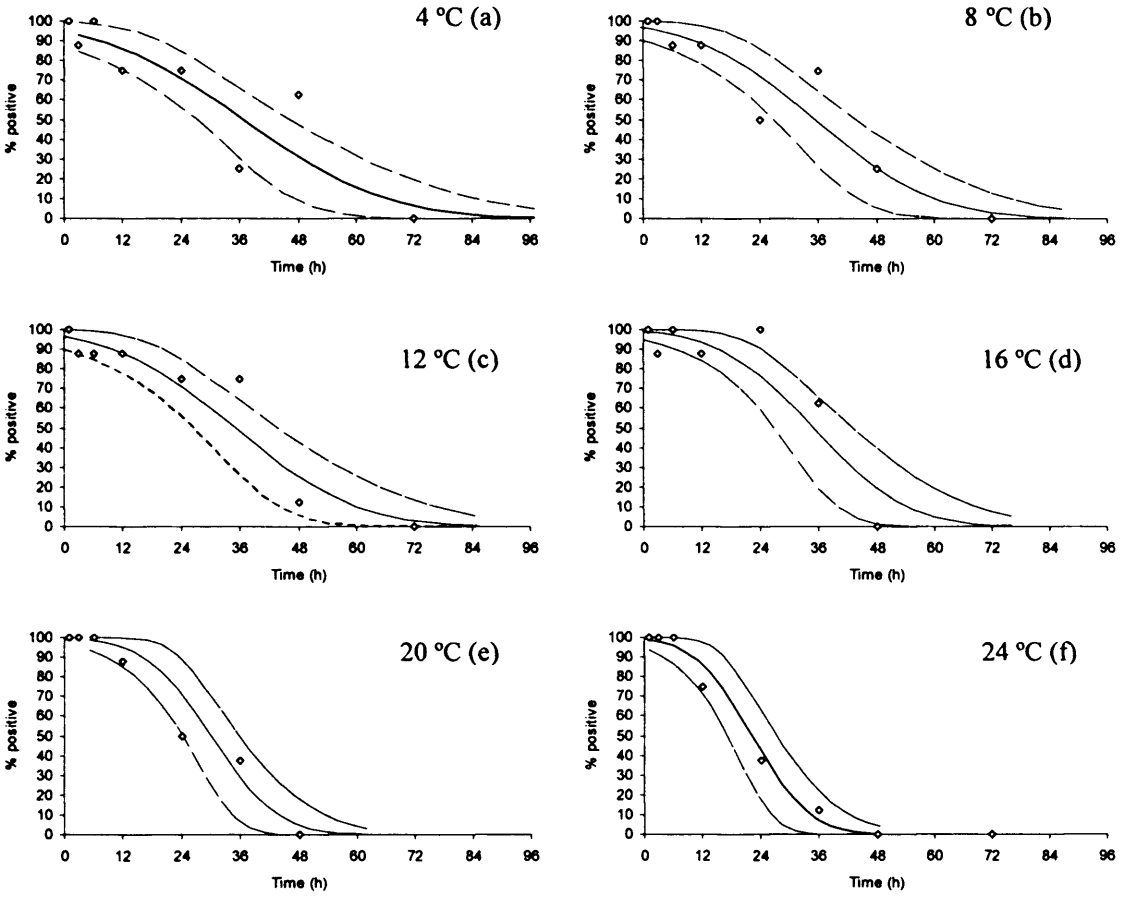


Figure 3.3.3 Median detection times of *Phasmarhabditis hermaphrodita* DNA in the gut of *Folsomia candida*. Bars represent 83.5% fiducial confidence intervals. Letters (A-B) represent groups with significant differences at $\alpha = 0.05$.

3.3.5. Effect of ambient temperature on the detection of nematode DNA in the gut of the collembolan *Folsomia candida*

Figures 3.3.4. a, b, c, d, e and f show the results of the feeding trials carried out at six different temperatures. Fitted Probit models described the data well for all feeding trials with the exception of the feeding trial carried out at 16 °C (Table 3.3.2). The poor fit of the model in this case is probably explained by the unusually high number of positives at the 24 h sampling point. Only the median detection time from the 24 °C feeding trial was significantly different to the other median detection times at the 95% level (Table 3.3.3). In all models, time had a highly significant impact on the probability of detecting *P. hermaphrodita* DNA ($P < 0.001$).

Plotting the median detection times against temperature (Figure 3.3.5) allowed any trend in the change in median detection time against temperature to be observed. Due to the poor fit of the Probit model at 16 °C, the median detection time calculated from this model was excluded from the regression. The data could not be transformed to obtain randomly distributed residuals, so a polynomial regression was carried out (Figure 3.3.5), which showed a significant correlation ($F_{2,2} = 32.82$, $P \leq 0.05$) between median detection time and temperature ($y = 34.31 + 0.6410x - 0.04636x^2$, $R^2 = 0.97$).



Figures 3.3.4 (a) (b) (c) (d) (e) and (f), Detectability of prey DNA in the gut of the collembolan *Folsomia candida* fed with *Phasmarhabditis hermaphrodita* at six ambient temperatures, using primers *PH-F-(271-291) and PH-R-(479-501)*. Figures represent feeding trials carried out at; 4 °C (a), 8 °C (b), 12 °C (c), 16 °C (d), 20 °C (e) and 24 °C (f). Solid lines represent fitted Probit models with dashed lines representing 95% fiducial limits. Points represent the percentage of individuals positive for *P. hermaphrodita* DNA at that time point.

Table 3.3.2 Goodness of fit tests for Probit models describing the detection of *Phasmarhabditis hermaphrodita* in the gut of *Folsomia candida* at six different ambient temperatures. Highlighted rows (grey) indicate a non-significant fit at the 95% level.

Temp (°C)	Pearson Chi-Sq	Deviance	df	P-Value
4	8.963	10.165	6	0.176
8	5.643	6.318	6	0.464
12	4.972	5.418	6	0.547
16	11.420	11.845	5	0.044
20	1.979	2.226	5	0.852
24	1.884	2.333	6	0.93

Table 3.3.3 Mean detection times of *Phasmarhabditis hermaphrodita* DNA in the gut of *Folsomia candida* with upper and lower 83.5% fiducial confidence limits

Temp (°C)	Estimate (hours)	StDev	Error	Lower 83.5% CI	Upper 83.5% CI
4	36.8	22.8	4.88	30.06	43.62
8	35.4	19.3	4.34	29.38	41.44
12	35.1	19.6	4.45	28.94	41.28
16	35.0	15.1	4.10	29.29	40.67
20	30.0	10.8	2.96	25.87	34.10
24	22.2	9.4	2.57	18.60	25.75

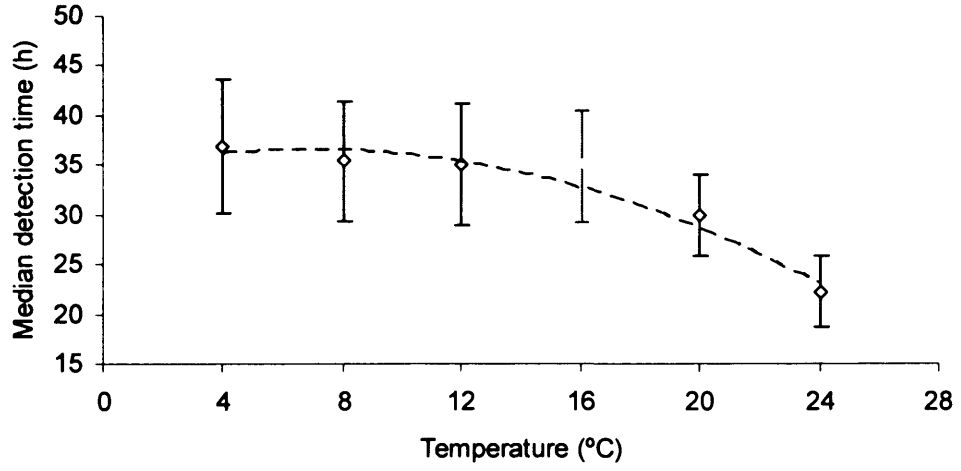


Figure 3.3.5 Median detection time (hours) calculated from fitted Probit models, plotted against temperature (°C) (◊, Error bars represent 83.5% confidence intervals). Dashed line represents a quadratic regression (excluding the data from the 16 °C feeding trial □).

3.3.6 Influence of decay on the detection of DNA from dead nematodes

The detectability of decaying nematode DNA over time is plotted on Figure 3.3.6. Regression analysis was used to plot the best fitting line on the detection data from soil ($y = 5E\text{-}05x^3 - 0.0132x^2 + 0.191x + 99.375$, $R^2 = 0.95$). The regression was highly significant ($P \leq 0.001$) and both the intercepts and slopes were significantly different from zero ($P \leq 0.001$). The median detection time was 94.7 h. Data from the water samples was not normally distributed and transformation would not correct this, so no regression could be carried out.

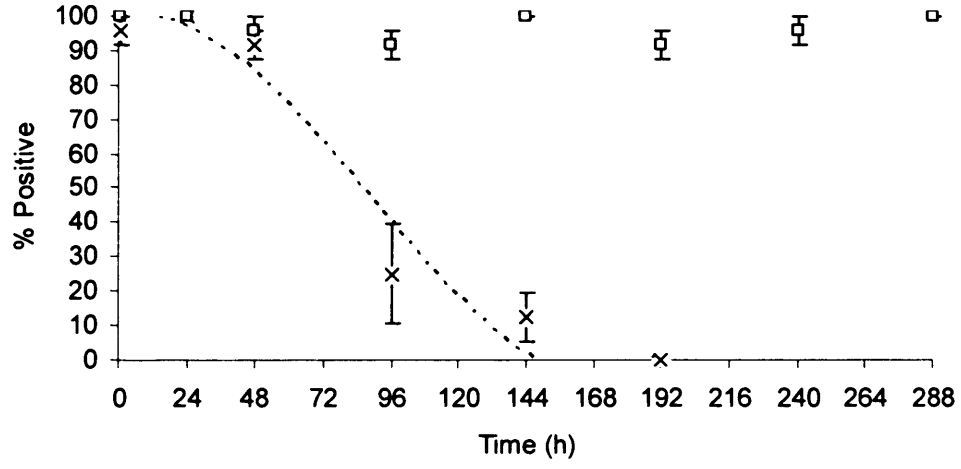


Figure 3.3.6 Detectability of *Phasmarhabditis hermaphrodita* DNA against time in distilled water (□) and soil (x) using primers *PH-F-(271-291) and PH-R-(479-501).* Bars ± S.E.

3.3.7 Effects of predation by collembola on nematode numbers

The initial experiments were conducted to investigate the numerical effects of predation by adult *F. candida* on nematode IJs. The effect of predation by as few as five *F. candida* on 100 nematodes over 48 h caused a decrease of over 80 % in numbers of nematodes recoverable from the plaster-of-Paris based feeding arenas compared to the control (Figure 3.3.7). There was a highly significant difference between the median numbers of nematodes for each collembola density (Kruskal-Wallis: H = 23.07, DF = 5, $P < 0.001$). Comparisons of the control vs. each of the collembola densities showed significant reductions in nematode numbers (Mann-Whitney: $P < 0.05$). The rate of *F. candida* predation upon *H. megidis* in plaster-of-Paris based feeding arenas is shown in Figure 3.3.8. There was no significant change in the numbers of nematodes over time in the controls arenas without predators (Linear regression: $P > 0.05$). The presence of the 10 collembola caused a significant decline ($F_{1,4} = 18.84$, $P < 0.05$) in the numbers of retrievable nematodes ($y = 60.51 - 2.60x$, $R^2 = 0.83$), with the numbers reduced by ~46 % in 10 h. The slopes of the regression lines were significantly different (ANCOVA: $F = 15.02$, $P < 0.05$).

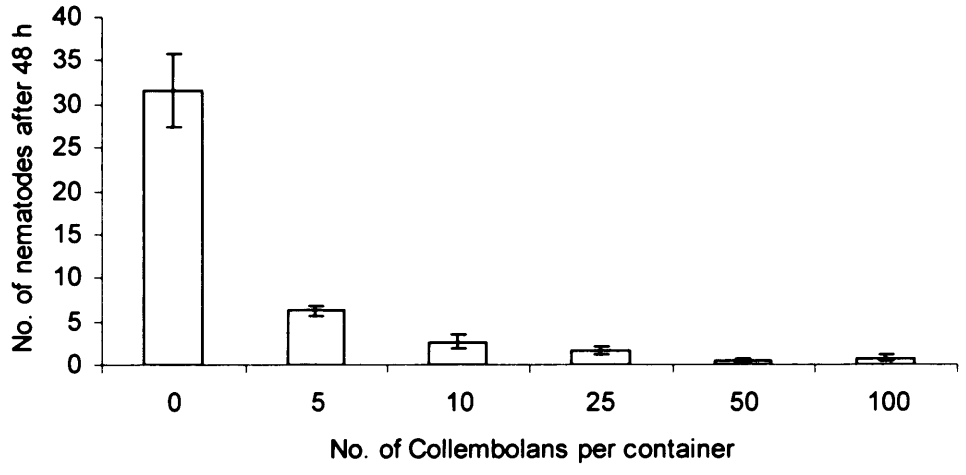


Figure 3.3.7 Number of *Heterorhabditis megidis* IJs counted after 48 h of exposure to predation by *Folsomia candida* at densities of 0, 5, 10, 25, 50 and 100 collembola per container. Bars ± S.E.

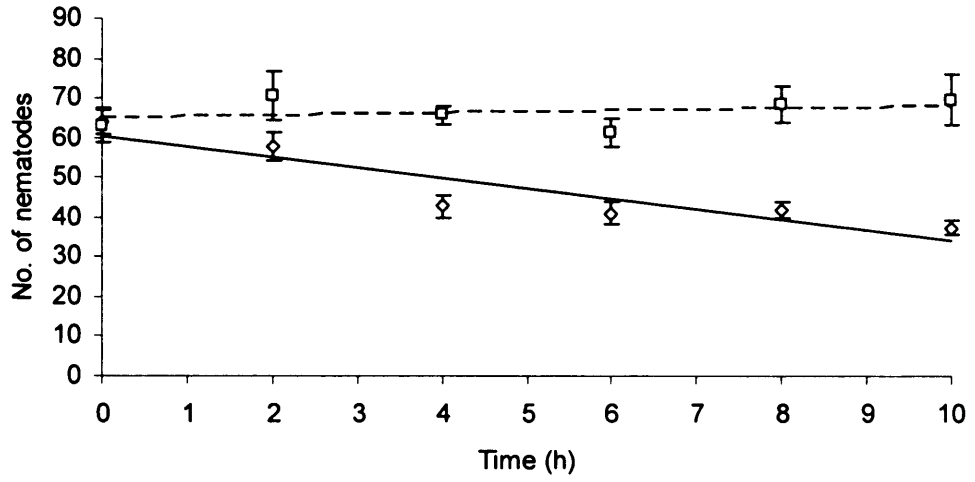


Figure 3.3.8 Number of *Heterorhabditis megidis* IJs remaining after predation by 10 adult *Folsomia candida* (—) (y = -2.6029x + 60.514) over a period of 10 h. The control line (---) shows numbers of nematodes remaining where no collembola were present. Bars ± S.E.

The complexity of the soil environment may influence the rate of predation by soil-dwelling microarthropods due to the presence of refuga for prey. In Figure 3.3.9, the change in nematode numbers within the soil over time, in the presence and absence of 20 adult *F. candida* is shown. In the control, regression analysis showed the number of nematodes retrieved declined significantly over time. The model was significant ($F_{1,4}$ = 27.51, $P < 0.05$) and the best fitting equation was y = 954 - 1.30x (R^2 = 0.87). The regression line fitted to the treatment data was also significant ($F_{1,4}$ = 16.45, $P < 0.05$), with the best fitting equation being y = 807 - 3.86x (R^2 = 0.804). Analysis using ANCOVA showed a significant difference between the slopes of the regression lines (F = 6.75, $P \leq 0.05$), with the numbers of nematodes declining more rapidly in the presence of *F. candida* than the control. The number of nematodes at the soil surface in the presence and absence of 20 adult *F. candida* is shown in Figure 3.3.10. Transformation of the y-axis with (1/x) allowed significant regression lines (treatment: $F_{1,7}$ = 49.07, $P \leq$ 0.001, control: $F_{1,7}$ = 15.62, $P \leq 0.05$) to be fitted to both the treatment (1/(y+1) = 0.0899 + 0.0398x, R^2 = 0.88) and the control data (1/(y+1) = 0.0410 + 0.00230x, R^2 = 0.69). Comparison of these lines using ANCOVA showed a highly significant difference between the slopes of these two lines (F = 27.58, $P \leq 0.001$), with the number of nematodes per cm^2 at the soil surface declining more rapidly in the presence of *F. candida* than in the control.

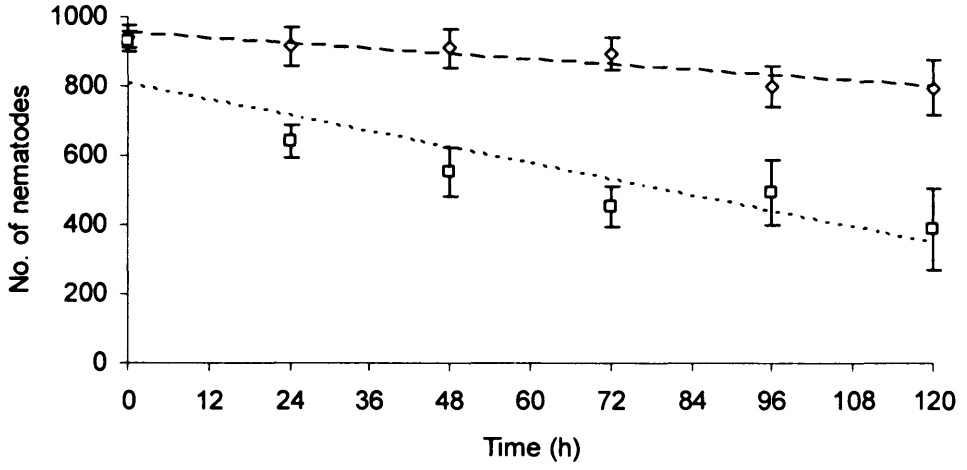


Figure 3.3.9 Number of live *Heterorhabditis megidis* remaining in the soil matrix, in soil-based feeding arenas over time, in the presence (□) and absence (◊) of 20 adult *Folsomia candida*. Bars ± S.E.

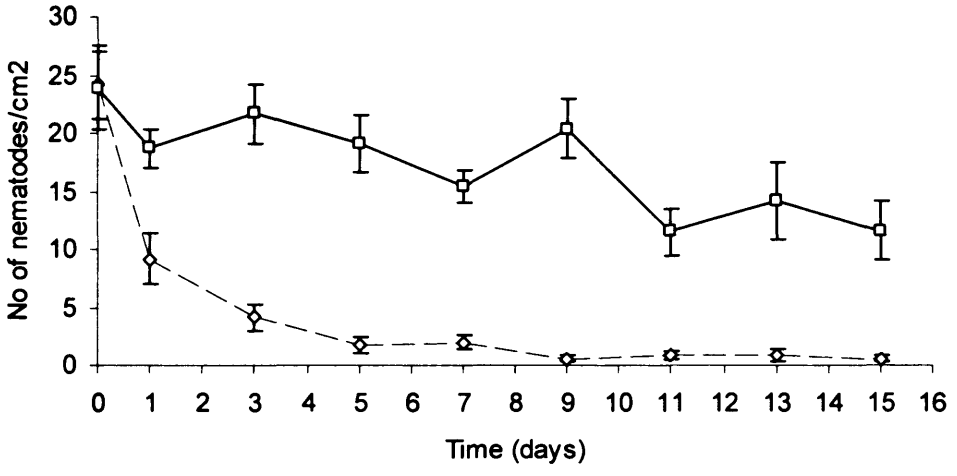


Figure 3.3.10 Number of live *Heterorhabditis megidis* remaining on the soil surface in soil-based feeding arenas over time, in the presence (◊) and absence (□) of 20 *Folsomia candida*. Bars ± S.E.

Predation by adult *F. candida* on *H. megidis* in soil arenas (Figure 3.3.11) caused a significant decline in the rate of infection of the mealworm *T. molitor* over time. Transformation of the y-axis with $1/\sqrt{(y+1)}$ allowed significant regression lines (treatment: $F_{1,7} = 31.82$, $P \leq 0.001$. control: $F_{1,7} = 22.83$, $P \leq 0.05$) to be fitted to the treatment ($1/\sqrt{(y+1)} = 0.110 + 0.00308x$, $R^2 = 0.82$) and control data ($1/\sqrt{(y+1)} = 0.101 + 0.000802$ Day, $R^2 = 0.762$). Comparison of these lines using ANCOVA showed a highly significant difference between the slopes ($F = 15.87$, $P \leq 0.001$), with the decline in number of infected hosts over time being faster in the presence of *F. candida*. Predation by adult *F. candida* on plaster-of-Paris (Figure 3.3.12) caused a significant decline in the rate of infection of the mealworm *T. molitor* over time. Transformation of the y-axis with $1/\sqrt{(y+1)}$ allowed significant regression lines (treatment: $F_{1,8} = 104.46$, $P \leq 0.001$. control: $F_{1,7} = 58.42$, $P \leq 0.001$) to be fitted to the treatment ($1/\sqrt{(y+1)} = 0.0730x$) and control data ($1/\sqrt{(y+1)} = 0.0999 + 0.00343x$, $R^2 = 0.89$). Comparison of these lines using ANCOVA showed a highly significant difference between the slopes ($F = 22.86$, $P \leq 0.001$), with the decline in number of infected hosts being faster in the presence of *F. candida*. Comparison of the decline in infection rates on the soil substrate with the plaster-of-Paris substrate using ANCOVA showed a highly significant difference between the slopes ($F = 23.13$, $P \leq 0.001$), with the decline in infection being more rapid on the plaster-of-Paris substrate.

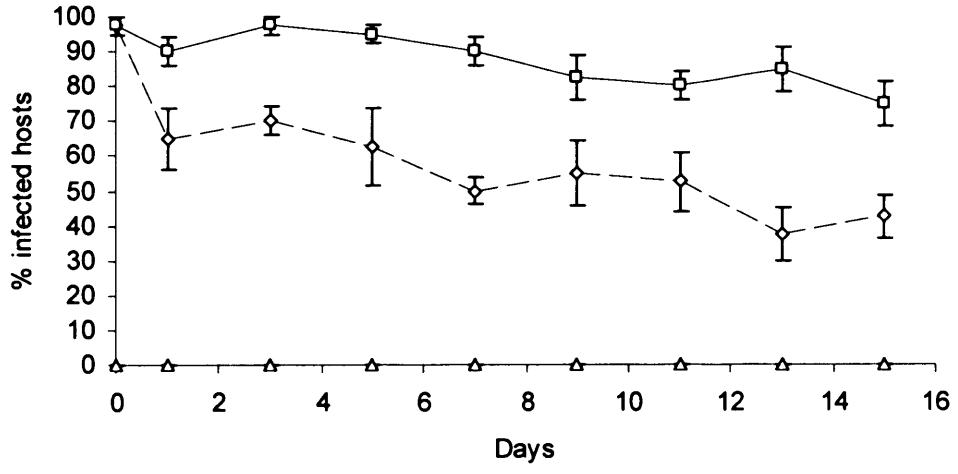


Figure 3.3.11 Percentage of infected host insects, *Tenebrio molitor*, over time in the presence of the nematode *Heterorhabditis megidis* in the presence (◊) or absence (□) of the collembolan *Folsomia candida* in soil based infection arenas.

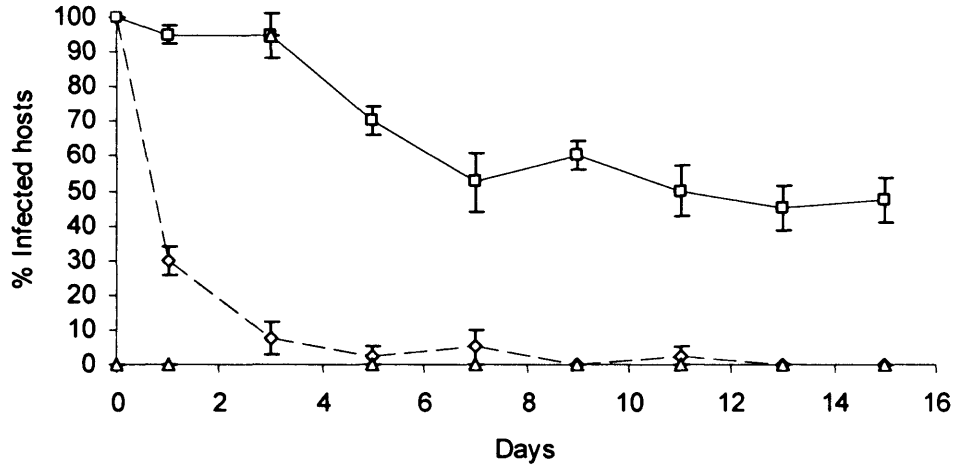


Figure 3.3.12 Percentage of infected host insects, *Tenebrio molitor*, over time in the presence of the nematode *Heterorhabditis megidis* in the presence (◊) or absence (□) of the collembolan *Folsomia candida* in plaster-of-Paris based infection arenas.

3.4 Discussion

The aim of this study was to investigate the viability of using PCR-based methods for determining the diet of soil-dwelling microarthropods and to identify potential sources of error during screening that may cause false positives or negatives and changes in median detection times. No signs of infection were observed in either the mites or collembola that had been exposed to four species of mollusc- and insect-parasitic nematodes (Section 3.3.1). This is not entirely unexpected in the case of *Phasmarhabditis hermaphrodita*, as it is a specialist parasite of molluscs (Wilson *et al.* 1993). The natural host range of *Steinernema feltiae* and *Heterorhabditis megidis* is much larger and, in the laboratory, these species are known to infect a wide range of species spanning nearly all insect orders (Kaya and Gaugler 1993). However, the range of insects that can be successfully infected in the laboratory is far higher than in the field (Bathon 1996). Despite this, there have been no records of these nematode genera infecting and killing collembola or mites and it has been concluded that parasitism of all forms is not a significant cause of mortality amongst collembola (Hopkin 1997).

Phoresy by parasitic nematodes has been observed frequently on invertebrates and has been recorded on earthworms (Shapiro *et al.* 1995; Campos-Herrera *et al.* 2006), Japanese beetles (Lacey *et al.* 1995), bees (Giblin-Davis *et al.* 2005), pseudoscorpions (Curcic *et al.* 2004) and isopods (Eng *et al.* 2005). It has been speculated that phoresy may play an important role in the dispersal of some species of entomopathogenic nematodes (Lacey *et al.* 1995). Records of external parasitism amongst collembola are very rare, one record being the observation of unidentified nematodes attached to the external surface of *Orchesella villosa* (see Miles 1971). In the present study, no phoresy on *Folsomia candida* was observed under the microscope and no viable IJs were passed from collembola to the nematode's host (Section 3.3.2). These results are consistent with previous observations using *F. candida* and the entomopathogenic nematode *Steinernema carpocapsae* (see Gilmore and Potter 1993). The experiment in section 3.3.2 ruled out the possibility of phoresy by live nematodes and external contamination of nematode DNA from nematode fragments causing false positives when screening using PCR. These results permit for the screening of whole microarthropods for nematophagy without a significant risk of contamination and false positives caused by phoresy.

A range of collembolan species have been observed to feed upon nematodes, including *Isotoma* sp. *Hypogastrura packardi*, *Sinella caeca*, *Entomobryoides dissimilis*, *Tomocerus vulgaris*, *T. flavescens*, *F. candida*, *Isotomurus palustris*, *Isotoma viridis* and *Isotoma cinerea*, *Onychiurus* sp. *Heteromurus nitidus*, *Protaphorura fimata* and *Proisotoma minuta* (see Brown 1954; Gilmore 1968; Osman *et al.* 1988; Gilmore and Potter 1993; Lee and Widden 1995; Chamberlain *et al.* 2006a). In this study (Section 3.3.3), nine out of the 11 species of collembola and two of the two species of mite, tested positive for predation on nematodes. One of the species that was not positive for nematode DNA was *E. nivalis*, a species that lives on plant surfaces (Meier and Zettel 1997), an environment with high levels of desiccation and a situation in which nematodes are rarely found. *Lepidocyrtus cyaneus* is a common species in the UK found in a wide range of habitats. It is unknown why this species did not feed on nematodes in this study. The results do, however, provide evidence for prey choice amongst collembolan species. Although the results indicate that nematophagy may be widespread amongst collembola, care needs to be taken interpreting the results, as feeding trials in the laboratory may not represent food choices in the field (Mckemey *et al.* 2003).

Due to the large size and/or heavy pigmentation of some of the predator species in this study, it was not possible to observe if food was present in the gut. This may explain why only 62.5 % of *O. villosa* and 75 % of *P. minuta* individuals tested positive. Collembola stop feeding prior, during and immediately post moult (Hopkin 1997) and, as a result, a certain proportion of a non-synchronous population will be always non-feeding. It has been speculated that large epiedaphic collembolan species that have high growth rates and higher moulting rates and spend more time with empty guts. Conversely, the smaller euedaphic species with slower metabolisms and lower moulting rates will spend proportionally more time with a full gut (Werner and Martin 1987). Using visual gut analysis, the proportion of collembola with empty guts has been shown to range from 37.7 % to 90 % (Anderson and Healey 1972; Addison *et al.* 2003). Although this high value may be a consequence of the method of gut analysis that was used, it seems likely that it can be expected to have a significant proportion of the population not feeding at any one time. Another factor that may influence the percentage of empty guts in field-caught collembola is their position in the substrate;

Vegter (1982) found a correlation between the vertical distributions of populations of *Tomocerus minor* and *Orchesella cincta* with the proportions of individuals having an empty gut.

Once food is consumed, a number of factors may influence the digestion rate and hence the median detection period of DNA in the gut of a predator. Digestion in invertebrates is temperature-dependant, whereby increasing temperature increases the rate of digestion (up to a point) (Frazier *et al.* 2006). Although the effect of temperature on nutrient release in the insect gut has been modelled (see Logan *et al.* 2002; Wolesensky *et al.* 2005), few studies have been carried out to investigate the effect of ambient temperature on prey DNA detection times. Previous investigations with invertebrate predator-prey systems have shown that increasing the ambient temperature from 20 to 27 °C decreased the detection period (Hoogendoorn and Heimpel 2001). This has also been shown using monoclonal antibodies in coccinellids (Hagler and Cohen 1990) and hemipterans (Hagler and Naranjo 1997). In the present study, only the median detection time from the feeding-trial carried out at 24 °C was significantly different at the 95% level. Despite this, in a comparison of median detection times against the feeding trial temperature, there is a clear correlation, with detection periods declining at higher temperatures (Figure 3.3.5). These results have potential implications when screening predators from the field, as natural fluctuations in temperature will influence median detection times. As a result, the impact of predation in a colder environment may be over estimated when compared to a warm environment. Therefore, comparison of samples from different sampling dates needs to be accompanied by temperature data, allowing fluctuations to be accounted for.

The effect of body mass and size on digestive process amongst invertebrates has not been well studied. The standard metabolic rate (SMR) has been shown to increase with body size in most animals, but the exact relationship may vary amongst species (Heusner 1985). In a study on the effects of body mass and age on the SMR in house crickets, *Acheta domesticus* (Orthoptera: Gryllidae), increasing body mass was found to result in increased SMR and increasing age was found to decrease SMR (Hack 1997). In the present study, decreasing body size resulted in decreasing median detection times, from 24.5 h for the largest size class (+ 400 μm), to 6.5 h for the 250-200 μm size class. Explaining the reasons for these results is complicated, as they are a consequence of the

interaction of diet choice, feeding capability, quantity of food eaten, digestive rate, metabolic rate and the rate of the passage of food in the gut. For example, the shorter median detection times in smaller individuals may purely be a result of the fact that a smaller quantity of nematode material was initially consumed, or it may be that smaller collembola have shorter gut lengths and therefore the rate of food turnover in the gut is faster. The result of all these interacting factors will effect the overall detection time of DNA in the gut of the collembola used in this study. Until further research is carried out, the relative importance of these factors will remain unknown.

There are other potential factors influencing detection periods that have not been considered in this study. Dodd (2005) using a carabid-slug predator-prey system, found that the consumption of alternative prey, after the consumption of the target prey, could significantly increase the detection period of prey in the gut. This may be caused by the presence of a larger quantity of food material in the gut and therefore a slower passage of food or a slower rate of digestion. The activity levels of the predator may also influence digestion rates, for example, high activity was shown to increase the production of haematin (a by-product of blood digestion) in tsetse flies (Loder *et al.* 1998). Other factors not considered include food quality, predator fitness, predator species, prey species and quantity of food eaten.

A potential source of false positives in field studies is the detection of scavenging rather than predation. DNA-based methods cannot distinguish between the two, so the potential for error caused by scavenging must be quantified. Scavenging has been shown to be a potential error in carabids feeding on slugs (Foltan *et al.* 2005) and in carabids feeding on chafer larvae (Juen and Traugott 2005). In this study, no decline was observed in the detection of DNA in distilled water, presumably due to the lack of decay-causing organisms. Decline in partially sterilized soil was more rapid, with a median detection period of 98.7 h (approx 4 d) and a maximum detection limit of 144 h (6 d). This value is likely to be a large underestimation of the rate of decay that would actually occur in the field, due to the use of partially sterilized soil (Section 3.2.6). Unsterilized soil, such as would be found in the field, contains a large abundance of detritivores in the form of microbes and fungi that would most likely increase the decay rate of nematode cadavers significantly. However, similar study was conducted by MacMillan *et al.* (2006), in which the persistence of *P. hermaphrodita* DNA from dead

nematodes in unpasteurized soil was determined. In this case, DNA could also be detected for a maximum of 6 days using real time qPCR and primers amplifying a region of 18S DNA.

The influence of predation by collembola was investigated using a series of experiments designed to study the effect on nematode numbers and nematode efficacy, on both plaster-of-Paris and soil substrates. As few as ten adult *F. candida* reduced a starting population of 100 nematodes by almost 50 % in 10 h (Figure 3.3.7). With an extraction efficiency of 66.6% (= the number retrieved from control arenas) it can be estimated that the adult *F. candida* were each eating a mean of 0.44 nematodes per hour over the 10 h test period on plaster-of-Paris. With densities of over 100,000 collembola per m^2 recorded in arable fields (Koehler 1999), a population this size could theoretically consume the entire population of nematode IJs applied at a density of 30 per cm^2 within 6.8 h. This estimate only applies to the reduction caused by collembola and the combined effects of all nematophagous soil biota could result in even greater reductions. The availability of nematodes on a plaster-of-Paris surface is, however, much greater than in the highly complex environment of the soil, resulting in higher levels of predation than would be experienced in the field (Hoddle 2003). Not all collembola are likely to be predatory towards nematodes (as the data demonstrated), with some species specializing on fungi and detritus (Filser 2002) and in the field alternative animal and vegetable food will also be available to the microarthropods, affecting predation rates.

Calculation of the rate of feeding on the soil substrate is more difficult as the Baermann funnel only allows for the extraction of live nematodes and the possibility of non-feeding mortality effects or wasteful killing cannot be ruled out. Wasteful killing often occurs when prey is present in high densities and is defined as killing without consumption. This has been observed amongst a wide range of invertebrates, including carabid beetles (Lang and Gsodl 2003), spiders (Samu and Biro 1993; Maupin and Reichert 2001), predatory mites (Metz *et al.* 1988) and damselfly nymphs (Johnson *et al.* 1975). The application of nematodes as a biocontrol agent results in an initial overabundance of food for nematophagous microarthropods and as a consequence may result in wasteful killing. This allows predator populations to exert a predation pressure beyond that would be expected for the population size.

The natural mortality of nematodes in the soil probably explains the significant decline in nematode numbers over time in the control (Figure 3.3.9). The IJs of pathogenic nematodes are completely non-feeding and rely entirely on their accumulated energy reserves for survival until a host is found (Hass *et al.* 2002). Because of this, starvation is thought to be one of the main causes of mortality in predator-free water (Qiu and Bedding 2000) and along with desiccation may explain the natural decline in the number of extractable nematodes IJs from the control. Despite the increase in the structural complexity of the soil substrate, *F. candida* were capable of reducing nematode numbers significantly over the 120 h sampling period. The number of nematodes at the soil surface showed a more rapid decline than overall nematode numbers (Figure 3.3.10). This is probably due to the predominantly epiedaphic lifestyle of *F. candida* (see Fountain and Hopkin 2004), resulting in higher levels of predation at the soil surface. As discussed previously, surface dwelling collembolan species are larger and have higher metabolic rates and an ensuing higher rate of food consumption than epiedaphic species. This has particular significance for the persistence of nematode biopesticides, as nematodes are generally applied to the soil surface. However, Wilson and Gaugler (2004) found no difference in the long-term persistence of the entomopathogenic nematode *Heterorhabditis bacteriophora* when applications were on the surface or sub-surface of turf plots.

Comparison of the rates of infection by *H. megidis* on *T. molitor* over time showed that the presence of *F. candida* had a significant effect on reducing rates of infection on both substrates. There was a more rapid decline in the infection rate on plaster-of-Paris compared to soil. This is probably due to the nematode availability on plaster-of-Paris being considerably higher than in soil, allowing the collembola to search for and find their nematode prey more easily. The highly complex soil environment provides areas that are inaccessible to large collembola such as *F. candida*, allowing a residual population of nematodes to remain and infect the host organism. Despite this, these experiments show that even a predominantly surface-dwelling collembolan species such as *F. candida* is capable of significantly reducing the efficacy of an insect-parasitic nematode within soil. Collembolan populations are made up of species that occupy a range of spatial niches. Although they tend to be broadly divided into the epiedaphic and euedaphic species, there are a range of intermediate forms (Werner and Dindal

1987). This study has mainly focussed on the larger surface dwelling epiedaphic species, as the euedaphic species proved to be difficult to culture in the laboratory. Because of this, little is known about their potential for nematophagy. If nematophagy is common amongst the smaller euedaphic species, refuga in which the nematodes are out of reach would be considerably less, resulting in higher levels of predation on nematodes within the soil.

Below ground food-webs are of particular interest to ecologists, as the interactions of soil organisms is an important factor in above-ground productivity. This study has demonstrated the potential for PCR-based methods for determining the diet of soil-dwelling microarthropods. Previous studies have mainly focused on the detection of the gut contents of relatively large invertebrates such as carabid beetles (Harper *et al.* 2005; Juen and Traugott 2005), linyphiid spiders (Agustí *et al.* 2003) and predatory bugs (Ma *et al.* 2005). The ability to detect the gut contents of small, soil-dwelling invertebrates opens up new avenues of research, helping with the understanding of the complex below-ground food-webs.

Chapter 4

Effects of nematode biopesticide inundation on the soil food-web

Abstract

Parasitic nematodes are used to control a wide range of soil-dwelling pests in a variety of different agroecosystems. Despite their relatively widespread use, little is known about the effects that inundation with nematodes has on the soil food-web and which components of the soil food web they directly interact with. This work describes a field study on the impact of the addition of the infective juveniles (IJs) of a mollusc parasitic nematode, *Phasmarhabditis hermaphrodita*, to a soil food-web. The abundance of microarthropods on and below the soil surface was recorded before and after nematode application, in nematode-treated plots and untreated control plots. This was used to identify microarthropods that responded either positively or negatively to nematode application. The surface-dwelling collembolan, *Isotoma viridis*, was found to respond positively by increasing in numbers in nematode treated plots when compared to non-treated control plots. The below-ground microarthropods, with the exception of the mites (Acari spp.) did not respond numerically to nematode addition to the soil. In addition, species-specific PCR-probes for *P. hermaphrodita* were used to screen for predation by the surface-dwelling microarthropods. High levels of predation by the collembola *I. viridis* and *Isotomurus palustris* indicated that predation by this group may have a significant effect on nematode persistence. Information on food-web interactions may allow for a better understanding of the factors that influence pest control and the persistence and recycling of these introduced biological control agents in the environment.

4.1. Introduction

Inundative applications of nematode biopesticides are used to control a wide range of insect and mollusc pests (Kaya and Gaugler 1993) in a variety of different crops including fruit trees, mushrooms, greenhouse and nursery plants, and vegetable and cereal crops (Ester *et al.* 2003; Kim *et al.* 2004; Lola-Luz and Downes 2007; Rica *et al.* 2006). Introductions of biological control agents in agroecosystems have been shown to have a diverse range of effects, both on the target pest-species and other non-target species of invertebrate (Frank and McCoy 2007). Understanding the processes behind these effects is important, both for the conservation of biodiversity within agroecosystems (Samways 2007) and in determining the success and persistence of introduced biological control agents (Hajek *et al.* 2007). Previous introductions of invertebrates for biological control have indicated that non-target effects can occur in a range of different systems (Wajnberg *et al.* 2001). However, such research has focussed on non-target effects in above-ground ecosystems, where interactions are generally easier to observe (e.g. Frank *et al.* 2007) and manipulation of predator and prey densities are technically more feasible (e.g. Schmidt *et al.* 2004). Below-ground food webs are highly complex and due to the concealed nature of the ecosystem, determining trophic interactions can be difficult (Wardle 2006).

The inundative introduction of nematode infective juveniles (IJs) to the soil can have a number of direct and indirect effects on the soil biota. Applications of nematode IJs for the control of soil-dwelling pests are usually at densities in the range of 300,000 to 500,000 per m^2 (Kaya and Gaugler 1993) although they can be applied at densities as high as 3,850,000 per m^2 (Wright 1988) and may involve repeated applications over time (Belay *et al.* 2005). These nematode inundations will result in temporarily inflated nematode populations within the soil. This influx of nematode IJs in the soil may have a wide range of different effects on the existing soil biota, due to the increased level of mollusc or insect pathogens in the environment, an increase in food and nutrient levels in the soil and increased competition with nematode species already present in the soil.

The infection of target and non-target macroarthropods can cause temporary or permanent suppression of invertebrate populations (Grewal *et al.* 2004). Although many entomopathogenic nematodes have been shown to infect a wide range of

macroarthropods in the laboratory, the range of species that are infected and controlled by nematode application in the field is considerably smaller (Bathon 1996). Effects of entomopathogenic nematodes on macroarthropods have been investigated in a number of field trials. Wang *et al.* (2001) showed that applications of the entomopathogenic nematodes *Heterorhabditis bacteriophora*, *Steinernema carpocapsae* and *S. riobravis* caused significant increases in the number of Staphylinidae. Campbell *et al.* (1995) found that application of *H. bacteriophora* caused increases in surface catches of mobile arthropods, particularly Araneae and Lithobiomorpha, whereas no change in catches was observed in plots sprayed with *S. carpocapsae*. Treatment with the entomopathogenic nematode *Heterorhabditis megidis* was found to cause temporary changes in the abundance of a range of soil-dwelling macroarthropods (Chevalier and Webster 2006). However, Georgis *et al.* (1991) found that applications of the nematode *S. carpocapsae* had no effect on non-target soil organisms compared to the control, and Ropek and Jaworska (1994) found that *S. carpocapsae* had no effect on the abundance of adult carabid beetles.

The response of microarthropods and other micro-fauna to nematode inundation has received less attention, possibly because they are not potential hosts for the nematode biopesticides commonly in use (see Chapter 3, Section 3.3.1). Plant parasitic nematodes have been shown to be adversely affected by applications of entomopathogenic nematodes, causing changes in the overall nematode community structure (Somasekhar *et al.* 2002; Jagdale *et al.* 2002; Lewis *et al.* 2001). Microarthropods, including collembola and mites, are important functional groups in the soil ecosystem, playing a role in nitrogen mineralisation, soil respiration, leaching of dissolved carbon and plant growth (Filser 2002). Previous studies have shown increases in the number of oribatid mites (Wang *et al.* 2001) and a decline in the number of collembola (Chevalier and Webster 2006) in response to nematode biopesticide application. The numerical responses of microarthropods to nematode inundation are potentially important due to changes in the aforementioned soil processes with which they are involved.

One potential effect of nematode application is caused by the increase in food for nematophagous soil-dwelling organisms. A wide range of soil biota have been recorded feeding on nematodes, including turbellarians, other nematodes, tardigrades, oligochaetes, mites, collembola and insects (Muaoka and Ishibashi 1976; Jatala 1986;

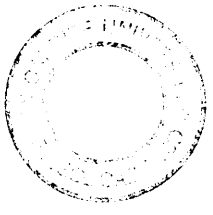
Sayre and Walter 1991; Kaya and Koppenhofer 1996) and some groups of microarthropods have been recorded feeding specifically on the nematode species commonly used to control soil-dwelling pests (Read *et al.* 2006; Epsky *et al.* 1988; Gilmore and Potter 1993). Interactions of this type may play an important role in the persistence of nematodes as biological control agents and hence influence efficacy and effectiveness of pest control. The poor persistence of nematode IJs in the soil has often thought to be one of the main reasons for a lack of prolonged pest control (Smits 1996; Koppenhofer *et al.* 1997; Georgis *et al.* 2006). Identifying predators of nematodes may provide a biotic explanation for the poor persistence of nematode biopesticides in some situations.

Although direct observations of the abundance of soil-dwelling microarthropods may provide information about the numerical effects of nematode application to soil ecosystems, they provide little information about the factors involved in driving population changes. Identifying trophic interactions, as well as numerical responses, allows for a better understanding of these processes.

In this study, field trials were conducted using the mollusc-parasitic nematode *Phasmarhabditis hermaphrodita* (Rhabditida: Rhabditidae), which is an obligate parasite of slugs and snails. It has been widely used for the control of the field grey slug, *Deroceras reticulatum* (Mollusca: Pulmonata), in asparagus (Ester *et al.* 2003), ornamentals (Grewal *et al.* 2001; Iglesias *et al.* 2001a), winter wheat (Wilson *et al.* 2004; Hass *et al.* 1999), Chinese cabbage (Hass *et al.* 1999) and lettuce crops (Wilson *et al.* 1995; Iglesias *et al.* 2001b). As with entomopathogenic nematodes, the nematode IJs are applied directly to the soil surface to control pest species living within and on the soil.

Field-based experiments were conducted to investigate a number of different aims, including to:

1. Determine the response of both euedaphic and epiedaphic microarthropods to an inundation of IJs of the mollusc parasitic nematode *P. hermaphrodita* to the soil ecosystem.



2. Identify the species of microarthropod that prey on nematode IJs after nematode inundation.

3. Determine the effect of an inundation of *P. hermaphrodita* on other soil-dwelling nematodes.

4.2. Materials and Methods

4.2.1. Microarthropod predation on nematodes in the field – Preliminary study

Field work was carried out at Burdens Farm, an arable farm at Wenvoe, nr. Cardiff, UK (Grid ref: ST120731), on the 22-23 June 2005 in fields of barley. The commercially available nematode *Phasmarhabditis hermaphrodita* (BeckerUnderwood, Littlehampton, UK) was applied in the form of IJs to the soil surface in four treatment plots (100 × 45 cm in the tram lines created by tractor wheels) alternating with four control areas (water applied but no nematodes), with a 1 m buffer area between plots. The nematodes were applied at the recommended field rate of 300,000 IJs in 250 ml of water per m^2, using a watering can. This was followed with a second irrigation of a further 250 ml per m^2 of water 10 min later to facilitate nematode penetration of the soil. Sites were left for 12 h overnight starting at 20:00 h. After this period, 5 min manual searches for microarthropods were carried out in each patch using a pooter, searching under loose soil and vegetation. The collected microarthropods were stored in 20 ml plastic tubes on ice in the field until they could be frozen at -80 °C at the laboratory.

Identification was carried out under a microscope using the keys from Hopkins (2007). The four most abundant microarthropod taxa encountered were: *Isotoma viridis*, *Isotomurus palustris*, *Lepidocyrtus cyaneus* and mesostigmatid mites (Acari: Mesostigmata). These were screened for the presence of *P. hermaphrodita* DNA within their guts. Five individuals of each taxa from each treatment area and control plot (n = 40) were washed in sterile water, placed in clean Eppendorf tubes and stored at -80 °C until later screening.

4.2.2. Detecting microarthropod predation on *Phasmarhabditis hermaphrodita* in the field and the effect of nematode application on microarthropod abundance

Field work took place in an arable field planted with wheat, at Wenvoe, nr. Cardiff starting on the 21st March 2006. A total of 30 treatment and 30 control plots (1 x 1 m) were arranged on a 6 x 10 grid, with 2 m between each plot. Plots were arranged in the field at least 10 m from the field margins to prevent any edge effects on the distribution of microarthropods (Ferguson 2004). As the field site was on a slope, plots were

arranged in 6 horizontal 'blocks' to allow the influence of position on the slope to be examined. On day zero, treatment plots were sprayed with the nematode *P. hermaphrodita* at a rate of 300,000 per m^2 in 1 L of dechlorinated tap water using a watering can during the evening (19:00-21:00). Control plots were sprayed with 1 L of water and no nematodes.

4.2.2.1 Surface-dwelling microarthropod sampling

Six treatment and six control plots were sampled two days before nematode application (-2 d) and then on 1, 6, 12 and 24 days after the initial application of nematodes (Figure 4.2.1). Samples (see Section 4.2.2 and 4.2.3) were then taken from one treatment and one control plot from each of the blocks on each sampling day, allowing for an even distribution of block samples on each day.

Block 1	24	1	12	6	1	-2	12	24	-2	6
Block 2	12	-2	6	24	12	6	-2	1	24	1
Block 3	1	24	6	-2	1	12	12	6	-2	24
Block 4	12	1	6	24	6	12	-2	24	1	-2
Block 5	6	-2	24	12	-2	24	1	12	1	6
Block 6	1	1	12	6	24	12	-2	-2	24	6

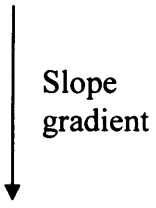


Figure 4.2.1 Experimental layout of the field trial. Numbers -2 to 24 represent sampling days, shaded boxes represent treatment plots (sprayed with *Phasmarhabditis hermaphrodita*), unshaded boxes represent control plots sprayed with water. Blocks represent groups of plots on a slope.

Collection of surface-dwelling microarthropods for abundance monitoring and for screening for predation, consisted of a 5 min manual search of the surface of each plot with an electric pooter. Any microarthropods on the surface or under loose vegetation and soil were collected and placed immediately on ice to preserve the gut contents. Once back in the lab, samples were stored at -80 °C until further processing commenced. Surface-dwelling collembola were identified to species where possible, using the keys in Hopkins (2007). Where identification to species level was not possible, unidentified collembola were identified to order or family level. As mites were present in low number on the soil surface, they were placed together as one group under the name Acari spp. The numbers of surface-dwelling microarthropods in all plots were

counted and 24 randomly selected individuals from each treatment plot were selected for gut contents screening for *P. hermaphrodita* (Section 4.2.4). A further four randomly selected microarthropods from each of the control plots (total 24 on each sampling day) were also selected for screening for evidence of predation on *P. hermaphrodita* (Section 4.2.4).

4.2.2.2. Soil-dwelling microarthropod sampling

In each of the six treatment and six control plots on each of the sampling days, five soil cores (4 cm diameter x 10 cm deep) were taken from random points within each plot. The cores from each plot were pooled together in rigid plastic containers to prevent microarthropods from being crushed. In the laboratory, the soil cores were gently mixed by hand to obtain a composite sample, reducing the influence of variable spatial patterns of soil biota in the sample soil. To determine total microarthropod numbers within the soil samples, 200 g of soil from each plot was placed in a stainless steel Tullgren funnel apparatus (Burkard Manufacturing Ltd, UK). Soil samples were left for 48 h in the funnels, under 40 W bulbs. The collected invertebrates were stored in 70 % EtOH. The total number of collembola and mites in each sample were counted under a stereo microscope. As identification of euedaphic microarthropods is difficult, due to small size and a lack of features, the samples were not identified to species level. Instead, the number of collembola in each sample belonging to the orders Poduromorpha, Entomobryomorpha and Symphypleona were recorded. The Entomobryomorpha, were further subdivided into two size classes: (1) individuals less than 1.0 mm and (2) individuals greater than 1.0 mm. Mites were again recorded as a single group.

4.2.2.3. Nematode sampling

To determine overall nematode numbers within the soil, a 50 g sample of soil from each plot was placed in a modified Baermann funnel apparatus, (adapted from Southey 1970) and left for 48 h at room temperature (22 ± 2 °C) (see Chapter 2, Section 2.1.2 for methodology). Nematodes in the collection chamber were removed and numbers were counted in a Sedgewick rafter counting cell (Graticules Limited, Tonbridge, UK) under a stereo microscope. After counting the total number of nematodes of all types in each sample, the nematodes from each plot were placed in 1.5 ml centrifuge tubes. The tubes

were centrifuged for 15 min at 13,000 rpm to pellet the nematodes. The supernatant was then removed using a pipette and the pelleted nematodes were stored at -80 °C for later extraction and screening.

4.2.2.4. Creation of a standard curve for estimation of *Phasmarhabditis hermaphrodita* density from soil

In order to assess the density of *P. hermaphrodita* in experimental plots and to check for its absence from control plots, a nematode density series, in soil, was produced. Soil from the field site at Wenvoe nr. Cardiff was defaunated by heating (at 60 °C for 48 h) in order to destroy any naturally occurring nematodes and other soil biota. The defaunated soil was then rehydrated to 25 % w/v prior to the start of the experiment. Following rehydration, 2000 *P. hermaphrodita* IJs were applied to 200 g of soil. A serial dilution of nematodes was then achieved by mixing half the soil with an equal weight of defaunated nematode-free soil. Prior to each soil dilution the soil was mixed thoroughly, by hand, to ensure even distribution of the nematodes through the sample. The dilution process was repeated six more times, creating a nematode dilution series of approximately 2000, 1000, 500, 250, 125, 62.5, 32.3 and 15.6 nematodes per 200 g of soil. Three replicates of each dilution were created and stored in sealed plastic bags.

The number of *P. hermaphrodita* in the nematode dilution soil samples was estimated using a modified baiting method commonly used for entomopathogenic nematodes (Curran and Heng 1992; Hass *et al.* 1999). This involved baiting soil samples with uninfected hosts (in this case, the slug *D. reticulatum*) for a set time period and then dissecting and counting the number of nematodes that established in each host. Preliminary experiments using live slugs as hosts resulted in inconsistent numbers of nematodes establishing in each host, probably due to varying host defence and health levels. Instead, pre-killed slugs (stored at -80 °C and then defrosted) were used to bait each soil sample, resulting in less variation between replicates. Five dead *D. reticulatum* weighing between 100-150 mg were placed in each soil sample and left for 72 h at 16 ± 1 °C in the dark. After this time, the rear mantle of each of the slugs was removed and digested for approximately 2 h using pepsin solution (see Chapter for details). As the pepsin solution was not entirely effective at digesting slug tissue, the remains of the mantle were broken apart in 0.1% Tween (Sigma-Aldrich, UK) solution using forceps,

in a 10 cm Petri dish. The nematodes IJs in each mantle were then counted. The number of nematodes in each host after exposure to different concentrations of IJs was then used to create a calibration curve, from which the concentration of nematodes in unknown samples could be calculated.

4.2.2.5. Determining the persistence of *Phasmarhabditis hermaphrodita* in field

To determine the rate of *P. hermaphrodita* persistence in the field, 200 g samples of soil from each plot were placed in resealable plastic bags. In each of these, three dead *D. reticulatum* weighing 100-150 mg were placed and the bags were stored for 72 h at 16 ± 1 °C in the dark. As slugs were difficult to obtain during the course of the experiment, the control samples were only baited with one slug each, to check for the presence *P. hermaphrodita*. The number of IJs in each host were recorded and used to infer the concentration of viable *P. hermaphrodita* IJs in the field-collected soil samples, using the calibration curve described in Section 4.2.2.4.

4.2.2.6. Environmental variables

The percentage moisture content of the soil was calculated by weighing 100 g of soil, drying it for 72 h at 60 °C and re-weighing. The difference in weight was assumed to be loss of water, which was then calculated as a percentage of the original soil sample. The average temperature in the field was recorded by placing two temperature data-loggers in the field site, one at the top of the sampling grid and one at the bottom. The data-loggers were placed on the soil surface and protected from direct sunlight by a vented plastic, housing. Temperature data was interpreted using Boxcar Pro software (MicroDAQ, Contoocook, USA).

4.2.3. DNA extraction

Microarthropod samples collected on the soil surface were placed individually on a 100 micron sieve and washed first with 70 % HPLC grade EtOH and secondly, with DNA free PCR water (Sigma, UK) to reduce external contamination. Microarthropods were then placed individually into clean 1.5 ml centrifuge tubes before following the extraction protocol outlined in Chapter 3, Section 3.2.7.

4.2.4. Screening for predation on *Phasmarhabditis hermaphrodita*

Screening of all field collected microarthropods was a two-stage process. The first stage involved screening the DNA extract with general primers to confirm the success or failure of the DNA extraction. Any samples that did not amplify using the general primers were tested one more time. If no band was observed for a second time, the sample was discarded from the analysis. General invertebrate primers *C1-J-1718* and *C1-J2191* (Simon *et al.* 1994) that amplified a 525-bp region of mtDNA from Subunit I of the Cytochrome Oxidase (*COI*) coding region were used (see Chapter 2, Sections 2.1.5.1 and 2.2.1 for primer sequences and PCR protocols). The second stage of the screening process involved screening for the presence of *P. hermaphrodita* DNA in the gut of microarthropods using primers *Ph-F-(271-291)* and *Ph-R-(479-501)* (see Chapter 2, Sections 2.1.5.2 and 2.2.1 for primer sequences and PCR protocols).

4.2.5. Statistical Analysis.

All data on soil biota abundance were analysed using a series of Generalized Linear Models (GLM) using the ASReml2 (VSN international Ltd, Hertfordshire, UK) statistical package. Analyses addressed the following questions. Does application of *P. hermaphrodita* alter the abundance of (1) microarthropods living on and within the soil and (2) soil-dwelling nematodes?

In order to assess the affect of the application of *P. hermaphrodita* IJs on the abundance of surface-dwelling microarthropods, soil-dwelling microarthropods and soil-dwelling nematodes, GLMs were conducted with the abundance of each of these groups incorporated as the dependent variable and treatment and a range of environmental variables as the independent variables (see Tables 4.2.2 - 4.2.4 for independent variables). Both the surface and soil-dwelling microarthropod groups were further analysed using the most abundant constituent taxa and these were included as dependent variables using GLM. The dependent variables for the surface-dwelling microarthropods were, the abundance of *Isotoma viridis* and *Isotomurus palustris* and the dependent variables for the soil-dwelling microarthropods were the abundance of

Acari spp, Entomobryomorpha (< 1mm), Poduromorpha and Symphypleona (see Tables 4.2.2 - 4.2.4 for all dependent variables).

In all GLM models where the microarthropod abundance data were treated as the dependent variables, running the models with a normal distribution and identity link function, gave non-normal residual distributions. However, GLMs with a normal distribution and a log link function were found to give rise to normal residual distributions and were therefore used throughout the subsequent analyses. Histograms of the independent variable soil moisture, showed underdispersion and so a power transformation (x^2) was used to normalise this data prior to analysis. Histograms of nematode abundance, surface microarthropod abundance and soil-dwelling microarthropod abundance showed overdispersion, therefore a base log 10 transformation was used to normalise these data prior to analysis where they were included as independent variables. Histograms of all other independent variables showed normal distributions for the data.

Limitations of sample size prevented the use of a single model for each dependent variable, including all possible independent terms and their interactions, as this would have resulted in extremely low degrees of freedom. The main model, for each of the dependent variables, was therefore formed from a number of submodels. These submodels analysed related data types and their interactions. For example, a range of summary data for temperature (i.e. average temperature, cumulative temperature, variance of temperature, temperature as a factor) and the interactions between those components was analysed in one submodel (Table 4.2.1). Non-significant terms were removed from these submodels in a stepwise manner, until a minimal model was produced. The restriction for significance was relaxed in these models and terms were retained if the *P*-value was < 0.1. The terms from the resulting models were then placed together in a combined model; this process of submodel combination was repeated for each dependent variable. No more than two submodels were undertaken for each dependent term. The abundance of *P. hermaphrodita* was not used as an independent term as its presence caused heteroscedasticity patterns of residuals in the final models which could not be overcome with any of the standard distributions and link functions.

The term 'plot' was initially included in all the combined models as a random factor, to control for any variation due to location that could not be explained by the measured environmental variables. However, the inclusion of this term caused poor residual distribution and as a result this term was removed from the final models. The model terms were removed in a stepwise manner until the final significant model was reached. All models were checked for the spread and pattern of the residuals to assess model fitting. The initial terms placed in the combined models are shown in Tables 4.2.2, 4.2.3 and 4.2.4.

Table 4.2.1 Terms initially included in submodels used to examine the influence of temperature on all the dependent variables (v denotes a continuous variable and c denotes a categorical variable).

Environmental measure	Dependent variables	Independent continuous/categorical variables
All variables in Table 4.22., 4.2.3 and 4.2.4	All dependent variables in Table 4.22., 4.2.3 and 4.2.4	Average temperature (v) Cumulative temperature (v) Variance of temperature (v) Average temperature (c) Average temperature (v) x Cumulative temperature (v) Average temperature (v) x Variance of temperature (v) Cumulative temperature (v) x Variance of temperature (v)

Table 4.2.2 Terms initially included in the abundance of soil-dwelling microarthropod models (v denotes a continuous variable and c denotes a categorical variable).

Environmental measure	Dependent variables	Independent continuous/categorical variables
Total number of microarthropods within the soil matrix	Log number of microarthropods within the soil matrix Log number of Acari within the soil matrix Log number of Entomobryomorpha ,< 1mm within the soil matrix Log number of Poduromorpha within the soil matrix Log number of Symphypleona within the soil matrix	Treatment (c) Average temperature (c) Moisture^2 (v) Day (v) Block (c) Plot (c) random Log nematodes (v) Log surface microarthropods (v) Treatment x Moisture^2 Treatment x Day Treatment x Log surface microarthropods Treatment x Log nematodes Moisture^2 x Day Moisture^2 x Log surface microarthropods Moisture^2 x Log nematodes Day x Log surface microarthropods Day x Log nematodes Log surface microarthropods x Log nematodes

Table 4.2.3 Terms initially included in the abundance of surface-dwelling microarthropod models (v denotes a continuous variable and c denotes a categorical variable).

Environmental measure	Dependent variable	Independent continuous/categorical variables
Total number of microarthropods on the soil surface	Log number of microarthropods on the soil surface Log number of *Isotoma viridis* on the soil surface Log number of *Isotomurus palustris* on the soil surface	Treatment (c) Moisture^2 (v) Day (v) Block (c) Plot (c) random Average temperature (c) Log within soil microarthropods (v) Log nematodes (v) Treatment x Moisture^2 Treatment x Day Treatment x Log within soil microarthropods Treatment x Log nematodes Moisture^2 x Day Moisture^2 x Log within soil microarthropods Moisture^2 x Log nematodes Day x Log soil microarthropods Day x Log nematodes Log within soil microarthropods x Log nematodes

Table 4.2.4 Terms initially included in the abundance of soil-dwelling nematode model (v denotes a continuous variable and c denotes a categorical variable).

Environmental measure	Dependent variable	Independent continuous/categorical variables
Total number of nematodes within soil matrix	Log number of nematodes within the soil matrix	Treatment (c) Moisture^2 (v) Day (v) Block (c) Plot (c) random Average temperature (c) Log within soil microarthropods (v) Log surface microarthropods (v) Treatment x Moisture^2 Treatment x Day Treatment x Log within soil microarthropods Treatment x Log surface microarthropods Moisture^2 x Day Moisture^2 x Log within soil microarthropods Moisture^2 x Log surface microarthropods Day x Log soil microarthropods Day x Log surface microarthropods Log within soil microarthropods x Log surface microarthropods

4.3. Results

4.3.1. Microarthropod predation on nematodes in the field – Preliminary study

Analysis of microarthropod predators captured in the field was used both to identify natural enemies of *Phasmarhabditis hermaphrodita* and to measure, under field conditions, the proportions of predators within each taxon that had fed on these nematodes. PCR-based gut screening of five individuals per plot of each of the four dominant taxa of soil microarthropod, showed evidence of feeding on the nematodes by the collembola *Isotoma viridis* and *Isotomurus palustris,* and by mesostigmatid mites (Figure 4.3.1). No predation was recorded by *Lepidocyrtus cyaneus* on *P. hermaphrodita,* demonstrating strong evidence of differences in prey choice. Microarthropods collected from sites not sprayed with nematodes were all negative, suggesting that natural populations of *P. hermaphrodita* were not present (or present in low densities) at this field site.

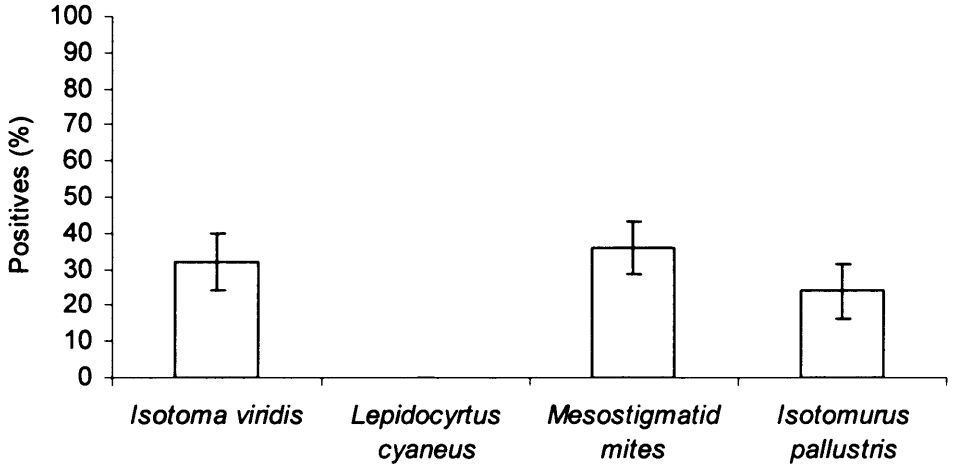


Figure 4.3.1 Percentage of field-caught microarthropods (the collembola *Isotoma viridis*, *Lepidocyrtus cyaneus* and *Isotomurus palustris*, and mesostigmatid mites) (n = 20 per taxa) testing positive for predation on the nematode *Phasmarhabditis hermaphrodita*. Bars represent ± S.E.

4.3.2. Detecting microarthropod predation on nematodes in the field and the effect of nematode inundation on microarthropod abundance

4.3.2.1. Estimating persistence of *Phasmarhabditis hermaphrodita* infective juveniles (IJs) in soil samples

In order to assess the density of *P. hermaphrodita* IJs in the collected field soil samples, a calibration plot was created. The number of *P. hermaphrodita* in infected hosts, *D. reticulatum*, was regressed against the number of nematodes in the soil dilution series (described in Section 4.2.2.4). The data was well described by a simple linear regression ($F = 290.69$, $df = 1$, $P < 0.001$; with the linear equation, $y = 0.0323\ x$) (Figure 4.3.2). The number of *P. hermaphrodita* per sample infecting each slug was then translated to a number of nematode IJs per 200g of soil using the calibration curve.

There was no evidence of *P. hermaphrodita* in any of the control plots. In the treatment plots, the number of infective *P. hermaphrodita* declined with time and by day 24 of the trial only two of the six treatment plots still showed evidence of the presence of *P. hermaphrodita* (Figure 4.3.3).

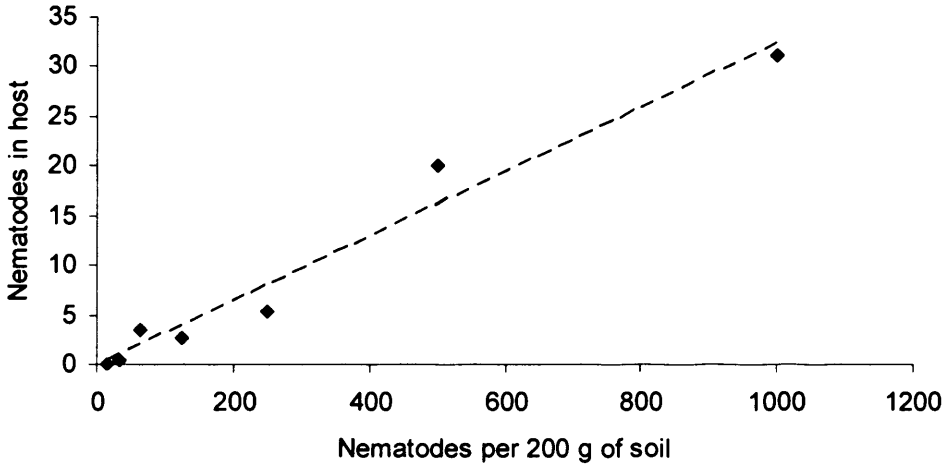


Figure 4.3.2 Calibration plot showing the number of *Phasmarhabditis hermaphrodita* in dead bait hosts, *Deroceras reticulatum*, in a concentration series of nematode IJs in sterilised soil.

Table 4.3.1 Average number of *Phasmarhabditis hermaphrodita* in dead bait *Deroceras reticulatum*, in soil treated with *P. hermaphrodita*. Numbers in parenthesis show standard errors.

Sample	Day -2		Day 1		Day 6		Day 12		Day 24	
1	0.0	(±0.0)	9.3	(±2.60)	3.3	(±1.86)	0.3	(±0.33)	0.0	(±0.00)
2	0.0	(±0.0)	15.0	(±4.36)	1.0	(±0.58)	5.0	(±2.00)	0.0	(±0.00)
3	0.0	(±0.0)	5.0	(±3.00)	14.7	(±3.48)	0.3	(±0.33)	0.0	(±0.00)
4	0.0	(±0.0)	4.7	(±1.67)	2.7	(±1.67)	3.7	(±2.19)	7.3	(±0.88)
5	0.0	(±0.0)	11.0	(±5.13)	13.0	(±2.08)	0.0	(±0.00)	1.3	(±0.88)
6	0.0	(±0.0)	9.3	(±2.67)	4.3	(±1.86)	1.7	(±1.20)	0.0	(±0.00)
Average	0.0		9.1		6.5		1.8		2.8	
StError	0.00		1.6		2.37		0.84		2.52	

Table 4.3.2 Calculated number of *Phasmarhabditis hermaphrodita* IJs per 200 g of soil from field collected samples, after transformation of raw values by x = y / 0.0323.

Sample	Day -2	Day 1	Day 6	Day 12	Day 24
1	0.0	303.0	108.2	10.8	0.0
2	0.0	487.0	32.5	162.3	0.0
3	0.0	162.3	476.2	10.8	0.0
4	0.0	151.5	86.6	119.0	238.1
5	0.0	357.1	422.1	0.0	43.3
6	0.0	303.0	140.7	54.1	0.0
Average	0.0	294.0	211.0	59.5	46.9
StError	0.00	51.3	77.0	27.3	38.9

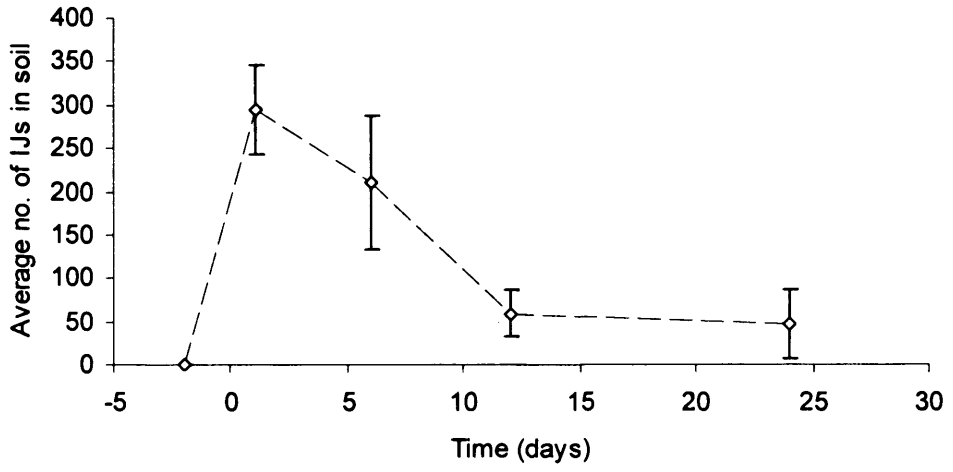


Figure 4.3.3 Calculated number of *Phasmarhabditis hermaphrodita* IJs in treated plots over time after transformation of raw values by x = y / 0.0323 from the nematode dilution series. Bars represent ± S.E.

4.3.2.2. Effect of application of *Phasmarhabditis hermaphrodita* infective juveniles on the abundance of soil-dwelling microarthropods and nematodes

Over the five sampling days, a total of 5018 microarthropods were collected from within the soil matrix and a further 2296 microarthropods were collected from the soil surface. Eight species of collembola from the soil surface samples were identified to species level (*Isotoma viridis*, *Isotomurus palustris*, *Entomobrya multifasicata*, *E. nivalis*, *Lepidocyrtus curvicolis*, *L. cyaneus*, *Orchesella cincta* and *O. villosa*). The remaining collembolan groups consisted of *Sminthuridae* spp., *Dicrtomidae* spp., and *Bourletiellidae* spp. Of the surface samples, *Isotoma viridis* (52.1 %) and *Isotomurus palustris* (27.2 %) represented the most common species, whilst Acari (2.9 %) and all other species of collembola (17.7 %) made up the rest. From the microarthropods collected within the soil matrix, Entomobryomorpha smaller than 1 mm made up the majority of samples collected (65.7 %), whilst Acari (22.7 %), Poduromorpha (4.5 %), Symphypleona (4.4 %) and Entomobryomorpha larger than 1 mm (2.6 %) made up the rest. A total of 26,513 nematodes of all types were counted from all the soil samples. The raw data for the abundance of surface- and soil-dwelling microarthropods is shown in Appendix 3, Tables 3.1 and 3.2, and Figures 3.1 to 3.11.

Microarthropod abundance on the soil surface

Treatment with the nematode *P. hermaphrodita* was the only independent variable that had a significant association with the total abundance of microarthropods on the soil surface (Table 4.3.3). Prediction from the model showed that treatment with nematode IJs increased the abundance of surface-dwelling microarthropods in each plot by 35.7 %, from an average of 31.6 to 42.9 individuals (Figure 4.3.4).

Analysis of the numerically dominant species of surface-dwelling microarthropod, the collembolan *Isotoma viridis*, revealed a significant affect of the application of *P. hermaphrodita*, with application predicted to increase *I. viridis* abundance by 41.8 % compared to the control (Figure 4.3.5). Additionally both time and soil moisture were associated with *I. viridis* abundance. Abundance of *I. viridis* was negatively associated with time, such that a 35.7 % decrease in abundance was predicted by day 24 (post-application) in both treated and control plots. Moisture had a positive association with abundance, with a 68.5 % increase in abundance predicted between the soils with lowest

and highest recorded moisture content (16.6 % and 34.6 %, respectively). Within this range, abundance of *I. viridis* is predicted to rise by 3.8% for every 1% rise in soil moisture content. *Isotomurus palustris* showed no significant association with any of the independent variables included in the model.

Table 4.3.3 Analysis of: a) total microarthropod abundance and b) abundance of *Isotoma viridis*. Generalised Linear Model outputs.

Dependent variable	Model terms	*F* statistic	d.f.	*P*
a) Total abundance of surface-dwelling microarthropods (log link)	Treatment	10.47	1,58	0.002
b) Abundance of *Isotoma viridis* on the soil surface (log link)	Treatment	6.76	1,56	0.012
	Moisture^2	10.62	1,56	0.002
	Day	5.11	1,56	0.028

Table 4.3.4 Analysis of: a) total number of microarthropods within the soil matrix, b) total number of Acari within the soil matrix, c) total number of Poduromorpha within the soil matrix, and d) total number of Entomobryomorpha < 1mm within the soil matrix. Generalised Linear Model outputs.

Dependent variable	Model terms	*F* statistic	d.f.	*P*
a) Total number of microarthropods within the soil matrix (log link)	Moisture^2	9.95	1,57	0.003
	Day	4.42	1,57	0.045
b) Total number of Acari within the soil matrix (log link)	Treatment	2.94	1,57	0.047
	Moisture^2	2.03	1,57	0.005
c) Total number of Poduromorpha within the soil matrix (log link)	Moisture^2	11.21	1,56	0.001
	Day	7.93	1,56	0.007
	Log number of surface-dwelling microarthropods	6.42	1,56	0.014
d) Total number of Entomobryomorpha < 1mm within the soil matrix (log link)	Moisture^2	6.91	1,57	0.011
	Day	4.61	1,57	0.036

Table 4.3.5 Analysis of the total number of nematodes within the soil matrix. Generalised Linear Model output.

Dependent variable	Model terms	*F* statistic	d.f.	*P*
Total number of nematodes within the soil matrix (identity link)	Treatment	0.22	1,56	0.637
	Day	6.27	1,56	0.015
	Treatment x Day	4.41	1,56	0.040

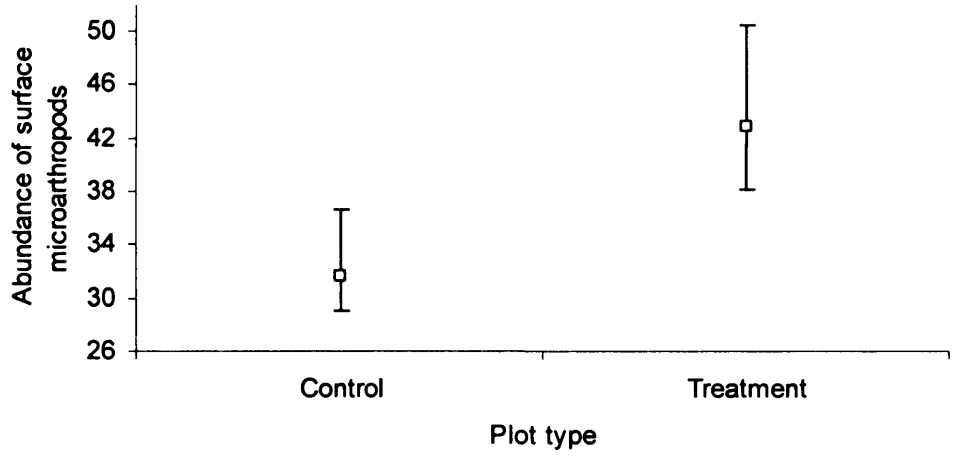


Figure 4.3.4 Predicted effect of application of *Phasmarhabditis hermaphrodita* IJs on the abundance of the combined total of all groups of surface-dwelling microarthropods when compared in untreated, control plots. Back-transformed values with 95 % confidence intervals are shown.

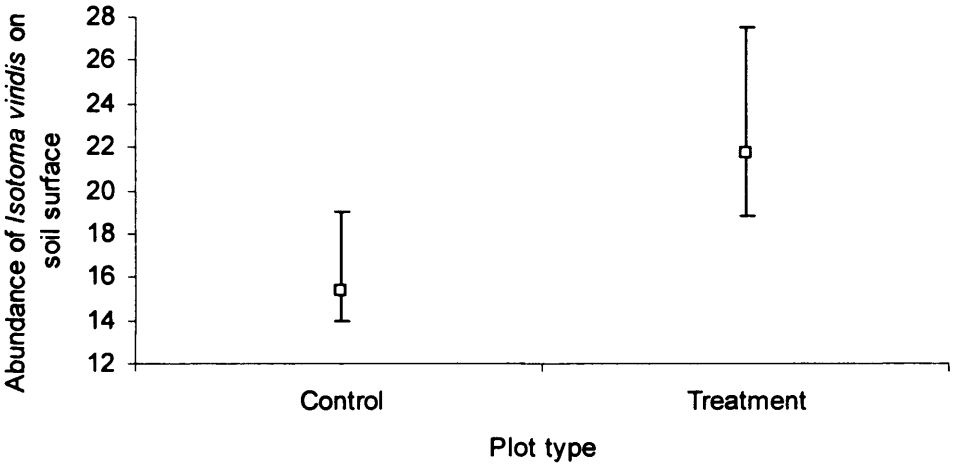


Figure 4.3.5 Predicted effect of application of *Phasmarhabditis hermaphrodita* IJs on the abundance of the collembolan *Isotoma viridis* when compared in untreated, control plots. Back-transformed values with 95 % confidence intervals are shown.

Microarthropod abundance within soil matrix

The minimal model for the total abundance of soil-dwelling microarthropods revealed that moisture had a positive association with total abundance, and day number had negative association (Table 4.3.4). Treatment was not significantly associated with the combined abundance of microarthropods in the soil. Only one group of soil-dwelling microarthropods, the mites (Acari spp.), had a significant association with treatment, with the addition of *P. hermaphrodita* predicted to cause a 24.5 % increase compared to control plots (Figure 4.3.6). Within all the soil-dwelling groups (Acari spp., Poduromorpha and Entomobryomorpha), soil moisture had a significant association with the total abundance of each of the three groups (Figure 4.3.7). Day was negatively associated with the number of Poduromorpha and Entomobryomorpha within the soil, and the number of Poduromorpha was negatively associated with the log of the number of microarthropods on the soil surface. None of the recorded variables were significantly associated with the number of Symphypleona within the soil matrix.

Nematode abundance within the soil matrix

The minimal model describing the abundance of nematodes within the soil showed that day had a significant effect on the abundance of nematodes within the soil matrix (Table 4.3.5). Treatment was only associated with the abundance of nematodes in interaction with day, such that the model predicted an increase in nematode numbers in the control plots, and a slight decline in the treatment plots (Figures 4.3.8 and 4.3.9). As a result, treatment with the nematode *P. hermaphrodita* was predicted to cause a 43.9 % reduction in overall nematode numbers compared to the control, by day 24 (post-application) of the study.

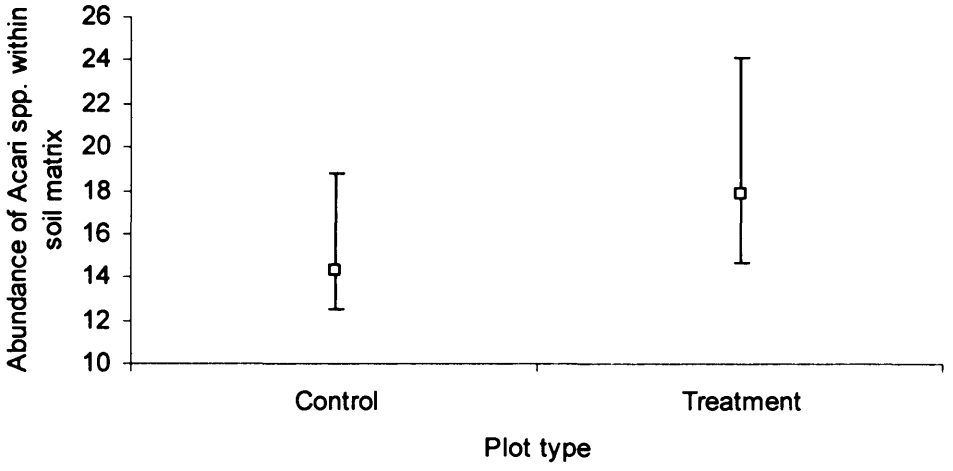


Figure 4.3.6 Predicted effect of application of *Phasmarhabditis hermaphrodita* IJs on the abundance of Acari spp. within the soil matrix when compared in untreated, control plots. Back-transformed values with 95 % confidence intervals are shown.

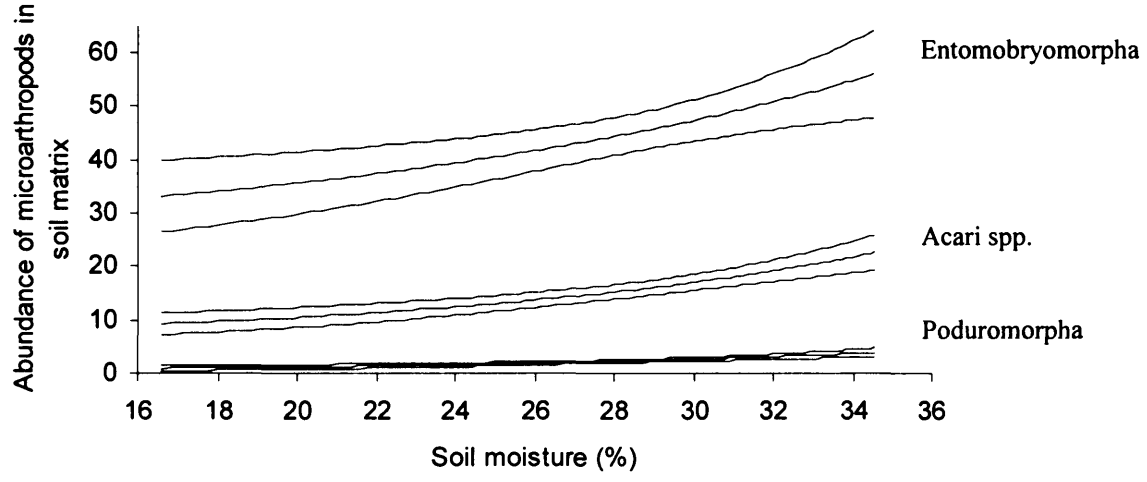


Figure 4.3.7 Predicted effect of moisture levels on the abundance of Acari spp., Poduromorpha and Entomobryomorpha (<1mm) when compared in untreated, control plots. Back-transformed values with 95 % confidence intervals are shown.

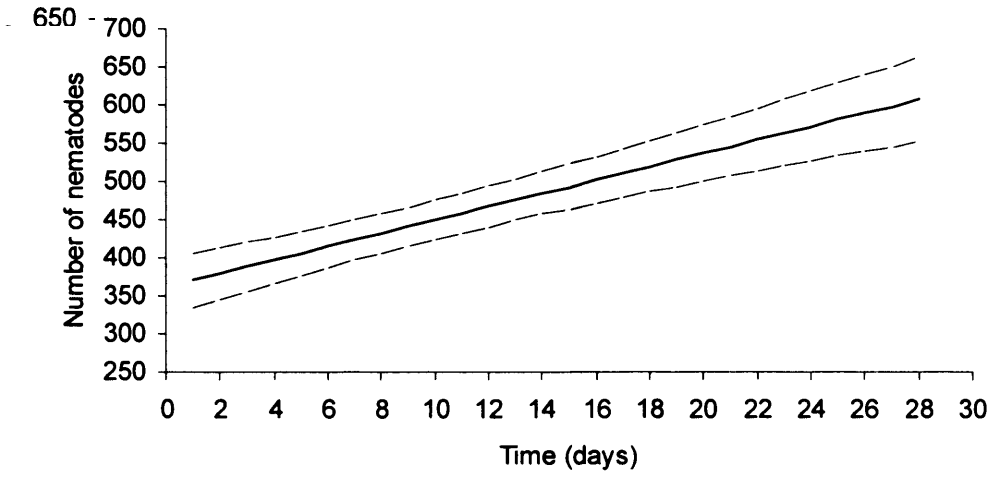


Figure 4.3.8 Predicted effect of time (days) on the abundance of nematodes within the soil matrix in untreated control plots. Back-transformed values with 95 % confidence intervals are shown.

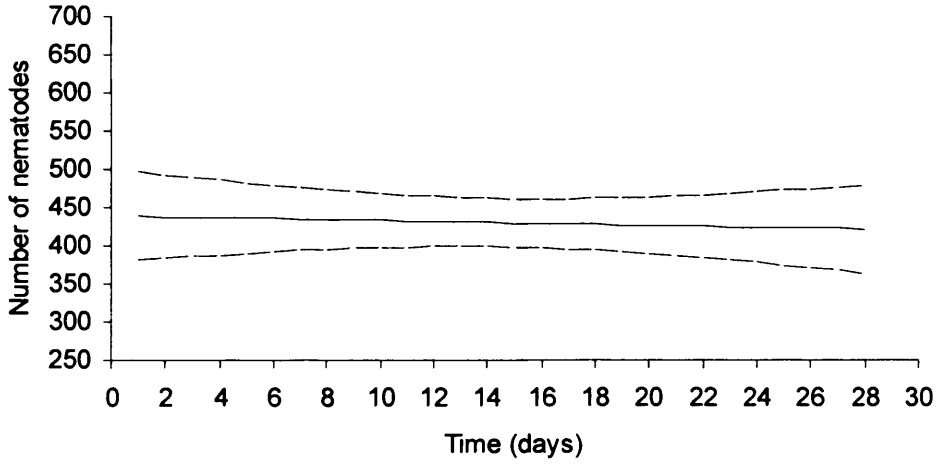


Figure 4.3.9 Predicted effect of time (days) on the abundance of nematodes within the soil matrix in plots treated with the nematode *Phasmarhabditis hermaphrodita*. Back-transformed values with 95 % confidence intervals are shown.

4.3.2.3. Detecting predation on *Phasmarhabditis hermaphrodita* infective juveniles in the field

Examples of ethidium-bromide stained agarose-gels showing the results of PCR-based screening for predation are shown in Appendix 3 (Figures 3.12 and 3.13 and Tables 3.3 and 3.4). A total of 166 microarthropods were screened on each of the sampling days for predation on the nematode *P. hermaphrodita*. The two most abundant species from the sub-samples of surface collected microarthropods were *Isotoma viridis* (55.1 %) and *Isotomurus palustris* (26.8 %). No feeding on *P. hermaphrodita* was detected before the addition of nematodes to the field site (Figure 4.3.10 a). There was no predation detected on *P. hermaphrodita* by any of the surface-dwelling microarthropods collected from the control plots on any of the sampling days. In the treatment plots on day one (one day after the application of *P. hermaphrodita*), 33 % of *Isotoma viridis* (n=88) and 35.9 % of *Isotomurus palustris* (n=39) tested positive for *P. hermaphrodita* (Figure 4.3.10. b). None of the other microarthropod species tested positive for *P. hermaphrodita* DNA. On day six (post-application) 10.6 % of *Isotoma viridis* (n=66), 10.5 % of *Isotomurus palustris* (n=38) and 33.3 % of the Acari spp (n=6) were positive for *P. hermaphrodita* DNA (Figure 4.3.10. c). No other species tested positive for the presence of *P. hermaphrodita* in their digestive tracts. On days 12 and 24, none of the surface collected predators tested positive for the presence of *P. hermaphrodita* DNA (Figures 4.3.10. d and e).

As temperature is shown to have a significant effect on prey detection times within the guts of collembola (Chapter 3, Section 3.3.5), the mean temperature in the 24 h prior to sampling was calculated and compared (Figure 4.3.11). The average temperature over 24 h on the five sampling days ranged from 9.6 °C to 13.3 °C. The temperature calibration curve described in Chapter 3, Section 3.3.5 (i.e. $y = 34.31 + 0.6410x - 0.04636x^2$, where y = median detection time, and x = temperature) calculated from feeding trials using the collembolan *F. candida* (a similar sized collembolan to *Isotoma viridis*), was used to calculate the estimated median detection times on each of the sampling days. The median detection times on days 1, 4, 10, 16 and 28 were 35.1, 34.6, 36.2, 35.6 and 35.3 h, respectively.

Figure 4.3.10 Graphs showing the number of microarthropods collected from six plots treated with the nematode *Phasmarhabditis hermaphrodita* (light bars) and the number of those individuals testing positive for *P. hermaphrodita* DNA (shaded bar). Figures represent: AC = Acari spp., IV = *Isotoma viridis*, IP = *Isotomurus palustris*, EM = *Entomobrya multifasicata*, EN = *Entomobrya nivalis*, LCU = *Lepidocyrtus curvicolis*, LCY = *Lepidocyrtus cyaneus*, OV = *Orchesella villosa*, OC = *Orchesella cincta*, SM = *Sminthuridae* spp., DI = *Dicrtomidae* spp., BO = *Bourletiellidae* spp., UN = Unidentified.

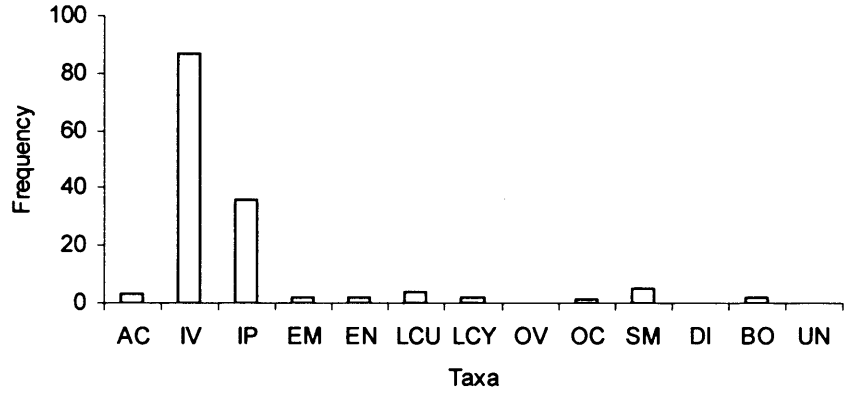


Figure 4.3.10 a) Day -2

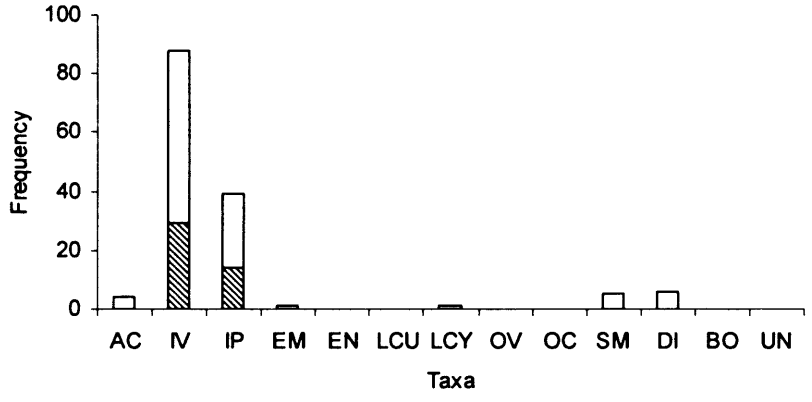


Figure 4.3.10 b) Day 1

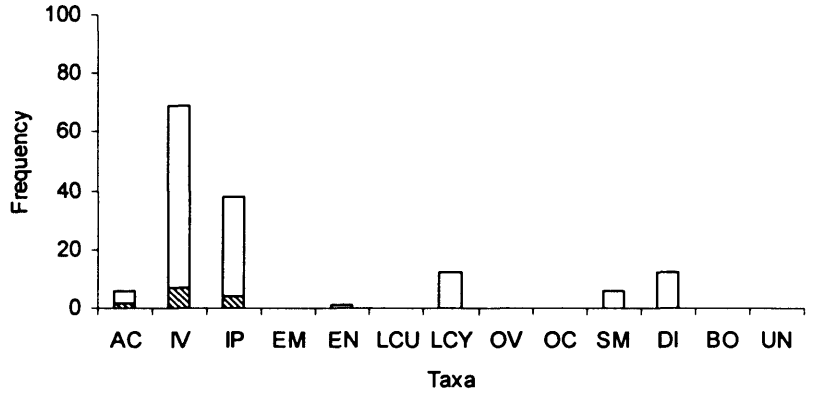


Figure 4.3.10 c) Day 6

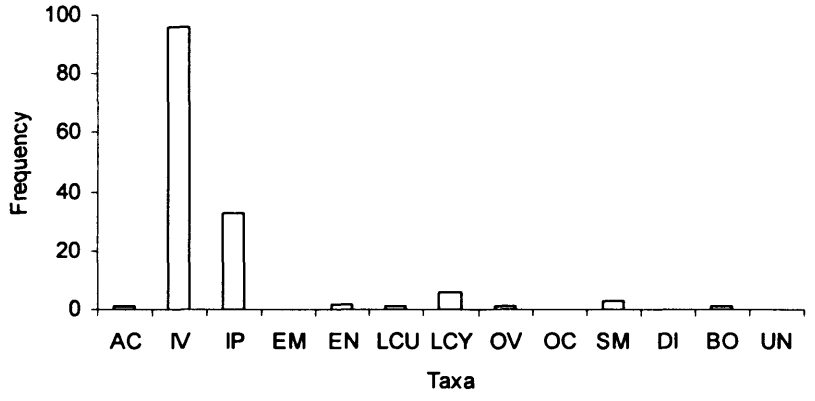


Figure 4.3.10 d) Day 12

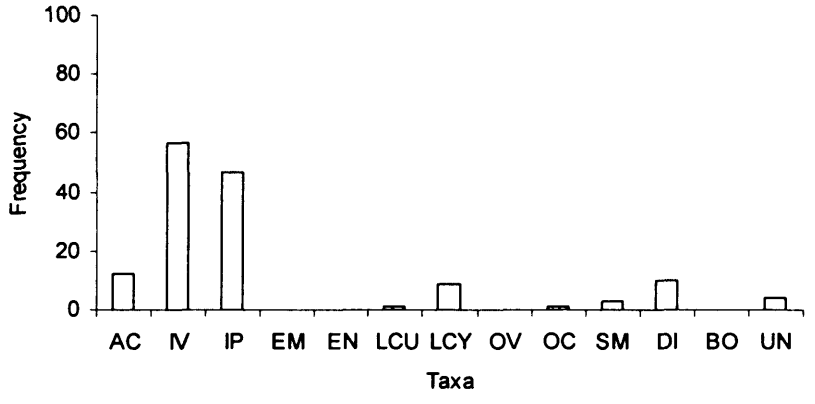


Figure 4.3.10 e) Day 24

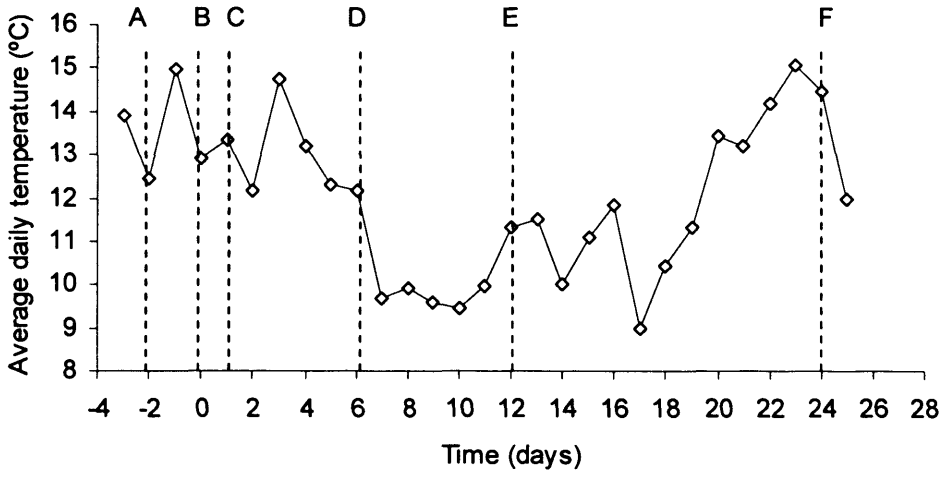


Figure 4.3.11 Average daily temperature over 24 h at Wenvoe nr. Cardiff, on day -2 to day 25 in °C. Symbols represent A = day -2 sampling. B = day 0 treatment with nematodes. C = day 1 sampling. D = day 6 sampling. E = day 12 sampling. F = day 24 sampling.

4.4. Discussion

This study demonstrated that the application of a mollusc-parasitic nematode to the soil ecosystem can cause changes to the abundance of microarthropods living on and within the soil. The preliminary field experiment (Section 4.2.1) provided valuable ecological information, identifying three taxa as significant predators of *Phasmarhabditis hermaphrodita*, under field conditions. Equally useful was the information that a fourth taxa, the collembolan *Lepidocyrtus cyaneus*, did not feed on these nematodes. A previous PCR-based study had shown that *L. cyaneus*, and two other collembola species, are themselves prey for linyphiid spiders (Agustí *et al.* 2003a). These data are, therefore, providing the first quantitative links in what must be a highly complex soil food web.

Rates of predation in the field ranged from 24 to 36 % of individuals testing positive. There have been relatively few other studies carried out using molecular detection of predation on field-caught invertebrates to use as a comparison, and none that address the interaction between microarthropods and their prey. Agustí *et al.* (2003a) found that rates of predation by field-caught linyphiid spiders on three species of collembola were 20-38 %. In other studies, of macroarthropods, predation levels by the carabid beetle *P. melanarius* on 15 different species of invertebrate prey ranged from 0% to 36% (Harper *et al.* 2005), predation by *Pterostichus melanarius* on the slug *Deroceras reticulatum* was recorded at 4.3% (in a year when very low slug populations densities were recorded: Dodd *et al.* 2003), and predation by *Nabis kinbergii* and *Lycosa* sp. on the diamondback moth *Plutella xylostella* in the field ranged from 10 - 67.6 % and 24.6 - 76.6 %, respectively (Ma *et al.* 2005). In the current study, the mites were analyzed as a single group, and therefore it is unknown which species were contributing the most to the reduction in nematode numbers. Many mesostigmatid mites are known to be predators of nematodes (Imbriani and Mankau 1983), being either exclusively nematophagous (Walter and Ikonen, 1989) or incorporating nematodes as a part of their diet (Inserra and Davis, 1983).

The second, more comprehensive field trial (Section 4.2.2), was designed to not only monitor which species of microarthropod were feeding on the introduced biological control agent over time, but to investigate how the inundative addition of nematode IJs

to the soil could affect the abundance of the soil biota, and how long the introduced nematodes persist in the soil in the short-term (24 days).

Soil bioassays are a well established method for assessing the persistence of entomopathogenic nematodes (Curran and Heng 1992; Hass *et al.* 1999), but they have rarely been used for assessing the persistence of *P. hermaphrodita*. In one study, Vernava *et al.* (2004) used live *D. reticulatum*, and an assessment of feeding inhibition and slug mortality to assess the persistence of *P. hermaphrodita* in field trials. The method used in the present study showed clear correlation between nematode numbers in the soil and nematodes within bait hosts, indicating that this method seemed to work well on soil treated with nematodes in the laboratory. However, there was no other method by which to compare the persistence of nematodes from the field collected soil, as direct counts of *P. hermaphrodita* proved difficult due to the large numbers of other nematodes sampled from the soil, and the conserved morphology of *P. hermaphrodita* IJs. The use of hosts to assess nematode persistence has some inherent problems, mostly due to the fact that only viably infective IJs will be detected. It has been shown that many pathogenic nematodes have phased infectivity (Campbell *et al.* 1999) where infection ability does not peak for several weeks after emergence from a host (Ryder and Griffin 2003). Because of this, the use of hosts to estimate persistence may underestimate nematode populations. The use of molecular methods, such as real-time quantitative PCR may overcome these issues (Torr *et al.* 2007; McMillan *et al.* 2006) as all individuals of the target species, regardless of infectivity, will be recorded.

In this study, the plots were not surrounded by barriers to prevent the movement of the surface and soil-dwelling organisms in and out of the plots, as it was not practical to construct and implement barriers for 60 plots each measuring 1 m x 1m. Because of this, any short term changes in microarthropod abundance were likely to be due to movement in and out of the plots, or increased mortality, rather than increases (or decreases) in reproduction rates. However, the alternative of placing barriers into the soil, as well as being expensive and time consuming, may have had significant effects on the soil moisture and temperature, especially in small plots. These changes may cause unexpected changes in the abundance of the soil-dwelling biota, affecting the conclusions of the study. An alternative would be to spray the nematodes over a larger area, although this may introduce further error due to large scale spatial patterns in

microarthropod distribution. However, the lack of barriers is a potential limitation of study, and needs to be taken into account when interpreting the results.

On the soil surface, treatment with *P. hermaphrodita* caused significant increases in the number of surface-dwelling microarthropods compared to control plots. This response was due to the numerically dominant species of surface-dwelling collembola *Isotoma viridis*, which increased in number significantly in treated plots. *I. viridis* is a large (> 4 mm) epiedaphic species of collembolan that is widespread and found in a range of habitats but particularly grassland (Hopkins 1997, 2007). *Isotomurus palustris*, the other dominant species of epiedaphic collembola, and similar in size and ecology to *Isotoma viridis*, did not respond to treatment or any of the other variables recorded in this study. This is possibly due to the relatively low abundance of this species, allowing population stochasticity to mask trends, or another unrecorded variable that influenced population size.

Predation screening using PCR identified both *I. viridis* and *Isotomurus palustris* as predators of the introduced nematode *P. hermaphrodita*. The low diversity of microarthropod species on the soil surface at this site reduced the information that could be obtained from this study, as fewer potential predators of nematodes were present on the soil surface. Of the 720 microarthropods that were screened, only 130 were not *Isotoma viridis* or *Isotomurus palustris*, and only two of these (Acari spp.) were positive for *P. hermaphrodita* DNA. However, only 54 microarthropods that were not *Isotoma viridis* or *Isotomurus palustris* were screened on days 4 and 10 (when predation was detected by *Isotoma viridis* and *Isotomurus palustris*), and of those 54, 13 were *L. cyaneus*, a species already identified as one that does not feed on *P. hermaphrodita*, and a further 29 were collembola from the order Symphypleona. The Symphypleona are thought to be herbivores (Salamon *et al.* 2004) and some species such as *Sminthurus viridis* may even be pests of crop plants (Hopkin 1997). It is possible that other species of collembola and microarthropod may play an important role in nematode predation at other sites and in other habitats. Although in this study the Acari on the soil surface did not play a significant role in nematode predation due to their low numbers, Acari are generally known as nematode predators (Walter 1989) and it may be that they would act as significant predators of *P. hermaphrodita* if present at higher densities.

The temperature at the site on the five sampling days did vary, but not enough to cause significant changes in the median detection times of prey DNA in the microarthropods tested in this study, with the median detection time varying by only 1 h across all days. This is not enough to cause significant changes in the interpretation of the field results, allowing comparisons of positives between sampling days to be valid.

The below-ground, euedaphic microarthropods did not respond to nematode application when all groups were interpreted as a whole, with only moisture and day being significant covariates in the minimal model. Only one group, the Acari spp. responded significantly to treatment. None of the euedaphic groups of collembola, the Poduromorpha, the Entomobryomorpha or the Symphypleona, responded to treatment in this study. The reason for this may be due to the epiedaphic lifestyle of these groups, which tend to be less mobile and thus feed only in the immediate vicinity, and feed on lower quality food (Lavelle and Spain 2001). Nematophagy would therefore be less likely in these groups of organisms, and if it does occur, these species are less likely to respond to increased food density by moving into food-rich areas. However, many species of microarthropod do not fall cleanly into the categories of euedaphic and epiedaphic; and there will be a continuous spectrum of intermediate species that may respond to nematode inundation in varying degrees. A study with a higher level of taxonomic resolution, and a higher degree of habitat separation (i.e. upper, middle and lower soil profiles) may identify these intermediate species and the role they play in nematophagy. Equally interesting in this study is the fact that none of the microarthropod groups declined in number in response to nematode IJ application. This gives clear indication that, at least in the short-term, the application of *P. hermaphrodita* does not have a negative effect on microarthropod communities.

Clearly this investigation would have benefited from gut contents screening of the euedaphic microarthropods for predation on the introduced nematode IJs. However, a number of methodological limitations prevented this from occurring. Extraction of microarthropods using Tullgren funnels is a lengthy process, involving elevated temperatures, which cause the gut contents of the collected microarthropods to have emptied or changed in composition before extraction is complete. Faster extraction methods of microarthropods from soil samples are available, including those using heptane and ethanol floatation (Walter 1987), although extraction from many samples is

often expensive and time consuming. Work would also need to be done to ensure these petrochemicals did not inhibit PCR.

There was a strong association between the abundance of all of the soil matrix-dwelling microarthropods (both collembola and mites) and the moisture content of the soil in the plots, both treatment and control. Soil moisture has previously been shown to be an important factor influencing the abundance and diversity of soil-dwelling microarthropods (Chikoski *et al.* 2006; Lensing *et al.* 2005; Tsiafouli *et al.* 2005), and it is clear that it plays an important role in determining below-ground abundance in this system.

The abundance of free-living nematodes was significantly affected by the addition of *P. hermaphrodita* to the soil in treated plots, causing a decline in the overall number compared to the control, in interaction with day. The models show that in the control plots the number of soil nematodes increased significantly with time, increasing by 63.7 % at day 28 post-application. The reason for this increase is unknown, as time was the only significant explanatory variable. Free-living nematodes within the soil contain a mixture of bacterivores, fungivores, herbivores, predators and omnivores (Blanc *et al.* 2006; Ferris and Bongers 2006) so the factors that influence overall nematode population numbers are likely to be numerous and highly complex. The minimal model in this study showed that the addition of *P. hermaphrodita* to the soil was predicted to prevent this increase in nematode numbers with time (observed in the control plots). The reason for this effect is unknown, although it has been shown that applications of entomopathogenic nematodes can suppress plant parasitic nematode populations (Jagdale *et al.* 2002), and that this may be due to an allelopathic response to substances produced by the symbiotic bacteria (Grewal *et al.* 1999). Therefore, it is possible that the application of *P. hermaphrodita* lowers the reproductive capacity or immigration of a group or groups, which are responsible for the increase in the control plots.

This study has indicated that the application of *P. hermaphrodita* IJs to the soil has a positive effect on the most abundant species of epiedaphic collembola (in this study, *Isotoma viridis*) and one group of euedaphic microarthropods, the Acari spp. Furthermore, these groups of microarthropods were identified as predators of the introduced nematode, so it is likely this positive response is due to increased migration

into a prey-rich environment. Collembola and mites are not the only potential predators of nematodes within the soil environment, and previous research has indicated that predatory nematodes, tardigrades and protozoa may also be significant predators of free-living nematodes in the soil (Hohberg and Traunspurger 2005). The molecular methods utilised in this study could be applied to these groups of organisms, providing suitable extraction methods can be developed. Examination of these groups could further highlight links in what must be a highly complex food web.

Chapter 5

Terminal Restriction Fragment Length Polymorphism (T-RFLP) for estimating nematode diversity within soils

Abstract

The high number and diversity of nematodes found in terrestrial soils makes them ideal indicator species for measuring both soil health and environmental disturbance. The highly conserved morphology of many nematode species means that morphological identification of nematode communities is a time consuming and specialist task. This study describes an investigation into the use of Terminal Restriction Fragment Length Polymorphism (T-RFLP) for the assessment of nematode community diversity. General primers amplifying a section of the SSU (18S) rRNA gene, and the restriction enzymes *Ase*I and *Mbo*II were used to assess a number of different 'communities' including a mix of identified species of mollusc and entomopathogenic nematode, and larger mix of unidentified species extracted from the field. Although this choice of gene and enzyme was not able to distinguish between all different species, a community profile was generated when a mixed nematode community was analyzed. Finally T-RFLP was used to assess a soil disturbance, in the form of the addition of the mollusc parasitic nematode *Phasmarhabditis hermaphrodita*, to the soil. T-RFLP was able to detect this disturbance in the form of a changed nematode community, and a reduced Molecular Operational Taxonomic (MOTU) richness.

5.1 Introduction

Nematodes are the most abundant and diverse of the soil Metazoa (Curry 1994) with densities of millions per m^2 and high diversity in soils worldwide (Yeates 1979). The ubiquitous nature of soil-dwelling nematodes means that they have been used as indicators of a number of soil variables. These include soil quality (Urzelai *et al.* 2000), levels of pollution and ecotoxicology (Heininger *et al.* 2007), vegetation succession (Goralczyk 1998), soil functioning (Ekschmitt *et al.* 2001) and ecological maturity (Imaz *et al.* 2002) and used to assess the effects of soil management practices (Okada and Harada 2007), including the use of conventional and organically farmed soils (Neher 1999). Within a nematode community there are typically a range of species that will respond in different degrees to changes in soil variables. Some species of nematode are resistant to change, and utilize stages such as dauer larvae or cysts to survive unfavourable conditions (Neher 2001). Other species are highly susceptible to change, making them useful as indicator species for environmental perturbation (Bongers 1999).

One of the underlying assumptions for the use of nematodes as bioindicators is that larger populations with a higher level of diversity represent healthier, more mature soils with low levels of disturbance (Yeates 2003). The number of nematode species recorded from temperate soils can be very high, with up to 154 species (Hodda and Wanless 1994) and 21 genera (Yeates and Bongers 1999) recorded in English grassland. The use of nematodes as soil indicators has traditionally revolved around the use of morphological methods of nematode identification. Since soil-dwelling nematodes are often less than 2 mm in length and highly conserved morphologically, identification to species level requires extensive training and a wide range of specialist texts (Yeates and Bongers 1999). Identification is further hindered by the fact that it is often only possible to correctly identify adult nematodes based on male- or female-specific structures (Floyd *et al.* 2002), thus excluding a large proportion of the nematode community from a survey. Accurate assessment of nematode biodiversity as an environmental indicator requires assignment of individuals to genus or even species level (Yeates 2003), but there is a lack of trained individuals to carry out this type of work, resulting on many surveys being carried out at the guild or functional group level (Bongers and Ferris 1999). Because of the shortcomings of morphological identification, molecular methods

offer an alternative approach that does not involve specialist nematode identification skills to assess nematode diversity.

Molecular methods can be used to generate a genetic profile or a molecular barcode for nematode community comparison. These methods utilize sequence variation in the nuclear or mitochondrial genome to differentiate between nematode species, subspecies or haplotypes. The term Molecular Operational Taxonomic Unit (MOTU) has been used to describe molecular derived, experimentally relevant Operational Taxonomic Units (OTUs) (Floyd *et al.* 2002), which can be used to measure the diversity of a population without making any assumptions about the taxonomic rank of the units. Molecular methods have the advantage that they can be applied to nematodes at all levels in the life cycle, allowing juveniles to be included in a community analysis.

One possible molecular approach is to obtain nuclear or mitochondrial sequence information from a nematode community, and use it to differentiate between nematode populations. These sequences can be identified to species level, although more frequently, differencing sequences are designated as MOTUs. Floyd *et al.* (2002) described this method as 'molecular barcoding' and utilized it to separate nematode MOTUs from field samples. However, this method is relatively time and cost intensive due to the fact that nematodes need to be extracted from the soil whole, separated individually, and sequenced to obtain clean sequence data. Griffiths *et al.* (2006) overcame this problem by cloning a mixed source of nematode DNA and sequencing 192 clones from each soil sample. This method allowed nematode MOTUs to be compared to a database of identified nematode sequences and samples identified to family or species level.

Another possible approach for assessing nematode biodiversity is to employ species-specific primers and probes to identify particular indicator species of nematodes, rather than the whole community. This approach has been utilized for the identification of single or small groups of plant parasitic nematodes from soil samples (Atkins *et al.* 2005; Cao *et al.* 2005; Holeva *et al.* 2006; Leal *et al.* 2005), but this provides no information about the whole nematode community. Jones *et al.* (2006a, b) took this a stage further by designing a set of species and group-specific primers and Taqman probes to identify 16 different species of microbial feeding nematodes from a Kansas

prairie. This approach involved extracting nematodes from soil samples and screening each nematode individually with the suite of species-specific probes and primers. Although this approach proved to be effective, with over 90% of the nematode samples identified, it is necessary to have prior knowledge of the species present at a site before primer design can proceed.

Methods involving the comparison of sequence data from nematodes in field samples will provide the highest level of resolution, and therefore will be the most sensitive measure of nematodes as bioindicators. However, the cost and time of sequencing each nematode from multiple field samples is currently prohibitive. Molecular approaches that do not involve the extraction and sorting of individual nematodes into separate samples have a significant advantage due to the time saving involved. Methods such as Denaturing Gradient Gel Electrophoresis (DDGE), Single-Strand Conformation Polymorphism (SSCP) and Terminal Restriction Fragment Length Polymorphism (T-RFLP) have previously been used to analyze mixed sources of bacterial and fungal DNA (Hori *et al.* 2006; Marsh 1999). DGGE is based on the application of a denaturing gel to discriminate between DNA fragments of the same or similar length, but with different sequence composition. The variable electrophoretic motility of partially denatured DNA fragments in a gradient of urea and formamide causes separation of fragments based on sequence composition and size. Foucher and Wilson (2002) used this technique to study nematode biodiversity, and were able to differentiate between species of nematode mixed in the laboratory. This technique was taken one stage further and used to assess nematode biodiversity in natural nematode populations (Foucher *et al.* 2004). Although the technique tended to underestimate rare species within a mixed sample, a positive relationship between the number of DGGE bands and morphologically identified taxa was obtained. Waite *et al.* (2003) also utilized DGGE to assess nematode community diversity by directly extracting whole community DNA from soil samples. This gave characteristic banding patterns that allowed different sites to be compared, although a lack of specificity in the nematode primers used for this investigation may have reduced the usefulness of this study (Foucher *et al.* 2004).

Terminal restriction fragment length polymorphism (T-RFLP) is a molecular method widely used in microbial studies to assess biodiversity and community composition. This method involves the amplification of a mixed DNA source comprising many

species, using general or group-specific primers. One or both of the oligonucleotides primers can be tagged with a fluorescent marker, allowing this to be incorporated into amplified fragments of DNA. The mixed DNA fragments are then subjected to restriction with an enzyme chosen with a restriction site that varies between species or OTUs. The terminal restriction fragments (T-RFs) are then run on a high resolution electrophoresis system that detects the fluorescently labelled fragments. Typically ABI gels are used to achieve this, and as a result the fragments can be automatically sized to the nearest base pair, and the size and area of each peak can be recorded. As restriction enzymes consistently restrict a specific sequence of DNA, fragment profiles from an unknown mixture of DNA sources can be compared to theoretical restriction profiles to identify T-RFs in a mixed sample. T-RFLP has been used to assess microbial communities in a wide range of situations, including the screening of soils in landfill sites (Stralis-Pavese *et al.* 2006), evergreen broad-leaved forests (Chan *et al.* 2006), *Sphagnum* bogs (Morales *et al.* 2006), watermelon rhizospheres (Park *et al.* 2006), mangrove sediments (Brito *et al.* 2006), marine sediments (Pereira *et al.* 2006) and in the gut of the earthworm *Lumbricus terrestris* (see Egert *et al.* 2004). T-RFLP does not reveal the whole diversity of a community unless the community is composed of very few OTUs. It does however provide a measure of diversity which can be used to discriminate between separate sites or different treatments (Blackwood *et al.* 2003). As the peak height and peak area is measured on an electropherogram, the relative abundance of each OTU may be compared within and between runs and samples. Despite T-RFLP proving to be an effective method for assessing both bacterial and fungal diversity, as of yet, there has been only one short example of this method being applied to the assessment of nematode diversity (Donn *et al.* 2007).

This study aims to test and develop T-RFLP as a method for assessing nematode community diversity. The aim is to achieve this by assessing the ability of T-RFLP as a technique to differentiate between known species of nematode in the laboratory. The accuracy of T-RFLP will then be assessed by comparing T-RFs from nematodes screened individually and as mixed samples. Finally, this method will be used in a preliminary field study to assess T-RFLP as a method for examining the impact of the application of a mollusc parasitic nematode, *Phasmarhabditis hermaphrodita* (Rhabditida: Rhabditidae), on a nematode community in the field.

5.2 Materials and Methods

5.2.1. Identification and separation of nematodes within the laboratory

Experiment one was carried out to test the ability of T-RFLP to distinguish between parasitic nematodes commonly used as biological control agents. Four species of parasitic nematode, a parasite of molluscs, *Phasmarhabditis hermaphrodita* (Rhabditida: Rhabditidae) and three insect parasites, *Steinernema feltiae* (Rhabditida: Steinernematidae), *Steinernema kraussei* (Rhabditida: Steinernematidae) and *Heterorhabditis megidis* (Rhabditida: Heterorhabditidae), were obtained from commercial suppliers (BeckerUnderwood, UK). The nematode infective juveniles (IJs) were rehydrated in tap water, and using a pair of fine tweezers, five individuals of each species were randomly selected and placed individually in 20 µl of DNA free water in a 1.5 ml centrifuge tube. Samples for T-RFLP consisted of five individuals of each of the four nematode species (n = 20). To test for the efficiency of T-RFLP to detect a mix of nematode DNA, a number of mixtures were also run, including: two samples consisting of a mix of all four nematode species (one individual of each) before DNA extraction; two samples consisting of a mix of all four nematode species post-DNA extraction but pre-PCR; and two samples consisting of a mix of all four nematode species DNA post-PCR. Samples were stored at -80 °C before T-RFLP analysis.

5.2.2. Sensitivity and accuracy of T-RFLP

Experiment two was carried out to investigate the ability of T-RFLP to distinguish between nematodes collected from the field. Unidentified nematode samples were collected from a field site at Burdens Farm, Wenvoe, nr. Cardiff. Soil cores (4 cm dia. 10 cm depth) were collected in an arable field planted with wheat (*Triticum* sp.). The cores were placed in sealed plastic bags and taken to the laboratory for processing within 2 h. Individual samples were broken up and the soil mixed in the laboratory, before extracting nematodes using a modified Baermann funnel method (see Chapter 2, section 2.1.2 for methodology) for a period of 72 h at room temperature 22 ± 2 °C. A total of 48 nematodes were randomly selected using fine tweezers under a stereo microscope, and placed individually in 1.5 ml centrifuge tubes with 20 µl of DNA free water (Sigma, UK) before storing at -80 °C. DNA was extracted from each nematode using a modified protocol from the PureGene DNA extraction kit (see section 5.4.2).

Samples for T-RFLP therefore consisted of 48 individually extracted nematode samples that were run one sample per lane. Two methods of comparing T-RFLP accuracy were carried out. The first was to mix extracted DNA from all 48 nematode samples prior to PCR. The second was to mix nematode DNA from all 48 nematodes post-PCR. Five mixes of each type were then submitted for T-RFLP analysis.

5.2.3. Preliminary screening of a nematode community from a disturbed habitat

The final experiment was carried out to investigate the ability of T-RFLP to reveal ecologically relevant changes in the nematode species composition from field collected soil samples. As part of another experiment investigating the effects of parasitic nematode applications on soil-dwelling microarthropods, the mollusc-parasitic nematode *P. hermaphrodita* was applied to the soil surface of an arable field containing wheat (see Chapter 4, section 4.4.2. for details of field trials, and section 4.2.2.3 for nematode sampling methods). The plots were sampled 12 days after the initial application of the parasitic nematode to the soil surface, and included six treatment and six control plots that are described in Chapter 4, plus one extra treatment and one extra control plot that were included for the purpose of this study. The samples for T-RFLP analysis therefore consisted of seven soil samples from plots treated with the nematode *P. hermaphrodita*, and seven plots from untreated soil, 12 days after the application of *P. hermaphrodita*.

5.2.4. DNA extraction

All nematode samples were extracted using a modified protocol from the PureGene Tissue extraction kit (Gentra, Minnesota, USA) as follows: 300 µl of cell lysis solution and 1.5 µl of Proteinase K solution were added to the nematode samples in 1.5 ml centrifuge tubes. The tissue was then broken up using a sterile plastic pestle, before incubating at 55 °C for 3 h. RNase solution (1.5 µl) was added to each sample, followed by incubation for 30 min at 37 °C. The samples were cooled to 4 - 10 °C on ice for 3 min, before adding 100 µl of protein precipitation solution and vortexing at high speed for 20 s. The samples were then centrifuged at 12,000 rpm for 5 min, and the supernatant was poured off into a clean 1.5 ml centrifuge tube containing 300 µl of 100% isopropanol and 0.5 µl of glycogen to act as a DNA carrier. The tube containing

the precipitated protein was discarded. The sample was mixed gently by inverting 50 times, before centrifuging at 12,000 rpm for 5 min. The supernatant was poured off, and the tube air dried for 2 min on clean absorbent paper. To clean the DNA pellet, 300 µl of 70 % ethanol was added and inverted gently three times. The samples were centrifuged a final time at 12,000 rpm for 2 min, after which the ethanol was discarded and the tubes air dried for 5-10 min. The DNA was rehydrated using 50 µl of DNA hydration solution, and incubated at 65 °C for no more than 1 h. The extracted DNA samples were stored at -20 °C until needed for screening.

5.2.5. Choice of DNA marker for T-RFLP

The choice of region for T-RFLP analysis is important, as the region must be conserved to allow for the use of primers that will amplify a wide range of different nematode species, and it must also be diverse between primers to allow for taxonomically relevant T-RFLP analysis. Mitochondrial regions COI and COII were examined, but the lack of suitable primer sites, and the relatively low diversity within the sequence did not allow for variable restriction sites to be identified. There have been a number of previous studies that have identified the small subunit rRNA (18S) gene as a suitable site for examining nematode diversity (Floyd *et al.* 2002; Wait *et al.* 2003; Jones *et al.* 2006a, b; Foucher *et al.* 2004). The 18S gene is present in 50-100 copies per genome, making it a better target than a single copy gene due to the increased sensitivity of primers targeting this region. There are a large number of 18S nematode sequences on GenBank (http://www.ncbi.nlm.nih.gov), allowing for the alignment of a wide range of nematode taxa and the identification of suitable restriction enzymes. The 18S gene forms a stem and loop structure, resulting in conserved stem regions and highly divergent loop regions (Floyd *et al.* 2002), making this region ideal for conserved primers that amplify variable stretches of DNA. A number of 18S general nematode primers were assessed for their suitability for the amplification of nematode DNA (Table 5.2.1 and Figure 5.2.1.) The primers were aligned to a number of potential non-target organisms to assess potential specificity. Based on specificity and product size, primer pair *Nem1* and *Nem2* (Foucher and Wilson, 2002) were chosen for use.

Table 5.2.1 Compendium of 18S general nematode primers that have been used for the amplification of nematode DNA in previous studies, including the size of the fragment, primer name, position on the 18S sequence of the nematode *Caenorhabditis elegans*, and the reference.

Size (bp)	Primer	Position on 18S (bp)	Primer sequence	Reference
900	*NemF*	1006-1028	GGCGATCAGATACCGCCCTAGTT	Mullin *et al.* (2003)
	rDNA1.58S	1.58S	ACGAGCCGAGTGATCCACCG	Cherry *et al.* (1997)
1000	*SSU18A*	51-69	AAAGATTAAGCCATGCATG	Blaxter *et al.* (1998)
	SSU26R	949-969	CATTCTTGGCAAATGCTTTCG	
630	*Nem1*	563-582	GCAAGTCTGGTGCCAGCAGC	Foucher and Wilson (2002)
	Nem2	1189-1209	CCGTGTTGAGTCAAATTAAG	
900	*Nem_18S_F*	99-121	CGCGAATRGCTCATTACAACAGC	Floyd *et al.* (2005)
	Nem_18S_R	1006-1023	GGGCGGTATCTGATCGCC	
800	*NemF1*	444-461	CGCAAATTACCCACTCTC	Waite *et al.* (2003)
	Nem896r	1184-1201	TCCAAGAATTTCACCTCTMACG	
345	*MN18F*	99-121	CGCGAATRGCTCATTACAACAGC	Bhadury *et al.* (2006)
	22R	424-442	GCCTGCTGCCTTCCTTGGA	
~1000	*SSU18A*	39-57	AAAGATTAAGCCATGCATG	Floyd *et al.* (2002)
	SSU26R	907-927	CATTCTTGGCAAATGCTTTCG	
~500	*SSU09R*	565-584	AGCTGGAATTACCGCGGCTG	

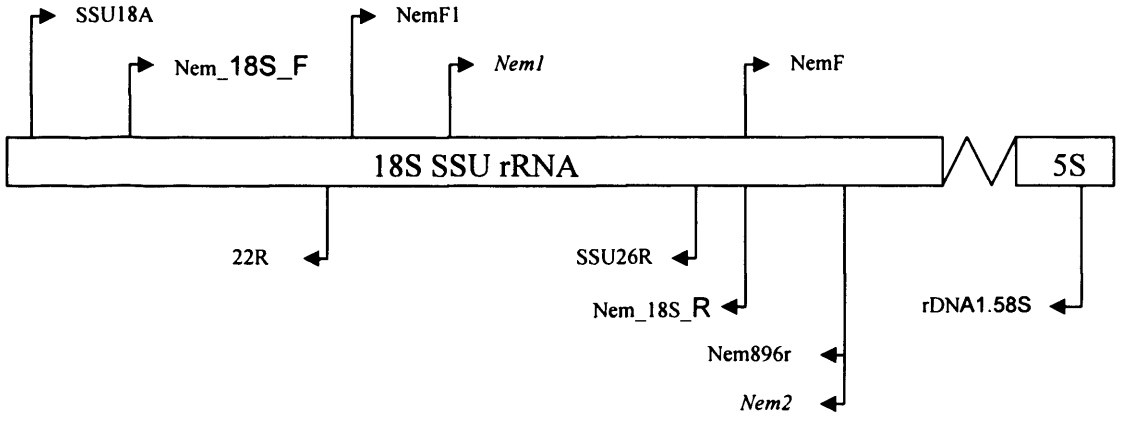


Figure 5.2.1 Position of 18S general nematode primers on 18S and 5.8S region

5.2.6. PCR, restriction enzymes and T-RFLP

A range of 18S nematode sequences that included the fragment covered by primers *Nem1* and *Nem2* (Foucher and Wilson, 2002) were selected from GenBank. These were then trimmed so that they were the same size as the 630 bp *Nem1* and *Nem2* fragment, and used to select diagnostic restriction enzymes (see Table 5.2.2). Restriction enzymes were chosen with the help of NEBcutter (New England Biolabs, Hitchin, UK: Vincze *et al.*, 2003) and RestrictionMapper (www.restrictionmapper.org), online restriction enzyme simulators that simulate restrictions by commercially available enzymes. The two most variable restriction enzymes were chosen for evaluation: *Mbo*II and *Ase*I (New England Biolabs, Hitchin, UK).

Table 5.2.2 Theoretical length of the Terminal Restriction Fragments (T-RFs) from a range of soil-dwelling nematodes after digestion by the restriction enzymes *Mbo*II and *Ase*I.

Nematode species	Accession no.	*Mbo*II	*Ase*I
Steinernema feltiae	AY035766.1	119	206
Steinernema glaseri	AY284682.1	117	204
Steinernema karii	AJ417021.1	77	206
Steinernema carpocapsae	AF036604.1	536	205
Steinernema abbasi	AY035764.1	511	181
Steinernema longicaudum	AY035767.1	117	204
Phasmarhabditis hermaphrodita	DQ639981.1	375	300
Rhabditis myriophila	U81588.1	113	403
Zeldia punctata	U61760.1	397	411
Acrobeles ciliatus	AF202148.2	397	411
Laxus oneistus	Y16919.1	139	414
Heterorhabditis zealandica	AJ920368.1	389	291
Heterorhabditis hepialus	AF083004.1	389	291
Heterorhabditis bacteriophora	AF036593.1	389	291

Primers *Nem1* and *Nem2* (Foucher and Wilson 2002) were used to amplify a 630 bp fragment of 18S nematode DNA. Forward primer *Nem1* was labelled with 6' carboxyflourescein (6-FAM) to enable the visualization of the 5' terminal restriction fragment. PCR was carried out on a GeneAmp 9700 thermal cycler (ABI Perkin Elmer, Beaconsfield, UK). Each reactions contained: 2 µl template DNA, 1x PCR buffer, 3.0 mM $MgCl_2$ (both provided by Invitrogen, Paisley, UK), 2 µl template DNA, 1x PCR buffer, 2.0 mM $MgCl_2$ (both provided by Invitrogen, Paisley, UK), 0.2 mM dNTP's

(Boehringer Mannheim, Indianapolis, USA), 0.2 units *Taq* polymerase (Invitrogen, Paisley, UK) and 0.5 µM of each primer (Operon, Cologne, Germany) and was made up to 25 µl with PCR water. Thermal cycle parameters included an initial denaturation step of 3 min at 94 °C followed by 35 cycles of 94 °C for 45 sec, 60 °C for 1 min 10 sec, 72°C for 1 min, and a final extension stage of 72 °C for 10 min.

Digestions for T-RFLP for experiments one and three were carried out using restriction enzymes *Ase*I and *Mbo*II (New England Biolabs, Hitchin, UK), and restrictions for experiment two were carried out using enzyme *Mbo*II only. Restriction digests for *Ase*I contained 4 U of enzyme, 12 µl of labelled PCR product, 1x NEB Buffer 3, and were made up to a total volume of 20 µl with 5.6 µl DNA free water. Restrictions were carried out at 37 °C for 1 ½ h, followed by an inactivation step of 65 °C for 20 min, and storage at 4 °C. Restriction digests for *Mbo*II contained 10 U of enzyme, 9 µl of labelled PCR product, 1x NEB Buffer 2, 10x Bovine Serum Albumin (BSA), and made up to a total volume of 15 µl with 1 µl DNA free water. Restrictions were carried out at 37 °C for 8 h, followed by an inactivation step of 65 °C for 20 min and storage at 4 °C. The success of the restrictions was checked on an ethidium-bromide stained 4.0 % agarose gel in 1 x TAE buffer (1mM EDTA, 40mM Tris-Acetate) and visualized by UV translumination. Gel images were analyzed using InGenius software (Syngene, Cambridge, UK) for PC. One microliter of digested PCR product was mixed with 0.25 µl of 500 bp *N,N,N',N'*-tetramethyl-6-carboxyrhodamine (ROX-500) and 10 µl of ice cold formamide. Analysis of samples was carried out on an ABI 3100 automated capillary DNA analyzer (ABI Prism model 3100, Beaconsfield, UK).

5.2.7 Sequencing of unidentified nematodes

The 48 individually extracted, unidentified nematodes from Section 5.2.2 were sequenced using PCR products amplified using primer pair *Nem1* and *Nem2* (Foucher and Wilson, 2002). Products were cleaned using the GeneClean Turbo for PCR kit (Qbiogene, Cambridge, UK), following manufacturer's instructions, and 25-50 pg aliquots of purified template DNA were used in sequencing PCR with a Big Dye ™ terminator sequencing kit (Promega, Madison, WI, USA). DNA was sequenced using the relevant forward and reverse primers for each reaction, and products were resolved

on an ABI 3100 automated capillary DNA analyzer (ABI Prism model 3100, Beaconsfield, UK).

5.2.8. T-RFLP sorting and statistical analysis

Raw data consisted of a series of peaks that represented the size of the T-RFs present in each sample. Fragment profiles were analyzed using Peak Scanner Software V1.0 (Applied Biosystems, Foster City, CA). T-RF peaks under 50 bp were excluded due to uncertainties in size determination and due to potential for interference from unincorporated labelled primers. Peaks under 50 fluorescent units were excluded from the analysis, due to potential interference from background fluorescence causing false positives. Fragment profiles were exported from Peak Scanner in to an Excel spreadsheet (Microsoft, Redmond, WA), and the T-RFLP profiles were sorted manually, by visually aligning fragment sizes into categories. The alignment of peaks was based on the size of the peaks in base pairs, and groups of peaks had no more than 2 bp difference between grouped fragments.

T-RFLP profiles from experiment 3 were analyzed using the methodology described in Rees *et al.* (2004). The peak data from the field samples was analyzed using XLSTAT (Addinsoft, New York, USA) plug-in for Microsoft Excel (Microsoft, Redmond, WA) and Primer V5 (Primer-E Ltd, Plymouth, UK) a multivariate statistics program for PC. Peak data from experiment one and two were both analyzed as presence-absence data (binary). Peak data from experiment three was analyzed as both binary data and after normalization of peak areas. Normalization of peak areas was conducted by dividing individual peak areas by the sum of all the peak areas on that gel, thus allowing different samples to be compared despite differences in overall peak intensity. Incorporating peak area into the analysis allows for the relative abundance of the different nematode MOTUs to be taken into account. Two different distance matricies were tested based on previous recommendations in the literature, including Bray-Curtis dissimilarity (Bray and Curtis 1957; Rees *et al.* 2004) and Jaccard distance (Blackwood *et al.* 2003), calculated using XLSTAT.

The similarity data was compared using XLSTAT by carrying out non-metric Multi Dimensional Scaling (MDS), a multidimensional ordination method that used data from

a similarity matrix and displays it in a two dimensional plot. To compare the statistical significance of the treatment and control groups an analysis of similarity (ANOSIM) was carried out in Primer V5. This was done to test the null hypothesis that the rank similarity between objects within a group is the same as the average rank similarity between objects between groups. The T-RFLP data was then tested for the average contribution of individual T-RFs to the average dissimilarity between samples using similarity percentage analysis (SIMPER) in Primer V5.

The diversity of MOTUs from experiment three was assessed using standard diversity indices, including the Shannon-Weaver diversity index (*H*), the Simpson diversity index (*1/D*), richness (*S*) and evenness (*E*). Richness (*S*) represented the total number of MOTUs in the sample. The Shannon-Weaver diversity index (*H*) was calculated using $H = -\Sigma(pi)(\log N pi)$, where *pi* = the number of unique fragments of an MOTU divided by the total number of fragments present in the sample. The reciprocal of Simpson's diversity index (1/D) was calculated using $1/D = 1/\ \Sigma(pi^2)$ and Evenness was defined as $E = H/(\log_2((S))$.

Species diversity indices were compared using a one-tailed randomisation test with 20,000 permutations, outlined in Solow *et al.* (1993) using a macro written in Microsoft Excel (Microsoft, Redmond, WA).

5.3 Results

5.3.1. Identification and separation of nematodes within the laboratory

In experiment one, four species of nematode, *Phasmarhabditis hermaphrodita*, *Heterorhabditis megidis*, *Steinernema feltiae* and *S. kraussei*, were used to assess the ability of T-RFLP to separate and identify different species of nematode. Figure 5.3.1 shows the size of the Terminal Restriction Fragments (T-RFs) produced after digestion by restriction enzymes *Ase*I and *Mbo*II (see Figures 4.1 and 4.2, in Appendix 4 for ethidium-bromide stained agarose gels showing digested nematode DNA). Both enzymes were capable of distinguishing between *P. hermaphrodita* and *H. megidis*, and also between these two species and the two steinernematid nematodes. Neither enzyme generated T-RFs that could distinguish between *S. feltiae* and *S. kraussei*.

Three different methods of mixing the nematode DNA were tested to ensure that all T-RFs were still present in the T-RFLP profile. All three methods (mixing prior to extraction, prior to extraction but pre-PCR, and post-PCR) resulted in the same number of peaks, although peak intensity varied between samples.

As 18S sequences were available on GenBank for *P. hermaphrodita* and *S. feltiae*, the theoretical T-RFs (determined by restriction simulator NEBcutter) could be compared to the actual T-RFs. The theoretical restrictions with *Mbo*II and *Ase*I on *P. hermaphrodita* (DQ639981.1) produced T-RFs of 375 bp and 300 bp respectively, compared to actual T-RFs of 373.9 bp and 301.7 bp. Theoretical restrictions with *Mbo*II and *Ase*I on *S. feltiae* (AY035766.1) produced T-RFs of 119 bp and 206 bp respectively, compared to actual T-RFs of 113.7 bp and 205.1 bp.

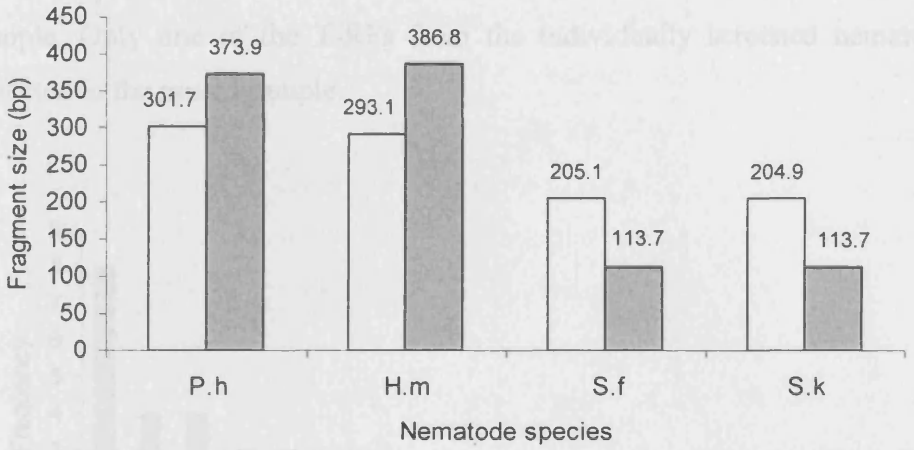


Figure 5.3.1 Average terminal restriction fragment (T-RF) size from four species of nematode (P.h = *Phasmarhabditis hermaphrodita*. H.m = *Heterorhabditis megidis*. S.f = *Steinernema feltiae*. S.k = *S. kraussei*) after digestion with restriction enzymes *Ase*I (light bars) and *Mbo*II (shaded bars).

5.3.2. Sensitivity and accuracy of T-RFLP

In experiment two, the ability of T-RFLP to identify all the nematode MOTUs in a mixture of nematode DNA was assessed using restriction by the enzyme *MboII*. When run individually, only 38 of the 48 samples produced clear T-RFs that were above the threshold detection limit. A total of 18 different T-RFs were identified from the 38 unidentified nematode samples. The frequency of the 18 unique MOTUs within the 38 samples is shown in Figure 5.3.2. In theory, each nematode species or MOTU should result in a single peak on the electropherogram. However, some individuals, when run singly on T-RFLP produced more than one band. Figure 5.3.3 shows the number of nematode samples with one, two or three terminal restriction fragments (T-RFs) per run.

All 48 nematode DNA samples were mixed in equal volumes pre-PCR, subjected to PCR, and then digested with *MboII* before ABI gel analysis. The T-RFLP profile generated from this had 13 different T-RFs, nine of which were found in single runs and four of which were unique to the pre-PCR mixed sample. Seven of the 16 T-RFs previously found from the individually screened nematode samples were not detected. DNA samples were also mixed post-PCR and then subjected to T-RFLP analysis.

Sixteen different T-RFs were detected in the T-RFLP profile, 15 of which were found in the individually screened nematode samples, and one which was unique to the mixed sample. Only one of the T-RFs from the individually screened nematodes was not detected in the mixed sample.

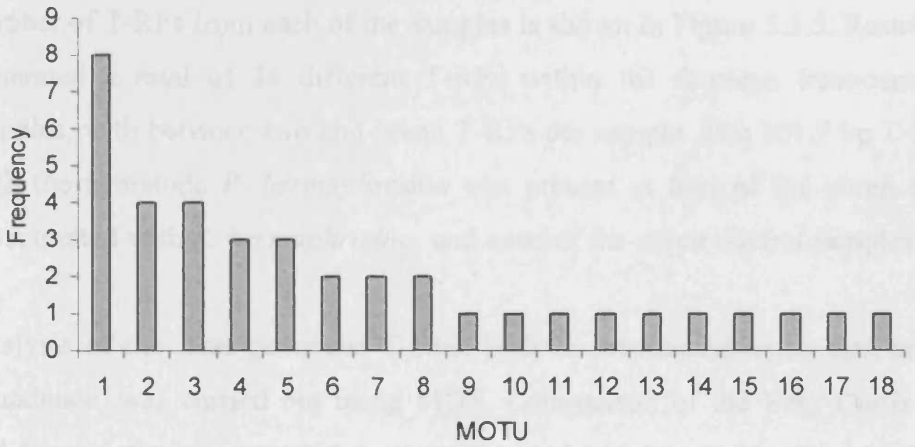


Figure 5.3.2 Frequency of unique terminal restriction fragments (T-RFs) from a set of 48 unidentified nematodes screened individually after digestion with the restriction enzyme *MboII*.

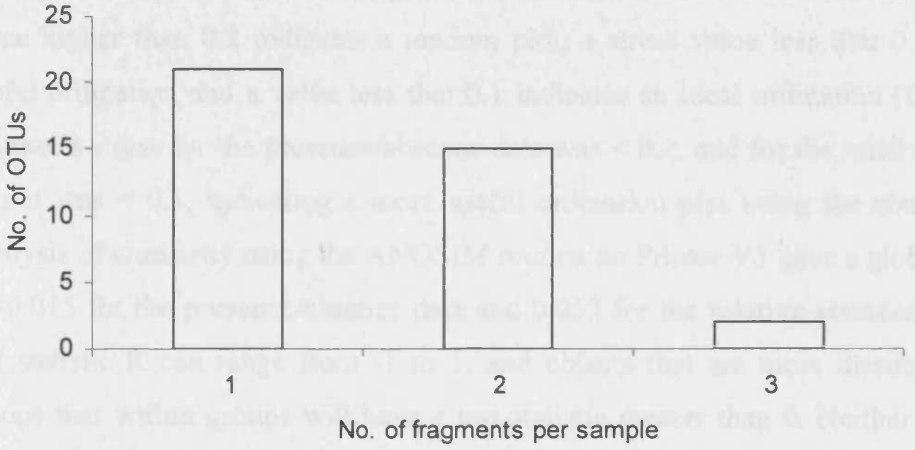


Figure 5.3.3 Number of nematode samples with one, two or three Terminal Restriction Fragments (T-RFs) per run.

5.3.3. Screening of a nematode community from the field

In experiment three, a mixed community of nematodes taken from the field were analyzed by T-RFLP. The first T-RFLP analysis was carried out using fragments restricted with *Ase*I. Figure 5.3.4 shows the frequency of the T-RFs from all seven samples in the control group, and all seven samples in the treatment group. The total number of T-RFs from each of the samples is shown in Figure 5.3.5. Restriction by *Ase*I generated a total of 24 different T-RFs within the fourteen treatment and control samples, with between two and seven T-RFs per sample. The 301.7 bp T-RF consistent with the nematode *P. hermaphrodita* was present in four of the seven samples from plots treated with *P. hermaphrodita*, and none of the seven control samples.

Analysis of the *Ase*I generated T-RFs, both as presence/absence data and as relative abundance, was carried out using MDS. Comparison of the Bray-Curtis dissimilarity and Jaccard distance revealed very similar results (data not shown). As Bray-Curtis is a widely used coefficient, and is available in a range of statistical packages, only the data from this analysis is shown. No clear clustering of the seven treatment and seven control samples was observed in the two dimensional plots using the presence-absence data (Figure 5.3.6), or the relative peak abundance data (Figure 5.3.7). The stress value of the MDS plot indicates how well the plot represents the distances between the data: a stress value higher than 0.2 indicates a random plot, a stress value less that 0.2 indicates a useful ordination and a value less that 0.1 indicates an ideal ordination (Clarke 1993). Kruskal's stress for the presence/absence data was < 0.2, and for the relative abundance data it was < 0.1, indicating a more useful ordination plot using the abundance data. Analysis of similarity using the ANOSIM routine on Primer V5 gave a global R statistic of -0.015 for the presence/absence data and 0.053 for the relative abundance data. The test statistic R can range from -1 to 1, and objects that are more dissimilar between groups that within groups will have a test statistic greater than 0. Neither of these test statistics were significant ($P \geq 0.05$), resulting in the acceptance of the null hypothesis that the rank similarity between nematode OTUs within treatment and control groups is the same as the average rank similarity between nematode OTUs between the treatment and control groups.

Similarity percentage analysis (SIMPER) on the presence/absence data indicated that the average similarity for the presence/absence data within the treatment group was 42.83 %, and in the control group it was 36.7 %. This level of similarity is comparable to the value calculated between the treatment and control group (37.4 %). Analysis of the relative abundance data using the same method did not change the results significantly, with the average similarity within the treatment group being 33.8 %, and within the control group it was 38.1 %. Pairwise similarity between the treatment and control groups was 33.3 %. Further analysis of the pairwise similarity showed that five T-RFs accounted for approximately 50 % of the similarity between the treatment and control groups with the presence/absence data, and two T-RFs accounted for more than 50 % of the similarity with the relative abundance data.

Multivariate Dispersion Indices (MVDISP) can be used to measure the level of dispersion within a population or sample. The higher the dispersion index is, the higher the level of dispersion is. Dispersion using presence/absence data was 0.94 for the treatment samples and 1.06 for the control, and the dispersion indices for the relative abundance data were 0.93 for the treatment and 1.07 for the control samples.

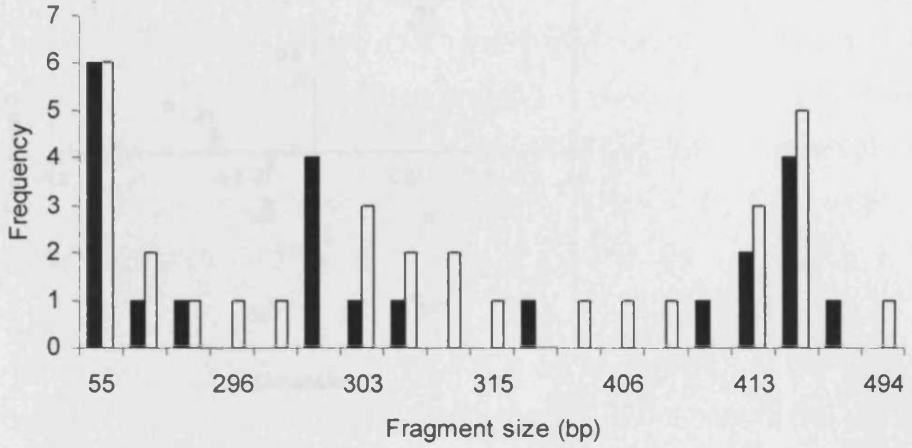


Figure 5.3.4 Frequency and size of terminal restriction fragments (T-RFs) generated by digestion with *Ase*I from both the treatment (dark bars) and the control (light bars) samples.

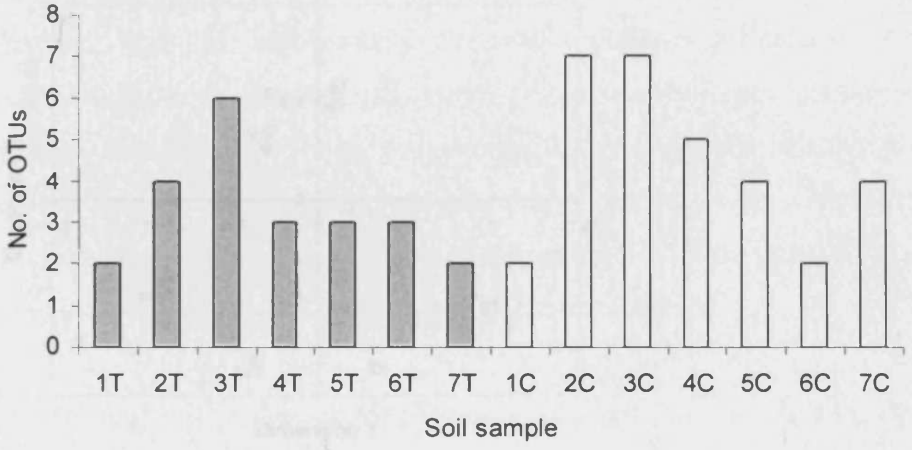


Figure 5.3.5 Total number of terminal restriction fragments (T-RFs) obtained by T-RFLP using enzyme *Ase*I from soil samples treated with the mollusc parasitic nematode *Phasmarhabditis hermaphrodita* (1T-7T) and from untreated soil samples (1C-7C).

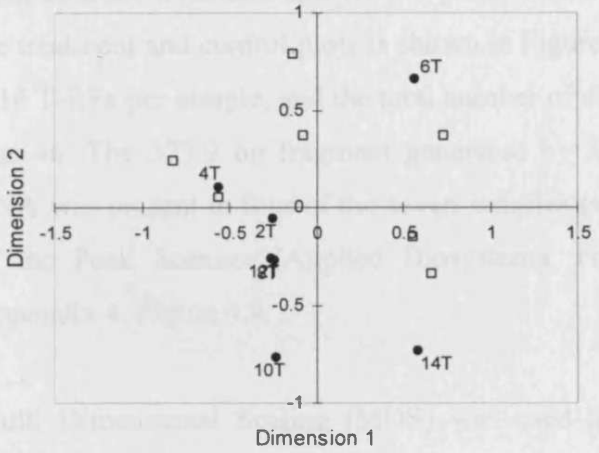


Figure 5.3.6 Multi Dimensional Scaling plot based on a Bray-Curtis similarity matrix of the T-RFLP data using restriction enzyme *Ase*I and presence-absence data (Kruskal's stress (1) = 0.165). Empty squares represent T-RFLP profiles from plots treated with *Phasmarhabditis hermaphrodita*; filled circles represent profiles from control (untreated) plots.

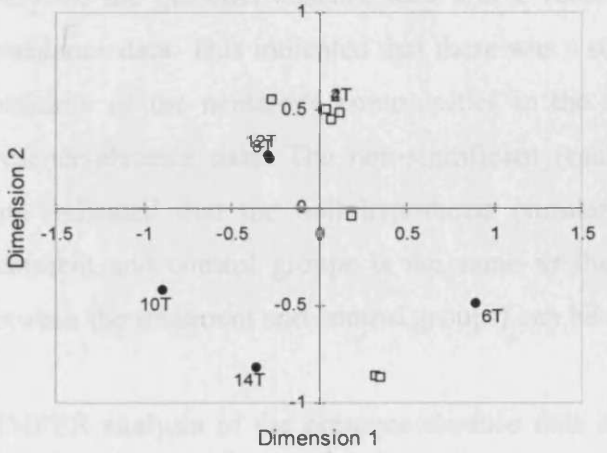


Figure 5.3.7 Multi Dimensional Scaling plot based on a Bray-Curtis similarity matrix from T-RFLP data using restriction enzyme *Ase*I based on normalized peak areas (Kruskal's stress (1) = 0.082). Empty squares represent T-RFLP profiles from plots treated with *Phasmarhabditis hermaphrodita*; Filled circles represent profiles from control (untreated) plots.

The second T-RFLP analysis of the field samples was carried out using restriction enzyme *MboII* (Figure 4.3 in Appendix 4 shows an ethidium-bromide stained agarose gel with digested nematode DNA). Figure 5.3.8 shows the frequency of all of the T-RFs

from both the treatment and control plots. The total number of T-RFs found in each of the treatment and control plots is shown in Figure 5.3.9. Restriction by *Mbo*II generated 6-16 T-RFs per sample, and the total number of different T-RFs found in all the samples was 46. The 373.9 bp fragment generated by *Mbo*II restriction of *P. hermaphrodita* DNA was present in four of the seven samples taken from treatment plots. An example of the Peak Scanner (Applied Biosystems, Foster City, CA) output is shown in Appendix 4, Figure 4.4.

Multi Dimensional Scaling (MDS) was used to visually analyze the relatedness of nematode populations taken from the treatment and control plots. Although no tight clustering occurred, there was some differentiation between the treatment and control group using both the presence/absence data (Figure 5.3.10) and the relative abundance data (Figure 5.3.11). Kruskal's stress values of < 0.2 indicated that both the presence/absence plot and the relative abundance plot gave useful two dimensional ordinations. Analysis of similarity (ANOSIM) gave a global R statistic of 0.204 ($P < 0.05$) for the presence/absence data and a value of 0.142 ($P \geq 0.05$) for the relative abundance data. This indicated that there was a statistically significant difference in the similarity of the nematode communities in the treatment and control plots using the presence-absence data. The non-significant result using the relative peak abundance data indicated that the null hypothesis (similarity between nematode OTUs within treatment and control groups is the same as the similarity between nematode OTUs between the treatment and control groups) can be accepted.

SIMPER analysis of the presence/absence data showed that the similarity within sites (Treatment = 43.13 %; Control = 41.30 %) was comparable, although higher, than the similarity between the treatment and control sites (36.38 %). Analysis of the pairwise similarity revealed that 12 T-RFs accounted for 50 % of the similarity between the treatment and control groups. Multivariate dispersion indices (MVDISP) can be used to measure the level of dispersion within a population or sample. In this case dispersion using presence/absence data was 0.99 for the treatment samples and 1.01 for the control, and the dispersion indices for the relative abundance data were 0.97 and 1.03 for the treatment and control samples.

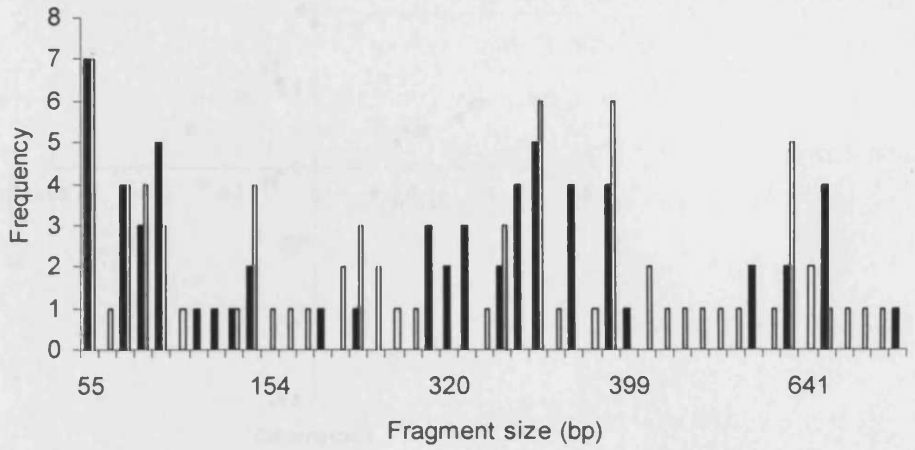


Figure 5.3.8 Frequency and size of terminal restriction fragments generated by digestion with *Mbo*II from both the control (light bars) and the treatment (dark bars).

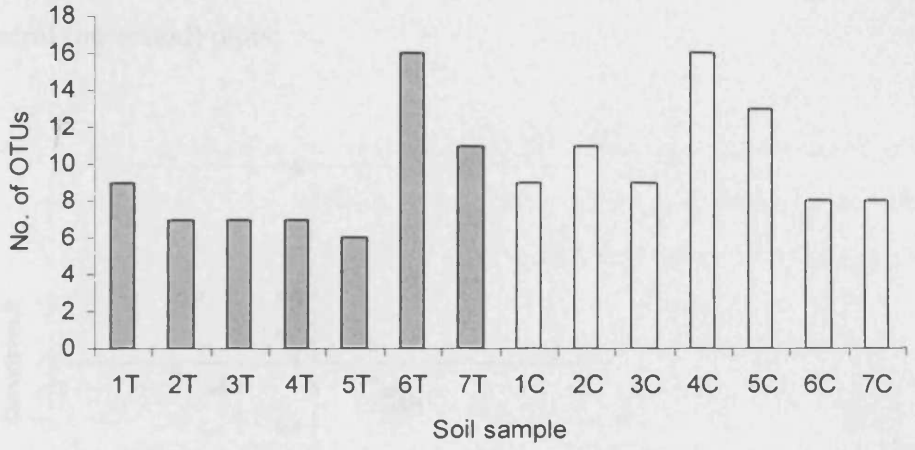


Figure 5.3.9 Total number of terminal restriction fragments (T-RFs) obtained by digestion with *Mbo*II, from soil samples treated with the mollusc parasitic nematode *Phasmarhabditis hermaphrodita* (1T-7T) and from untreated soil samples (1C-7C).

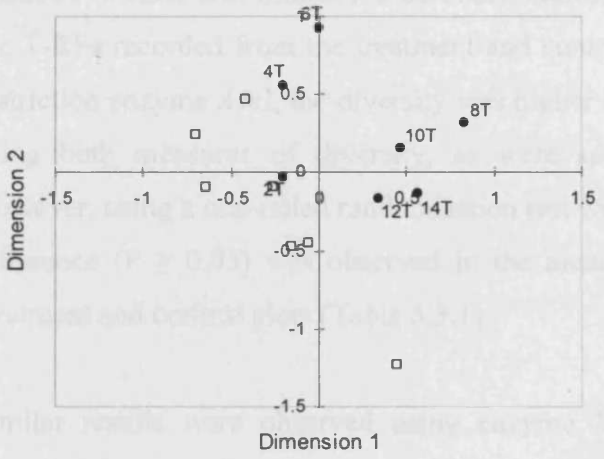


Figure 5.3.10 Multi Dimensional Scaling plot based on a Bray-Curtis similarity matrix of the T-RFLP data using restriction enzyme *Mbo*II and presence-absence data (Kruskal's stress (1) = 0.142). Empty squares represent T-RFLP profiles from plots treated with *Phasmarhabditis hermaphrodita*. Filled circles represent profiles from control (untreated) plots.

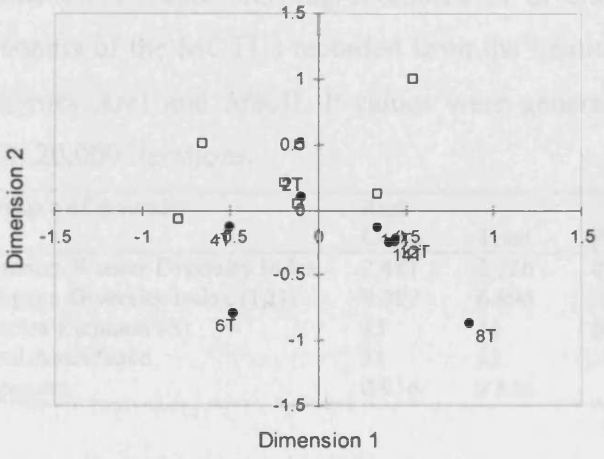


Figure 5.3.11 Multi Dimensional Scaling plot based on a Bray-Curtis similarity matrix from T-RFLP data using restriction enzyme *Mbo*II based on normalized peak areas (Kruskal's stress (1) = 0.139). Empty squares represent T-RFLP profiles from plots treated with *Phasmarhabditis hermaphrodita*. Filled circles represent profiles from control (untreated) plots.

Shannon-Weiner and Simpson's diversity indices were used to assess the diversity of the T-RFs recorded from the treatment and control plots for both *Mbo*II and *Ase*I. For restriction enzyme *Ase*I, the diversity was higher in the control than the treatment plots, using both measures of diversity, as were species richness and total abundance. However, using a one-tailed randomisation test with 20,000 permutations, no significant difference ($P \geq 0.05$) was observed in the measures of diversity and richness in the treatment and control plots (Table 5.3.1).

Similar results were observed using enzyme *Mbo*II, where diversity, richness and abundance were reduced in the treatment plots compared to the control. Again, using a one-tailed randomisation test with 20,000 permutations, no significant difference was observed in the measures of diversity in the treatment and control plots. However, there was a significant difference ($P < 0.05$) in the MOTU richness between the treatment and control plots (Table 5.3.1).

Table 5.3.1 Table showing measures of diversity, species richness, abundance and evenness of the MOTUs recorded from the treatment and control plots using restriction enzymes *Ase*I and *Mbo*II. P-values were generated using a randomization procedure with 20,000 iterations.

Measure of diversity	*Ase*I			*Mbo*II		
	Cont	Treat	P-Value	Cont	Treat	P-Value
Shannon-Wiener Diversity Index	2.481	2.126	0.194	3.287	2.964	0.060
Simpson Diversity Index (1/D)	9.707	6.696	0.200	21.224	17.034	0.183
Species Richness (S)	15	11	0.325	35	23	0.024
Total Abundance	31	23		74	63	
Evenness	0.916	0.886		0.925	0.945	

5.4 Discussion

The lack of skilled personnel with the ability to morphologically identify soil-dwelling nematodes, and the time needed for training, mean that molecular methods are becoming an important alternative for assessing nematode diversity. This study demonstrates that T-RFLP is potentially a viable method for assessing the diversity of nematode communities from within soil samples.

Using restriction enzymes *Mbo*II and *Ase*I to generate T-RFLP profiles from a 630 bp fragment of the 18S region of rDNA, it was possible to distinguish between *Phasmarhabditis hermaphrodita*, *Heterorhabditis megidis*, and the two steinernematid nematodes, *Steinernema feltiae* and *S. kraussei*. Neither enzyme produced T-RFs that could distinguish between *S. feltiae* and *S. kraussei*. Further analysis of nematode 18S sequences obtained from GenBank showed that neither of these enzymes was able to produce T-RFs that were completely diagnostic at the species level within the Steinernematidae, as some of the species within this genus could not be separated with any combination of commercially available enzymes for this DNA fragment. In addition, of the three Heterorhabditidae species theoretically tested, none could be separated to species level.

The aim of experiment two was to examine whether T-RFLP could reflect the diversity in sample of mixed nematode species. The usefulness of this investigation would have been significantly increased by using nematodes identified to species level, instead of the unidentified species used in this study. Attempts were made to sequence the 48 unidentified nematode samples using primers *Nem1* and *Nem2* (Foucher and Wilson 2002) that were originally used to amplify the 630 bp 18S fragment for T-RFLP. Unfortunately no clean sequence data could be obtained for the samples, despite several attempts, either due to the unsuitability of these primers for sequencing, or sample degradation. A future study, based on known, sequenced nematode samples would allow accurate measurement of how effective T-RFLP is at determining nematode diversity. However, by running nematodes individually through T-RFLP, something which has not often been done for bacterial or fungal analysis, the characteristic bands produced by individual nematodes could be examined. The results indicated that some of the nematode samples analyzed produced more than one band, something not

predicted by the theoretical analysis. One possible reason for this is that despite optimization, the primers *Nem1* and *Nem2* (Foucher and Wilson 2002) are amplifying a multi-banded product with bands faint enough to not be visible on an ethidium-bromide stained agarose gel. The high sensitivity of the ABI 3100 automated capillary DNA analyzer may pick up these extra digested bands, resulting in multiple bands for some individuals. Other sources of multiple bands may include 18S pseudogenes (Benevolenskaya *et al.* 1997) and intra-individual variation in the ribosomal gene cluster (Krieger and Fuerst 2002). As multiple (up to 3) bands were present in single samples, each unique combination of bands was counted as a unique MOTU. Based on this criterion, from the 38 random nematode samples that produced bands, a total of 17 unique fragments were produced, which resulted in 18 different MOTUs being identified. This level of diversity is very high from just 38 samples, however since 154 species have been identified from a single English grassland (Hodda and Wanless 1994), and there is the possibility that T-RFLP is recording not only different species, but cryptic species, subspecies and haplotypes (although this may be countered by reduced taxonomic resolution in other groups), this level of diversity is not entirely unexpected.

By combining extracted DNA from the 48 separate nematodes prior to PCR, the T-RFLP profile differed significantly from the expected profile in the individual runs. Only half the T-RFs were present in the mixed run, and four completely new T-RFs were recorded. Mixing DNA post-PCR produced an almost identical profile to the individual runs, with 15 of the 17 T-RFs present and only one new T-RF. The reduced number of T-RFs in the pre-PCR profile is probably due to selective amplification of DNA fragments in the PCR stage. Selective amplification may occur on those MOTUs that are present in higher concentrations in the nematode mix, or due to differences in the primer efficiency amongst different nematode taxa. Differential amplification using general primers has been encountered with nematodes in the past (Griffiths *et al.* 2006), where amplification of DNA from some genera was poor using general 'nematode' primers. This problem may be overcome in part by combining multiple PCR products from the same sample, reducing the effects of stochastic processes in influencing the final proportions of PCR products. The use of different general primers or the use of degenerate primers designed to overcome differences at primers sites may also help. The reason for the presence of new T-RFs in both the pre- and post-PCR samples is

unknown, as it is reasonable to assume that the T-RFs from the mixed sample would consist of the same T-RFs as the individually run samples. It is possible that these extra peaks were a result of background noise in the chromatogram, and did not represent genuine MOTUs, although care was taken to exclude noisy chromatograms, and the threshold level was set at a level recommended in the literature (Osborne *et al.* 2006). Setting a higher threshold may reduce the number of these errors, but at the same time reduce the number of T-RFs present in each run. Further work is needed to identify the cause of this discrepancy and identify an optimum screening threshold.

Experiment three was designed as a preliminary study to investigate whether T-RFLP could distinguish between an undisturbed and a disturbed soil-dwelling nematode community. Using the T-RFLP profiles generated by restriction enzyme *Ase*I, Multidimensional Scaling (MDS) produced valid ordination plots, but no clear differentiation between the nematode communities in the control and treatment samples. This was further confirmed by the results from the ANOSIM and SIMPER analysis. The species diversity indices showed that although there were differences in the diversity (using both Shannon-Weiner and Simpson's diversity index) and richness and abundance between the treatment and control plots (with the control being more diverse and MOTU rich that the treatment), these differences were not statistically significant. The choice of using either presence-absence (binary) or relative abundance of the peaks did have a significant effect on the analysis, with relative abundance producing a more useful MDS plot (shown by the lower Kruskal's stress value). However, both these methods of peak recording were non-significant using ANOSIM.

Using enzyme *Mbo*II generated significantly more T-RFs that *Ase*I (15 for *AseI* compared to 35 for *Mbo*II in the control plots), resulting in a higher degree of taxonomic separation of the nematode community. Because of this, T-RFLP using *Mbo*II was able to identify clear differences between untreated soil and soil that had been treated with the nematode *P. hermaphrodita*, as shown in the MDS plots. The use of presence-absence or relative abundance had a significant effect on the results, with the presence-absence data indicating higher levels of dissimilarity between the treatment and control groups than the relative abundance data. The use of presence-absence data allows all peaks to have the same impact, regardless of the relative abundance of that MOTU. Because of this, the presence or absence of rarer species can have a larger

impact on the analysis. Where relative abundance is used, the loss or addition of rare nematode MOTUs in the community may go unnoticed. The control plots were again more diverse than the treatment plots, although again these differences were not statistically significant. However, the control plots did have a significantly higher MOTU richness than the treatment plots, indicating that the addition of *P. hermaphrodita* did have a negative effect on the soil nematode community.

The lower level of taxonomic resolution using *Ase*I compared to *Mbo*II was responsible for the differing results. Where *Mbo*II can identify a higher number of MOTUs, any changes in the composition of nematode species in the soil are more likely to be highlighted. As stated previously, the level of taxonomic resolution of T-RFLP with these enzymes needs to be further investigated by conducting calibratory experiments with samples of known nematode species, or by comparing morphologically identified field samples with T-RFLP generated profiles. The use of different enzymes, and different gene regions with higher or lower taxonomic resolution, will enable the examination of environmental impact at different levels in the nematode community.

The results of the impact of *P. hermaphrodita* application on the soil nematode community are in line with previous studies on the effect of the application of nematode biopesticides to the soil. Grewal *et al.* (1997) found that applications of the entomopathogenic nematodes *Steinernema carpocapsae* and *S. riobravis* were capable of reducing the population density of the plant parasitic nematodes *Meloidogyne* sp., *Belonolaimus longicaudatus* and *Criconemella* sp. A similar result was caused by the application of the nematodes *S. feltiae* and *S. carpocapsae* on the potato cyst nematode *Globodera rostochiensis* (see Perry *et al.* 1998), and after the application of *S. feltiae* on the root knot nematode *Meloidogyne incognita* (see Lewis *et al.* 2001). Jagdale *et al.* (2002) discovered that both alive and dead *S. carpocapsae* were capable of reducing numbers of plant parasitic nematodes, leading to the suggestion that allelochemicals produced by the nematode or the bacterial symbiont were responsible for this effect. Application of two strains of *Heterorhabditis bacteriophora* and one of *H. indica* significantly reduced the abundance, species richness, diversity and maturity of the whole nematode community, although this effect was caused by the reduction of plant parasitic nematodes (Somasekhar *et al.* 2002). However, care needs to be taken interpreting the results in the present study, as this is the first time this tool has been

applied to the analysis of a nematode community. Further work needs to be conducted before any conclusions about the impact of *P. hermaphrodita* on soil nematode communities can be made.

Despite the promise of T-RFLP as a method, there are a number of experimental difficulties that must be overcome before it can be used reliably to measure nematode diversity as an indicator of soil health or disturbance. Yeates and Bongers (1999) stated that for a molecular method to replace morphological methods, it would need to "reflect the relative abundance of particular species or functional groups." Measuring the relative abundance of the peaks will reveal the relative abundance of the amplified DNA fragments that are run through the chromatogram. Work is needed to ensure that this accurately reflects the proportion of nematode MOTUs in the extracted DNA, and that this accurately reflects the proportion of nematode MOTUs in the soil. Extraction methods for removing nematodes from the soil have already been shown to produce biases in the nematode species composition (McSorley and Frederick 2004), although this problem may be overcome by extracting nematode DNA directly from the soil matrix (Waite *et al.* 2003). However, even if these stages were achieved without significant error, the peak height will be affected by the relative biomass of nematodes in the sample, not the number of individuals, due to the fact that soil-dwelling nematodes vary considerably in size and volume amongst species and life-stages.

The fact that this T-RFLP using this combination of DNA region and enzymes does not produce a unique T-RF for each species may reduce the usefulness of this method for the tracking of particular species of nematode in relation to a changing nematode community. In this study the diagnostic band for the presence of *P. hermaphrodita* was present in four of the treated samples using both *Ase*I and *Mbo*II. However, until a thorough survey of the existing nematodes is completed at this site, possibly by using a clone library, no assurances about the identity of this T-RF can be made. Despite this, these results indicate that T-RFLP can reveal changes in the nematode community, and as long as this is consistent between studies it is still a useful tool due to the high speed and low cost compared to conventional methods. There are many other potential target genes that could be used for nematode community studies, including those with faster rates of evolution to allow a higher level of taxonomic resolution. However, 18S is a logical choice due to the presence of a wide range of existing nematode sequences on

GenBank with which to base restriction enzyme choices on. New techniques such as multiplex T-RFLP (M-TRFLP) may allow for the use of multiple regions and enzymes in the same reaction (Singh *et al.* 2006), significantly increasing the amount of information that may be obtained from a single run.

Chapter 6

Molecular detection of intraguild predation between a generalist predator and nematode parasites in agroecosystems

Abstract

Intraguild interactions are thought to be widespread amongst invertebrates. Intraguild research in agroecosystems has mainly focussed on the trophic interactions between predators that share common prey. The potential interactions between parasites (in particular parasites in their hosts) and predators have received less attention, possibly due to the difficulties in quantifying this interaction in the field. This study describes the use of PCR-probes designed for the detection of the mollusc- and insect-parasitic nematodes *Phasmarhabditid hermaphrodita*, *Heterorhabditis megidis*, and *Steinernema feltiae*, within their invertebrate hosts *Deroceras reticulatum*, *Tenebrio molitor* and *Galleria mellonella*. Detection of parasite DNA within the host was possible at the early stages of infection (t = 1 h) and increased with time since infection, with 100 % of hosts testing positive after 96 h. These probes were then used to detect predation by a generalist invertebrate predator, the carabid beetle *Pterostichus melanarius*, on hosts of varying stages of infection. Detection of parasite DNA in the gut of a predator that had consumed the infected host was possible as early as 24 h after initial infection. The longer the host had been infected, the longer it was possible to detect parasite DNA in the predator gut, with maximum detection times being achieved after 216 h of infection. Detection times of host DNA in the predator gut was found to decrease as time since infection increased. These trends were found in all three nematode-parasite systems tested.

6.1. Introduction

Intraguild predation (IGP) occurs when two or more species that share the same host or prey engage in trophic interactions (Rosenheim *et al.* 1995). The prevalence of IGP in natural ecosystems is thought to be widespread (Brodeur and Rosenheim 2000), and may play an important role in the population dynamics of many predators and parasites, and consequently, due to cascade effects, their prey and hosts, and the plants that support them (Spiller and Schoener 1990). In this study the definitions set out in Polis (1989) are followed, in which the term guild is used in its broadest sense, and includes all the organisms that use the same resources despite differences in utilization. Because of this, a single guild may include predators, parasites and pathogens, and IGP may cover a wide range of trophic interactions. One particular form of IGP that has received relatively little attention occurs when predators or scavengers consume parasitized prey, thus consuming both the host or cadaver and the developing parasite within (Rosenheim *et al.* 1995). Holt (1997) described this interaction in the form of a community module (Figure 6.1.1), in which the interaction between the predator and the parasite could either be one or two way. A one-way interaction occurs when the predator eats the parasite whilst in the host organism, described as 'indirect IGP' (Brodeur and Rosenheim 2000), resulting in the predator being the intraguild predator, and the parasite being the intraguild prey (unidirectional IGP). The second option is a two-way interaction between the predator and parasite, in which the predator eats the parasite and the parasite can infect and kill the predator. This situation has been described as mutual or bidirectional IGP (Rosenheim *et al.* 1995). The effects of parasites on trophic interactions are widespread and diverse (for a full review of the subject, see Hatcher *et al.* 2006), and can result in almost all possible outcomes on the population dynamics of the species involved.

The role of the host during parasite infection, both alive (moribund) and dead (as a cadaver), is essential in the life-cycle of many parasites (Kaya and Gaugler 1993). The host can play a role in protecting parasites from desiccation (Koppenhofer *et al.* 1997; Perez *et al.* 2003), UV exposure (Gaugler *et al.* 1992), predation (Zhou *et al.* 2002), pathogens (Walsh and Webster 2003), extremes of temperature (Lewis and

Shapiro-Ilan 2002) and is important in supplying food for the developing parasite (Kaya and Gaugler 1993).

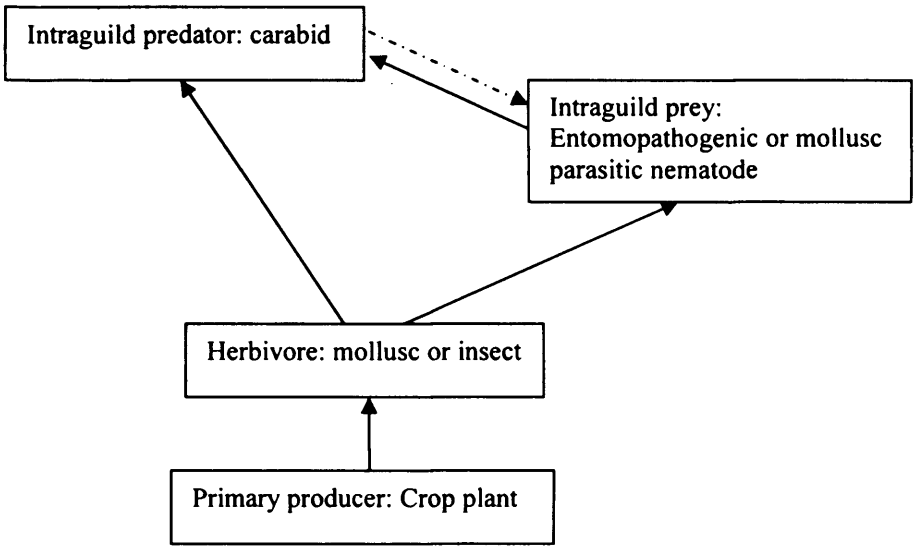


Figure 6.1.1 Potential trophic interactions involving a primary producer (a plant), a herbivore (a mollusc or insect), a predator (a carabid beetle) and a parasite (a mollusc or entomopathogenic nematode); expressed in the form of a 'community module' (after Hatcher et al. 2006). Solid lines represent energy flow, dotted line represents possible 'mutual' IGP caused by parasitism of carabids.

Examples of this type of community module have encompassed a wide range of predator-prey and parasite systems, with the majority of research focusing on interactions between predators, parasitoids and their hosts. As previously highlighted, the outcome of these interactions can be diverse. For example, the predatory ladybird *Hippodamia convergens* was observed to have a negative effect on populations of the parasitoid *Lysiphlebus testaceipes*, although this did not reduce aphid suppression (Colfer and Rosenheim 2001). Generalist predators were observed to reduce rates of parasitism by the parasitoid *Psyllaephagus bliteus* on the pest psyllid, *Glycaspis brimblecombei*, and this was strongly tied to the developmental stage of the psyllids (Erbilgin *et al.* 2004). Predation by the fire ant, *Solenopsis invicta*, was found to have no effect on either the density of adult parasitoids, or the rates of parasitism on the pest species, the diamondback moth larvae *Plutella xylostella* (see Harvey and Eubanks 2005).

The effect of IGP on biological control has both ecological and economic significance, and is relevant both with single predator/parasite introductions (where the introduced biocontrol agent may interact with natural ecosystem components) and multi-predator/parasite introductions (resulting in direct interaction between introduced agents). Intraguild interactions between natural components of ecosystems may also result in a suppression of predation or parasitism on pests (Lang 2003). Antagonistic interactions are the least desirable outcome in terms of successful pest control, and are a result of IGP causing reduced predation or parasite pressure on the herbivore population (Muller and Brodeur 2002). Understanding the role in which particular species or groups of organisms play in maintaining or reducing predator and parasite populations through IGP is important for the management of agroecosystems.

As with the study of all forms of invertebrate predation, scavenging and parasitism, there are a number of intrinsic difficulties that must be overcome to enable accurate determination of trophic links in the field. The majority of current research into the molecular detection of parasitism has focussed on insect parasitoids belonging to the orders Hymenoptera and Diptera (Greenstone 2006). Traditionally, rearing and morphological identification have been used to monitor rates of parasitism in the field, but this has been superseded by the use of molecular methods, including the use of allozyme electrophoresis and DNA based methods (reviewed in Greenstone 2006; Gariepy *et al.* 2007). The cryptic nature of IGP involving invertebrates means that direct observations are frequently difficult. *Post-mortem* analysis of predator gut contents and parasitized hosts allows the systems in question to be sampled after the interactions have occurred, resulting in reduced disturbance of the system under study. Although PCR-based approaches are being used increasingly to study predation (reviewed by Symondson 2002; Sheppard and Harwood 2005; Gariepy *et al.* 2007), none so far have investigated predation on parasitized prey.

This study investigated the use of species-specific PCR-probes to track infection and consumption of three species of parasitic nematode that are commonly used as biological control agents: *Phasmarhabditis hermaphrodita* (Rhabditida: Rhabditidae), *Steinernema feltiae* (Rhabditida: Steinernematidae) and *Heterorhabditis megidis*

(Rhabditida: Heterorhabditidae). Species-specific primers were used to amplify DNA from model host organisms, the field grey slug *Deroceras reticulatum* (Mollusca: Pulmonata), the waxworm *Galleria mellonella* (Lepidoptera: Pyralidae) and the mealworm *Tenebrio molitor* (Coleoptera: Tenebrionidae). These primers were used to assess the potential of DNA-based methods to detect predation and scavenging by the generalist ground beetle *Pterostichus melanarius* (Coleoptera: Carabidae) on hosts infected with the three previously mentioned species of nematode.

There are a number of objectives which this study aims to address, including to:

1. Determine the ability of PCR-based detection to identify infection by parasitic nematodes within insect and mollusc hosts and to use this method to describe the change in parasite detection ability as time since initial infection increases.

2. Investigate the effect of the insect- and mollusc- parasitic nematodes on a potential insect predator and scavenger of the infected hosts, and to measure the potential error caused by non-target infection of this predator.

3. Determine the ability of the PCR-based detection to detect predation and scavenging on nematode infected hosts, and to determine the processes that influence detection times within the gut of an invertebrate predator.

6.2. Materials and Methods

6.2.1. Molecular detection of parasitic nematode DNA within the host

To determine the efficiency of the species-specific PCR-probes (see Chapter 2, Section 2.1.5.1) to detect parasitism, the invertebrates *Deroceras reticulatum* (100–200 mg), *Tenebrio molitor* (100–200 mg), and *Galleria mellonell*a (100-150 mg) were used as hosts for the mollusc- and insect-parasitic nematodes *Phasmarhabditis hermaphrodita*, *Heterorhabditis megidis* and *Steinernema feltiae* respectively (see Chapter 2, Section 2.1.1 for host and nematode rearing methods). In three separate experiments, host organisms of each species were placed individually in 90 mm Petri dishes with a layer of moist 90 mm filter paper (Fisher Scientific, Leicestershire, UK) as substrate. For experiments using slugs, the sides and lids of the dishes were coated with Fluon® to prevent slugs climbing the sides of the container and avoiding the nematodes (see Symondson 1993). The nematode infective juveniles (IJs) were applied at the recommended rate (30 per cm^2 for *P. hermaphrodita*, and 50 cm^2 for *H. megidis* and *S. feltiae*) in 1 ml of dechlorinated tap water to each dish. Hosts were exposed to nematodes for a period of 72 h, after which they were removed and stored individually in clean 90 mm Petri dishes (Sterelin, UK), with a layer of damp filter paper as substrate. Food was supplied for the slugs in the form of cabbage disks, mealworms were supplied bran, and waxworms with commercial waxworm food (LivefoodUK, Somerset, UK). Infected *D. reticulatum* were stored at 16 ± 1 °C, and *T. molitor* and *G. mellonella* at 20 ± 1 °C. Twenty hosts were removed 1 h after initial exposure to the nematodes, and a further 20 hosts were removed every 24 h for a further 240 h. Hosts were weighed, and it was recorded whether they were dead or alive. To detect the presence of the nematode within the host tissue, half the *D. reticulatum* (10 from each sampling time) were washed in distilled water to remove any external nematodes, and a section of the posterior mantle weighing approx 20-25 mg was removed from each slug using a clean scalpel and placed in a 1.5 ml centrifuge tube. Half the *T. molitor* and *G. mellonella* (10 of each from each sampling time) were also washed in distilled water, and the caudal segment was removed using a clean scalpel and placed individually in clean 1.5 ml centrifuge tubes. The tissue samples were stored at – 80 °C for later screening.

The remaining hosts (10 from each sampling time) were used to assess the number of nematodes in each host at each sampling time. The hosts were dissected in an identical manner to the ones used for molecular screening, with the posterior mantle of the slugs and the caudal segment of the insect larvae being removed. These sections were then digested following the procedure outlined in Caroli *et al.* (1996). Briefly, host tissue was placed in 15 ml centrifuge tubes with 4 ml of pepsin solution (2.3 g of NaCl, 0.8 g of Pepsin (both from Sigma-Aldrich, UK)) in 100 ml of deionised water, (pH adjusted to 1.8-2.0 with HCl (Sigma-Aldrich, UK)). The tubes were incubated for at 37 °C for 2 h to digest the insect tissue. After this, the suspension was diluted in 10 ml of 0.1% Tween (Sigma-Aldrich, UK) solution, mixed thoroughly and poured into a 10 cm Petri dish. The nematodes of all stages in each host were counted under a high power stereo microscope. This procedure was conducted for the host cadavers collected for the first 96 h, after which the nematodes in each host became too numerous to count.

6.2.2. Resistance of the carabid *Pterostichus melanarius* to exposure to mollusc- and insect-parasitic nematodes

To determine the effects of mollusc- and insect-parasitic nematodes on the predatory carabid *Pterostichus melanarius*, beetles were collected and stored as described in Chapter 2, Section 2.1.1. The carabids were maintained for at least 10 days before the experiment to ensure they were healthy. Carabids were fed one *Calliphora vomitoria* larvae each, before the start of the experiment, to ensure similar nutritional states.

The carabids were then placed individually in soil-filled bioassay arenas. These arenas consisted of plastic containers (125 mm dia, 70 mm depth) each filled with 50 g of sandy silty loam soil from Long Ashton, Bristol. The soil was defaunated by air drying at room temperature for 20 days before the start of the experiment. The soil was then re-hydrated to a moisture level of 20 % (w/v) with dechlorinated tap water. Nematode IJs of *P. hermaphrodita, H. megidis* and *S. feltiae* were applied to these arenas at a rate of either 30 per cm^2 (*P. hermaphrodita*) or 50 per cm^2 (*H. megidis* and *S. feltiae*), in 2 ml of water to the soil surface. Additional containers had water but no nematodes added to the soil surface as a control. Ten carabids from each of the nematode treatment groups, and 10 carabids from the control group, were removed

24, 72, 120, 168 and 216 h after the start of the experiment and checked for mortality (total 200 beetles). The beetles were then killed and stored individually in clean 1.5 ml centrifuge tubes at -80 °C, for later screening with the respective nematode-specific primers (Section 6.2.5).

6.2.3. Post-ingestion detection of parasitic nematode DNA and host DNA within the guts of carabid scavengers

To assess the ability of PCR-probes to detect predation on parasitized prey, *D. reticulatum* (100–200 mg), *G. mellonella* (100-150 mg) and *T. molitor* (100-200 mg) were infected with the nematode parasites *P. hermaphrodita, S. feltiae* and *H. megidis* respectively, using the same procedure as described in Section 6.2.1. The hosts were killed at 24 h, 120 h and 216 h after first exposure to the nematodes, and frozen at -80 °C for logistical purposes. This created three feeding trials at three levels of infection for each nematode-host system tested (a total of nine feeding trials). From here on, the three feeding trials shall be referred to as the '24 h', the '120 h' and the '216 h' feeding trials.

Before the start of the feeding trials, infected hosts were removed from the freezer and allowed to thaw at room temperature for 1 h. Carabid beetles (*P. melanarius*) were starved for seven days, before being placed individually in 90 mm Petri dishes with moistened filter paper as substrate. A single host cadaver was placed in each arena and the beetles were allowed to feed. Any non-feeding carabids were removed and replaced with a fresh beetle until feeding commenced. After 2 h beetles were removed and placed into clean Petri-dishes with a filter paper substrate. Four male and four female beetles were removed 1, 3, 6, 12, 18, 24 and 36 h after the mid-point of the feeding period, for each of the nine feeding trials. A control consisted of a replicated feeding trial, but with unfed beetles. The beetles were placed individually in 1.5 ml centrifuge tubes and stored at –80 °C for later screening (Section 6.2.5).

6.2.4. Carabid foregut dissection and DNA extraction

Before dissecting the foregut, the beetles were allowed to defrost for a period of ½ - 1 h at room temperature. The foregut was removed by twisting the beetle apart between

the pronotum and elytra. This causes the foregut to be pulled out, separating at the proventriculus. Any extra gut was removed using a pair of sterile scissors and discarded. The foregut could then be removed from the thorax using a pair of forceps and placed into a pre-weighed 1.5 ml centrifuge tube containing 20 µl of Phosphate buffered saline (PBS). The tube was then re-weighed, and the starting value subtracted from the new value to give the weight of the foregut. The foregut was broken open and mixed by vortexing at high speed for 20 s. This stock solution of foregut contents was then stored at -80 °C for later DNA extractions.

Fifteen microlitres of the foregut content solution was removed to a clean 1.5 ml centrifuge tube for DNA extraction. DNA extractions were carried out using a modified protocol from the PureGene Tissue extraction kit (Gentra, Minnesota, USA) as follows: 300 µl of cell lysis solution and 1.5 µl of Proteinase K solution were added to the gut extract in 1.5 ml centrifuge tubes. The gut contents was then broken up using a sterile plastic pestle, before incubating at 55 °C for 3 h. RNase solution (1.5 µl) was added to each sample, followed by incubation for 30 min at 37 °C. The samples were cooled to 4 - 10 °C on ice for 3 min, before adding 100 µl of protein precipitation solution and vortexing at high speed for 20 s. The samples were then centrifuged at 12,000 rpm for 5 min, and the supernatant was poured off into a clean 1.5 ml centrifuge tube containing 300 µl of 100% isopropanol and 0.5 µl of glycogen to act as a DNA carrier. The tube containing the precipitated protein was discarded. The sample was mixed gently by inverting 50 times, before centrifuging at 12,000 rpm for 5 min. The supernatant was poured off, and the tube air dried for 2 min on clean absorbent paper. To clean the DNA pellet, 300 µl of 70 % ethanol was added and inverted gently three times. The samples were centrifuged a final time at 12,000 rpm for 2 min, after which the ethanol was discarded and the tubes air dried for 5-10 min. The DNA was rehydrated using 50 µl of DNA hydration solution, and incubated at 65 °C for no more than 1 h. The extracted DNA samples were stored at -20 °C until needed for screening.

6.2.5. Screening

Screening of all feeding trials was a two-stage process. The first stage involved screening the DNA extract with general primers to confirm the success or failure of the DNA extraction. Any samples that did not amplify using the general primers were

tested one more time. If no band was observed for a second time, the sample was discarded from the analysis. For stage one, general invertebrate primers *C1-J-1718* and *C1-J2191* (Simon *et al.* 1994) (Table 6.2.1) were used that amplified a 525-bp region of mtDNA from Subunit I of the Cytochrome Oxidase (*COI*) coding region. PCR reactions contained 2 µl template DNA, 1x PCR buffer, 2.0 mM $MgCl_2$ (both provided by Invitrogen, Paisley, UK), 0.2 mM dNTPs (Invitrogen, Paisley, UK), 0.2 units *Taq* polymerase (Invitrogen, Paisley, UK) and 0.5 µM of each primer (Operon, Cologne, Germany) and were made up to 25 µl with PCR water. Thermal cycle parameters included an initial denaturation step of 2 min at 94°C followed by 35 cycles of 94°C for 1 min 10 sec, 45°C for 1 min 10 sec, 72°C for 1 min 30 sec, and a final extension stage of 72°C for 7 min. Ultraviolet translumination was used to visualise 4 µl of PCR product following electrophoresis at 100 V for 1 h on a 1.8% (w/v) agarose gel stained with ethidium-bromide in 1 x TAE buffer (1mM EDTA, 40mM Tris-Acetate). Gel images were analyzed using InGenius software (Syngene, Cambridge, UK) for PC.

The second stage of the screening process involved screening for parasitic nematode DNA (Section 6.2.1, 6.2.2, and 6.2.3) belonging to *P. hermaphrodita*, *H. megidis* and *S. feltiae*, using the primers in Table 6.2.1. This was done on a GeneAmp 9700 thermal cycler (ABI Perkin Elmer, Beaconsfield, UK), in 10 µl reactions using the following conditions: 1 µl template DNA, 1x PCR buffer, 2.0 mM $MgCl_2$ (both provided by Invitrogen, Paisley, UK), 0.2 mM dNTPs (Invitrogen, Paisley, UK), 0.1 U *Taq* polymerase (Invitrogen, Paisley, UK) and 0.5 µM of each primer (Operon, Cologne, Germany). Thermal conditions included a first cycle of denaturation at 94°C for 3 minutes, followed by 35 cycles of 94°C for 30 sec, annealing for 45 sec, 72°C for 1 min, and a final extension stage of 72 °C for 10 min. Annealing temperatures for each of the primer pairs are shown in Table 6.2.1. Products were visualized as described previously.

The beetles described Section 6.2.3 were screened for host DNA belonging to *D. reticulatum*, *T. molitor* and *G. mellonella*. The primers for the host PCR reactions are shown in Table 6.2.1. All reactions were carried out on a GeneAmp 9700 thermal cycler (ABI Perkin Elmer, Beaconsfield, UK) in 10 µl reactions using the following conditions; 1 µl template DNA, 1x PCR buffer, 2.0 mM $MgCl_2$ (both provided by

Invitrogen, Paisley, UK), 0.2 mM dNTPs (Invitrogen, Paisley, UK), 0.1 U *Taq* polymerase (Invitrogen, Paisley, UK) and 0.5 μM of each primer (Operon, Cologne, Germany). Thermal conditions included a first cycle of denaturation at 94°C for 3 minutes, followed by 30 cycles of 94°C for 30 sec, annealing for 45 sec, 72°C for 1 min, and a final extension stage of 72 °C for 10 min. Annealing temperatures for each of the primer pairs are shown in Table 6.2.1. Products were visualized as described previously.

Table 6.2.1 Primers used in this study for the species-specific amplification of DNA from nematodes (*Steinernema feltiae*, *Phasmarhabditis hermaphrodita* and *Heterorhabditis megidis*) and hosts (*Deroceras reticulatum*, *Tenebrio molitor* and *Galleria mellonella*). The optimised annealing temperature is also shown.

Target species	Forward primer	Reverse Primer	Fragment length (bp)	Annealing temp (°C)
General	*C1-J-1718*	*C1-J2191*	525	50
S. feltiae	*SF-F-(429-447)*	*SF-R-(610-629)*	200	62
P. hermaphrodita	*PH-F-(284-304)*	*PH-R-(479-501)*	217	62
H. megidis	*HM-F-(288-307)*	*HM-R-(419-437)*	149	60
D. reticulatum	*DR11F*	*DR29RC*	109	55
T. molitor	*TM-F-(332-354)*	*TM-R-(526-548)*	216	60
G. mellonella	*GM-F-(295-315)*	*GM-R-(469-491)*	196	66

6.2.6. Statistical Analysis

Molecular detection of parasitic nematodes within the host

Linear regression analyses were conducted to test the relationships between the number of PCR-positives for parasite DNA against time, the host mortality against time, and the number of nematodes per host against time. Analyses only included data up to the point at which the asymptote was reached, as it is the rate of increase in positives or mortality that is relevant for this system. Transformations of the y-axis were conducted, with the transformation that resulted in the best fitting regression being chosen. As no nematode infection was found in the control (uninfected) hosts, all regressions were forced through zero, assuming zero mortality, nematodes within the host and PCR positives for nematode DNA at time zero. Analysis of covariance (ANCOVA) was used to test for

significant differences between the slopes and intercepts of the best fitting regressions between all three nematode-host systems.

Linear regressions, carried out using MINITAB 14, were conducted to test the hypothesis that the number of PCR-positives for parasite DNA would increase as the mortality of the host increased, and to test the hypothesis that the number of PCR-positives for parasite DNA would be positively related to the number of nematodes within the infected host.

Post-ingestion detection of parasitic nematodes within the host

Probit models, a form of generalized linear model, were used to test the hypothesis that the detection of parasite and host DNA within carabid foreguts would decrease as time since initial consumption increased. Analysis was conducted using MINITAB 14. Probit models are particularly appropriate for treating dichotomous and categorical variables, such as the positive/negative data recorded from feeding trials. The method from Payton *et al.* (2003), based on using 83.5% fiducial limits as an approximation of $\alpha = 0.05$ test, was used to determine significant differences between the median detection times (the time for 50 % of the predator foreguts to be positive for the prey DNA) calculated from the best fitting Probit model

.

To test the hypothesis that prey detection times (as reflected in the median detection time) of the consumed parasitic nematodes would increase, and that the prey detection times of the consumed hosts would decrease, as the time since initial infection of the consumed host increased, the median detection times were calculated from the fitted Probit models. These were again compared using the method from Payton *et al.* (2003).

To examine whether there was a correlation between foregut weight and the number of positives for prey DNA in the foregut contents, regressions were carried out using foregut weight as the predictor and number of positives as the independent variable, using MINITAB 14.

6.3. Results

6.3.1 Molecular detection of parasitic nematodes within the host

Detection of nematode DNA within the host reached an asymptote after approximately 96 h in all three host-nematode systems (Figure 6.3.1), so only data up to this point was analysed. Transformation of the y-axis by y^3 allowed linear regressions to accurately describe the increase in parasite detection over time. The best-fitting linear regression lines for the detection of *Phasmarhabditis hermaphrodita*, *Steinernema feltiae* and *Heterorhabditis megidis* DNA were therefore $y^3 = 9.84x$, $y^3 = 11.2x$, and $y^3 = 12.2x$ respectively. All these regressions were highly significant (*P. hermaphrodita*: $F = 158.15$, $P < 0.001$; *S. feltiae*: $F = 120.44$, $P < 0.001$: *H. megidis*: $F = 73.34$, $P < 0.001$). The slopes for the detection of nematode DNA in all three hosts were not significantly different at the 95 % level (ANCOVA: $F = 0.73$, $P \geq 0.05$).

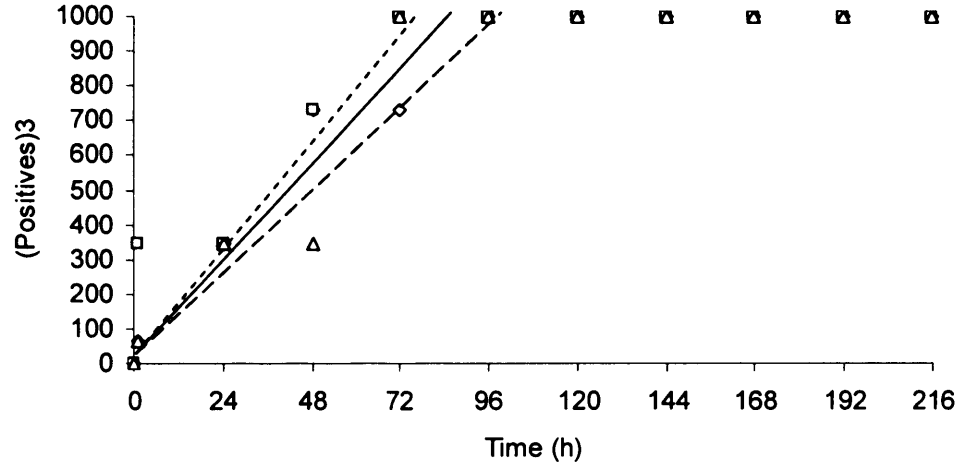


Figure 6.3.1 Number of insect and mollusc hosts testing positive for nematode DNA against time since infection, after transformation of the y-axis by x3. Detection of *Heterorhabditis megidis* within *Tenebrio molitor* (— □); detection of *Phasmarhabditis hermaphrodita* within *Deroceras reticulatum* (---- ◊); detection of *Steinernema feltiae* within *Galleria mellonella* (— — Δ). Lines represent best fitting linear regressions on the data before the asymptote is reached.

Data for the mortality of the hosts, against time since infection (Figure 6.3.2), was only analysed up to the point at which the asymptote in mortality was reached (*Deroceras reticulatum* = 240 h, *Galleria mellonella* and *Tenebrio molitor* = 192 h). Transformation of the y-axis by y^3 allowed linear regressions to be fitted to the data. The best fitting linear regressions for the mortality of *D. reticulatum*, *G. mellonella*, and *T. molitor* were $y^3 = 4.07x$, $y^3 = 5.13x$, and $y^3 = 5.98x$ respectively. All these regressions were highly significant (*D. reticulatum*: $F = 94.27$, $P < 0.001$; *G. mellonella*: $F = 220.87$, $P < 0.001$; *T. molitor*: $F = 191.29$, $P < 0.001$). There was no significant difference between the slopes for the mortality of the nematode infected hosts over time (ANCOVA: $F = 0.91$, $P \geq 0.05$).

The average number of nematodes of all stages was counted from the host tissue, in each of the three nematode-host systems (Figure 6.3.3). Linear regressions forced through zero (assuming no parasites at time zero) described the increase in nematode numbers over time, with the best fitting regression for the number of *P. hermaphrodita*, *S. feltiae* and *H. megidis* being $y = 0.167x$, $y = 0.251x$, and $y = 0.0799x$ respectively. All of these regressions were highly significant (*P. hermaphrodita*: $F = 54.41$, $P < 0.001$; *S. feltiae*: $F = 382.52$, $P < 0.001$; *H. megidis*: $F = 607.16$, $P < 0.001$). There was a significant difference between the slopes of the lines (ANCOVA: $F = 13.71$, $P < 0.05$).

After transformation of the x-axis by $1/x+1$, significant correlations were observed between the number of positives for nematode DNA and the mean host mortality for both the *P. hermaphrodita* and *S. feltiae* infected hosts (*P. hermaphrodita*: $y = 14.2 - 10.3\ 1/\sqrt{(x+1)}$; *S. feltiae*: $y = 13.3 - 11.4\ 1/\sqrt{(x+1)}$). Both these regressions were significant (*P. hermaphrodita*: $F = 10.78$, $P < 0.05$; *S. feltiae*: $F = 47.66$, $P < 0.001$). No correlation was observed between mortality of *T. molitor* infected with *H. megidis* and positives for *H. megidis* DNA.

Significant correlations were observed between number of positives for nematode DNA and the average number of nematodes recorded in the target host tissue in all three nematode host systems tested (*P. hermaphrodita*: $y = 10.2 - 8.21\ 1/(x+1)$; *S. feltiae*: $y = 2.14\ \sqrt{(x+1)}$; *H. megidis*: $y = 15.1 - 13.2\ 1/\sqrt{(x+1)}$). All of these regressions were highly significant (*P. hermaphrodita*: $F = 31.46$, $P < 0.05$; *S. feltiae*: $F = 152.97$, $P < 0.001$; *H. megidis*: $F = 19.70$, $P < 0.05$).

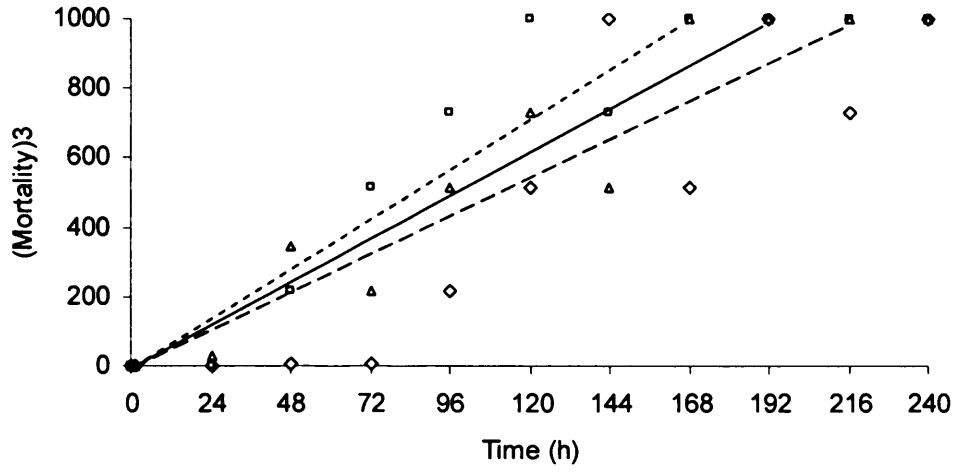


Figure 6.3.2 Mortality of insect and mollusc hosts after exposure to pathogenic nematodes after transformation of the y-axis by x3. Mortality of *Tenebrio molitor* infected with *Heterorhabditis megidis* (— □); mortality of *Deroceras reticulatum* infected with *Phasmarhabditis hermaphrodita* (---- ◊); mortality of *Galleria mellonella* infected with *Steinernema feltiae* (— — Δ). Lines represent best fitting linear regressions.

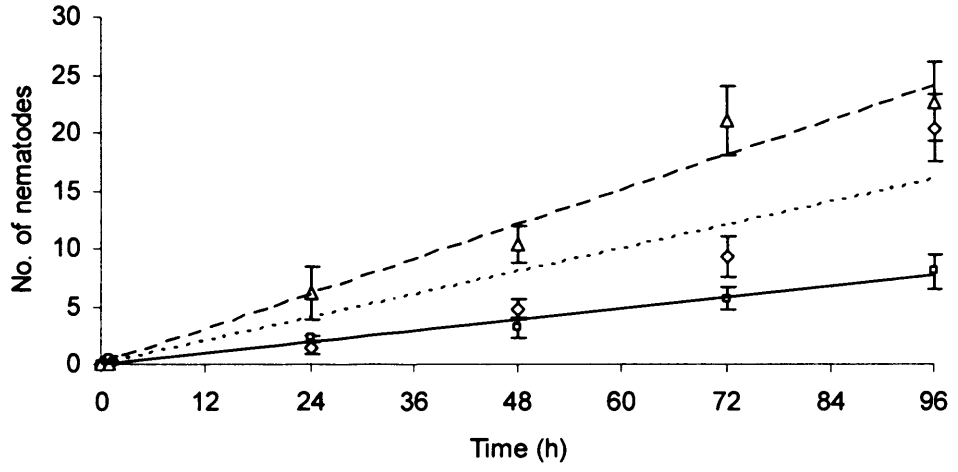


Figure 6.3.3 Number of nematodes in host tissue over time since infection. Number of *Heterorhabditis megidis* in *Tenebrio molitor* caudal segment (— □), number of *Phasmarhabditis hermaphrodita* in *Deroceras reticulatum* rear mantle (---- ◊); and number of *Steinernema feltiae* in *Galleria mellonella* caudal segment (— — Δ). Lines represent best fitting linear regressions.

6.3.2. Resistance of the carabid *Pterostichus melanarius* to exposure to mollusc- and insect-parasitic nematodes

A total of 200 carabid beetles were monitored for mortality and detection of nematode DNA in the foregut, in the presence of three species of mollusc- and insect-pathogenic nematode. No beetles, in the treatment bioassays or the controls, died within the 216 h test period. Two beetles, one exposed to *H. megidis* for 120 h, and one exposed to *S. feltiae* for 216 h, had foreguts which tested positive for their respective nematode DNA (10 % of beetles from that sample day). No individuals in the control tested positive for any of the three species of nematode.

6.3.3. Post-ingestion detection of parasitic nematode DNA and host DNA within the guts of carabid scavengers

It was possible to detect DNA from the nematode *P. hermaphrodita* and its mollusc host *D. reticulatum* within the foregut of the carabid beetle *Pterostichus melanarius* (Figures 6.3.4 (a) (b) and (c) and Figures 6.3.5. (a) (b) and (c)). The Probit models described the data well at the 95 % level ($P \geq 0.05$) (Tables 6.3.1 and 6.3.2). Time was found to cause a significant reduction in the probability of detecting host and nematode DNA within carabid foreguts in all feeding trials ($P < 0.001$), except for the detection of *P. hermaphrodita* in the '24 h' feeding trial ($P \geq 0.05$).

Median detection times for *P. hermaphrodita* DNA in carabid foreguts increased from 0.8 h in the '24 h' feeding trial to 15.4 h in the '216 h' feeding trial, and detection times of *D. reticulatum* DNA in carabid foreguts declined from 16.1 h after 24 h infection, to 8.9 h after 216 h of infection (Table 6.3.6). The median detection time of *P. hermaphrodita* was significantly shorter at the 95 % level ($P < 0.05$) in the '24 h' feeding trial in comparison to the '120 h' and '216 h' feeding trials. The median detection time for *D. reticulatum* DNA was significantly longer at the '24 h' feeding trial when compared to the '120 h' and '216 h' feeding trials ($P < 0.05$).

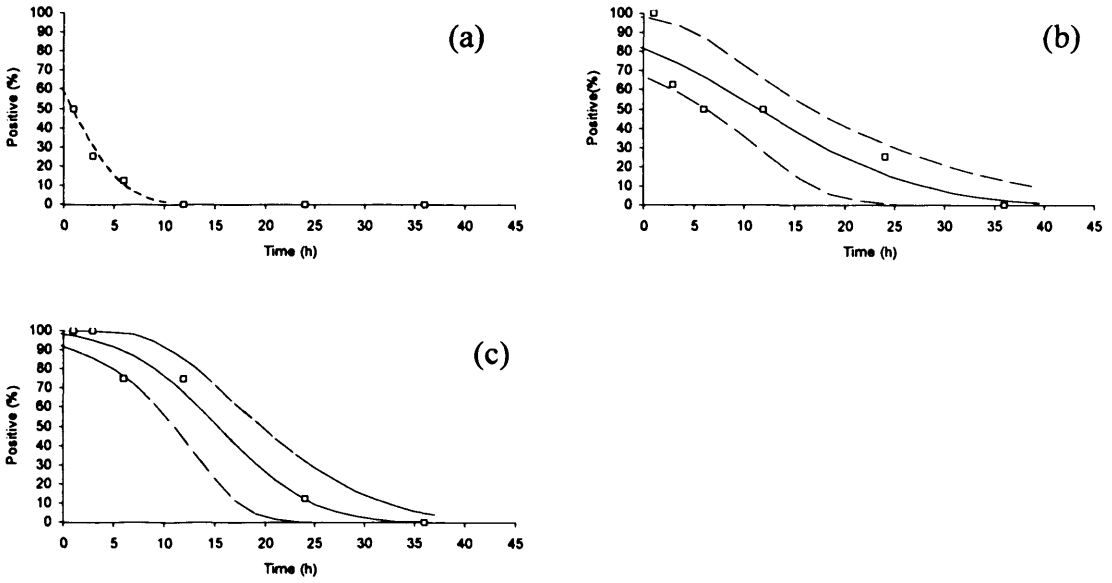
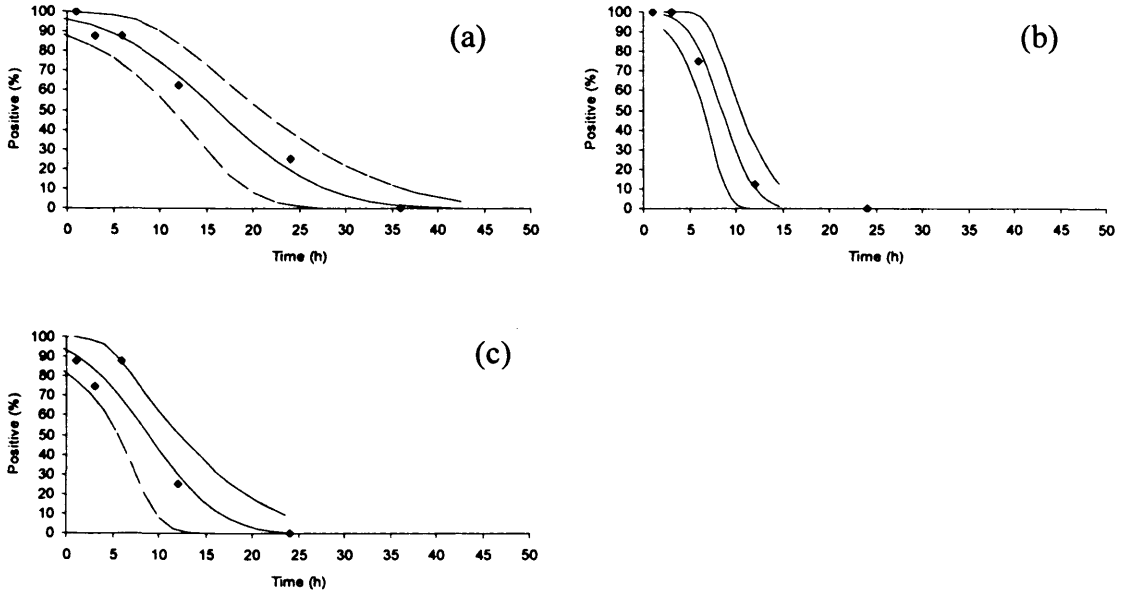


Figure 6.3.4 (a) (b) and (c) Symbols represent percentage of *Pterostichus melanarius* guts at each time point that were positive for *Phasmarhabditis hermaphrodita* DNA (8 replicates at each time point). (a): '24 h' feeding trial. (b): '120 h' feeding trial. (c). '216 h' feeding trial. Solid lines represent fitted Probit models with dashed lines representing 95% fiducial limits.

Table 6.3.1 Goodness of fit tests for the Probit models for the probability of detecting DNA from the nematode *Phasmarhabditis hermaphrodita* within infected slugs *Deroceras reticulatum* over time, after consumption by the beetle *Pterostichus melanarius*.

Feeding trial	Statistic	Chi-Square	Df	P-Value
24 h	Pearson	0.164958	4	0.997
	Deviance	0.186998	4	0.996
120 h	Pearson	4.39059	4	0.356
	Deviance	6.04105	4	0.196
216 h	Pearson	2.72405	4	0.605
	Deviance	2.85924	4	0.582



Figures 6.3.5 (a) (b) and (c) Symbols represent percentage of *Pterostichus melanarius* guts at each time point that were positive for *Deroceras reticulatum* DNA (8 replicates at each time point). (a): '24 h' feeding trial. (b): '120 h' feeding trial. (c). '216 h' feeding trial. Solid lines represent fitted Probit models with dashed lines representing 95% fiducial limits.

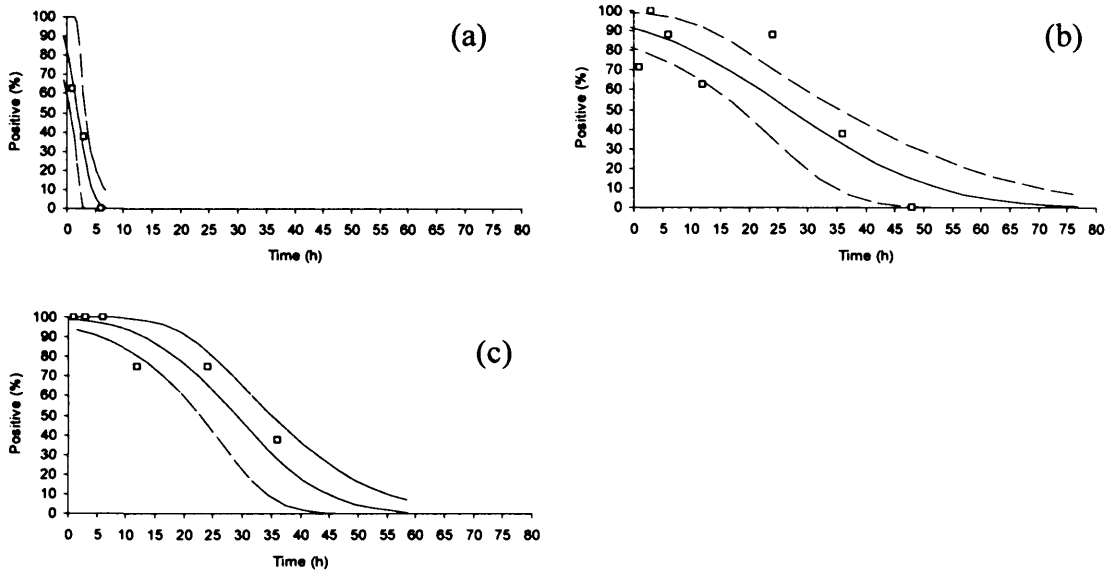
Table 6.3.2 Goodness of fit tests for the Probit models for the probability of detecting DNA from the slug *Deroceras reticulatum* infected by the nematode *Phasmarhabditis hermaphrodita* over time, after consumption by the beetle *Pterostichus melanarius*.

Feeding trial	Statistic	Chi-Square	df	P-Value
24 h	Pearson	1.07799	4	0.898
	Deviance	1.51924	4	0.823
120 h	Pearson	0.477058	3	0.924
	Deviance	0.690563	3	0.875
216 h	Pearson	2.01981	3	0.568
	Deviance	2.25357	3	0.521

Primers for the nematode *S. feltiae* and its lepidopteran host *G. mellonella* were used to detect prey DNA in the foregut of the carabid *P. melanarius* (Figures 6.3.6 (a) (b) (c) and Figures 6.3.7 (a) (b) (c)). Fitted Probit models described the data well at the 95 % level ($P \geq 0.05$) (Tables 6.3.3 and 6.3.4) with the exception of the detection of *G. mellonella* DNA in the 120 h feeding trial. This would appear to be caused by the unexpectedly large number of positives at the 24 h post-feeding sampling point. Time was found to cause a significant reduction in the probability of detecting host and nematode DNA in carabid foreguts in all feeding trials ($P < 001$).

Median detection times for *S. feltiae* DNA in carabid foreguts increased from 1.9 h in the '24 h' feeding trial to 28.9 h in the '216 h' feeding trial, and detection times of *G. mellonella* DNA in carabid foreguts declined from 21.4 h after 24 h infection to 8.7 h after 216 h of infection (Table 6.3.6). The median detection time of *S. feltiae* DNA at the '24 h' feeding trial was significantly shorter than in the '120 h' and '216 h' feeding trials (Table 6.3.7). Detection of *G. mellonella* DNA was significantly shorter in the '216 h' feeding trial when compared to the 120 h and 24 h feeding trials.

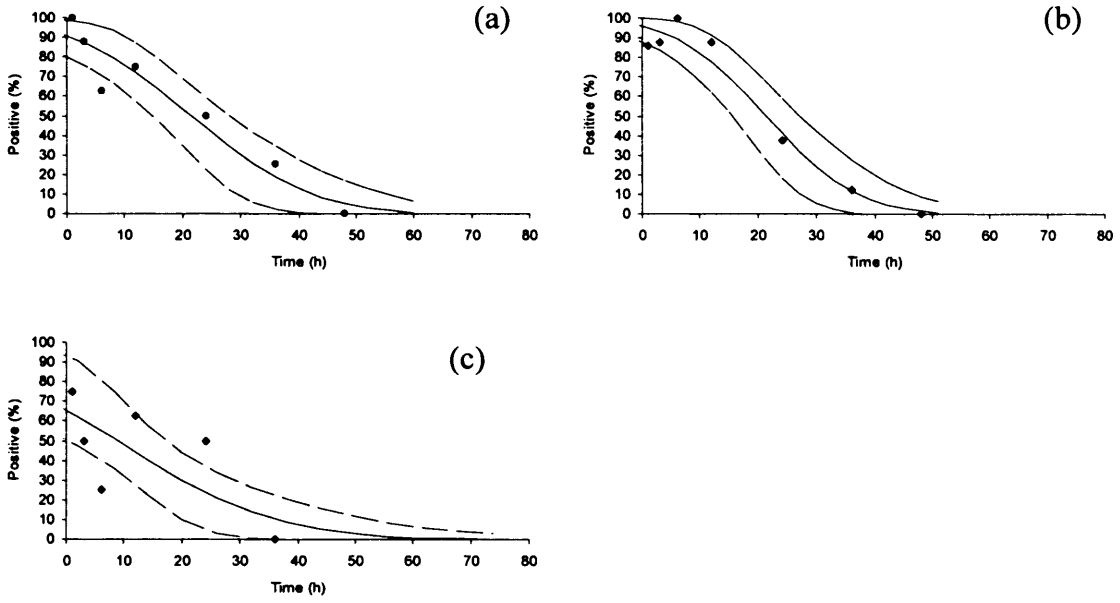
In the feeding trials involving *T. molitor* infected with *H. megidis*, the beetles did not eat any mealworms that had been infected for more than 24 h. As a result, no screening took place for the '120 h' or '216 h' feeding trials. Primers for *H. megidis* and *T. molitor* were used to screen for DNA in the foregut of the carabid *P. melanarius* in the '24 h' feeding trial (Figure 6.3.8 (a)). No positives were observed for nematode DNA at this level of infection. For the detection of mealworm DNA, fitted Probit models described the data well at the 95 % level ($P \geq 0.05$) (Table 6.3.5).



Figures 6.3.6. (a) (b) and (c) Symbols represent percentage of *Pterostichus melanarius* guts at each time point that tested positive for *Steinernema feltiae* DNA after the consumption of infected *Galleria mellonella* (8 replicates at each time point). (a): '24 h' feeding trial. (b): '120 h' feeding trial. (c). '216 h' feeding trial. Solid lines represent fitted Probit models with dashed lines representing 95% fiducial limits.

Table 6.3.3 Goodness of fit tests for the Probit models for the probability of detecting DNA from the nematode *Steinernema feltiae* within infected waxworms, *Galleria mellonella* over time, after consumption by the beetle *Pterostichus melanarius*.

Feeding trial	Statistic	Chi-Square	df	P-Value
24 h	Pearson	0.507921	5	0.992
	Deviance	0.668485	5	0.985
120 h	Pearson	9.6899	5	0.085
	Deviance	11.2253	5	0.047
216 h	Pearson	4.53672	5	0.475
	Deviance	4.57468	5	0.470



Figures 6.3.7 (a) (b) and (c) Symbols represent percentage of *Pterostichus melanarius* guts at each time point that tested positive for *Galleria mellonella* DNA after the consumption of infected *G. mellonella* (8 replicates at each time point). (a): '24 h' feeding trial. (b): '120 h' feeding trial. (c). '216 h' feeding trial. Solid lines represent fitted Probit models with dashed lines representing 95% fiducial limits.

Table 6.3.4 Goodness of fit tests for the Probit models for the probability of detecting DNA from the waxworm *Galleria mellonella* infected by the nematode *Steinernema feltiae* over time, after consumption by the beetle *Pterostichus melanarius*.

Feeding trial	Statistic	Chi-Square	df	P-Value
24 h	Pearson	4.10872	5	0.534
	Deviance	4.85770	5	0.433
120 h	Pearson	3.27983	5	0.657
	Deviance	3.83353	5	0.574
216 h	Pearson	8.99154	5	0.109
	Deviance	9.72630	5	0.083

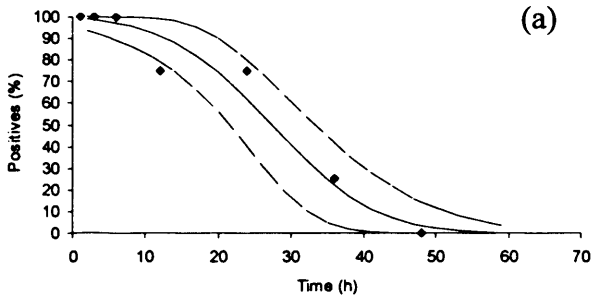


Figure 6.3.8 (a) Symbols represent percentage of *Pterostichus melanarius* guts at each time point that tested positive for *Tenebrio molitor* DNA after the consumption of infected *T. molitor* (8 replicates at each time point). (a): '24 h' feeding trial. Solid lines represent fitted Probit models with dashed lines representing 95% fiducial limits.

Table 6.3.5 Goodness of fit tests for the Probit models for the probability of detecting DNA from the mealworm *Tenebrio molitor* infected by the nematode *Heterorhabditis megidis* over time, after consumption by the beetle *Pterostichus melanarius*.

Feeding trial	Statistic	Chi-Square	df	P-Value
24 h	Pearson	4.04629	5	0.543
	Deviance	3.97002	5	0.554

Table 6.3.6 Median detection times of *Deroceras reticulatum* and *Phasmarhabditis hermaphrodita* in the foregut of the beetle *Pterostichus melanarius*, calculated from best fitting Probit regressions with upper and lower 83.5% fiducial confidence limits to show significant differences ($P < 0.05$).

Target	Trial	Parameter	Estimate	Standard Error	Lower 83.5% CI	Upper 83.5 % CI
Phasmarhabditis hermaphrodita	24 h	Mean	0.79	1.62	1.46	3.04
		StDev	4.07	2.10	1.99	8.32
	120 h	Mean	11.46	2.67	7.76	15.17
		StDev	12.67	3.57	8.58	18.73
	216 h	Mean	15.36	2.19	12.33	18.39
		StDev	7.39	1.81	5.26	10.39
Deroceras reticulatum	24 h	Mean	16.11	2.40	12.78	19.44
		StDev	9.09	2.16	6.53	12.65
	120 h	Mean	8.49	1.01	7.08	9.89
		StDev	2.80	0.79	1.89	4.15
	216 h	Mean	8.90	1.67	6.53	11.17
		StDev	5.97	1.85	3.88	9.18

Table 6.3.7 Median detection times of *Galleria mellonella* and *Steinernema feltiae* in the foregut of *Pterostichus melanarius*, calculated from best fitting Probit regressions with upper and lower 83.5% fiducial confidence limits to show significant differences ($P < 0.05$).

Target	Trial	Parameter	Estimate	Standard Error	Lower 83.5% CI	Upper 83.5 % CI
Steinernema feltiae	24 h	Mean	1.91	0.65	1.00	2.82
		StDev	2.02	0.86	1.12	3.66
	120 h	Mean	26.91	4.30	20.95	32.87
		StDev	19.83	4.96	14.00	28.07
	216 h	Mean	28.88	3.14	24.52	33.24
		StDev	12.24	2.69	9.02	16.61
Galleria mellonella	24 h	Mean	21.42	3.39	16.72	26.12
		StDev	16.21	3.71	11.79	22.28
	120 h	Mean	21.31	2.94	17.23	25.39
		StDev	12.32	2.62	9.18	16.55
	216 h	Mean	8.66	4.28	2.71	14.60
		StDev	21.68	6.81	14.01	33.54

Table 6.3.8 Median detection times of *Tenebrio molitor* and *Heterorhabditis megidis* in the foregut of *Pterostichus melanarius*, calculated from best fitting Probit regressions with upper and lower 83.5% fiducial confidence limits.

Target	Feeding trial	Parameter	Estimate	Standard Error	Lower 83.5% CI	Upper 83.5 % CI
Tenebrio molitor	24 h	Mean	27.32	2.96	21.52	33.12
		StDev	11.27	2.46	7.34	17.29

Effect of foregut weight on prey detection times

For the *D. reticulatum* - *P. hermaphrodita* feeding trial, there was a negative relationship between time since feeding and the weight of the foregut when consuming slugs infected for 24 h ($y = 0.119\text{-}0.00101x$, $R^2 = 0.91$) and 120 h ($y = 0.0992\text{-}0.000657x$, $R^2 = 0.78$) (Figure 6.3.9). Both these regressions were significant at the 95% level (24 h: $F_{1,4} = 42.08$, $P < 0.05$; 120 h: $F_{1,4} = 14.39$, $P < 0.05$). There was no relationship between time and foregut weight in the control, and the feeding trial using slugs infected for 216 h ($P \geq 0.05$).

A similar pattern was observed in the *G. mellonella – S. feltiae* feeding trial, where there was a negative relationship between time since feeding and the weight of the foregut when consuming waxworms infected for 24 h ($y = 0.115\text{-}0.00111x$, $R^2 = 0.84$) and 120 h ($y = 0.0946\text{-}0.000567x$, $R^2 = 0.68$) (Figure 6.3.10). Both these regressions were significant at the 95% level (24 h: $F_{1,4} = 20.90$, $P < 0.05$, 120 h: $F_{1,4} = 8.64$, $P < 0.05$). There was no relationship between time and foregut weight in the control, and the feeding trial using waxworms infected for 216 h ($P \geq 0.05$).

For the *T. molitor – H. megidis* feeding trial there was also a significant ($F_{1,4} = 20.81$, $P < 0.05$) negative relationship between time since feeding and the weight of the foregut in the feeding trial using mealworms infected for 24 h ($y = 0.114\text{-}0.00108x$, $R^2 = 0.84$). In the control, and the feeding trials using mealworms infected for 120 h and 216 h there was no relationship between time and foregut weight ($P \geq 0.05$).

Only in three feeding trials was there a significant relationship between foregut weight and number of positives within the guts of the beetles. These were; detection of *D. reticulatum* after 24 h of infection by *P. hermaphrodita* ($y = -210+2578x$, $R^2 = 0.89$), detection of *P. hermaphrodita* after 120 h of infection in *D. reticulatum* ($y = -224 + 3016x$, $R^2 = 0.82$) (Figure 6.3.11), and the detection of *G. mellonella* after 24 h of infection by *S. feltiae* ($y = -6.16 + 115x$, $R^2 = 0.79$) (Figure 6.3.12).

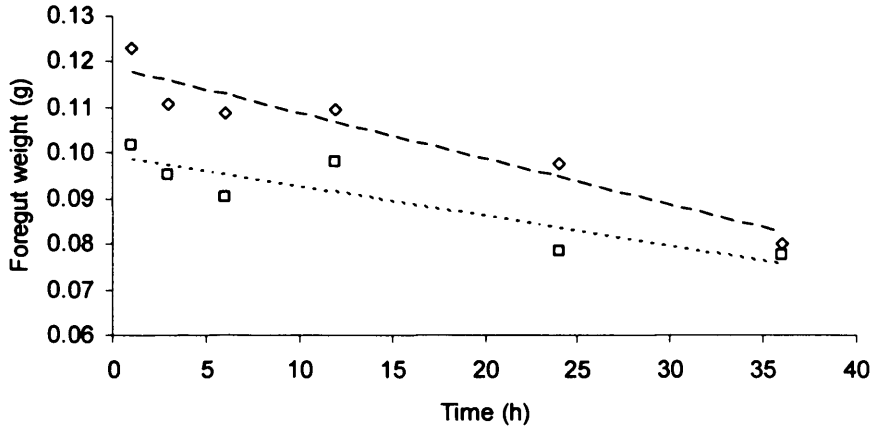


Figure 6.3.9 Weight of the foregut of the beetle *Pterostichus melanarius* against time since feeding on *Deroceras reticulatum* infected with the nematode *Phasmarhabditis hermaphrodita* for 24 h (◊ — —) and 120 h (□ ----). Line represents best fitting linear regression.

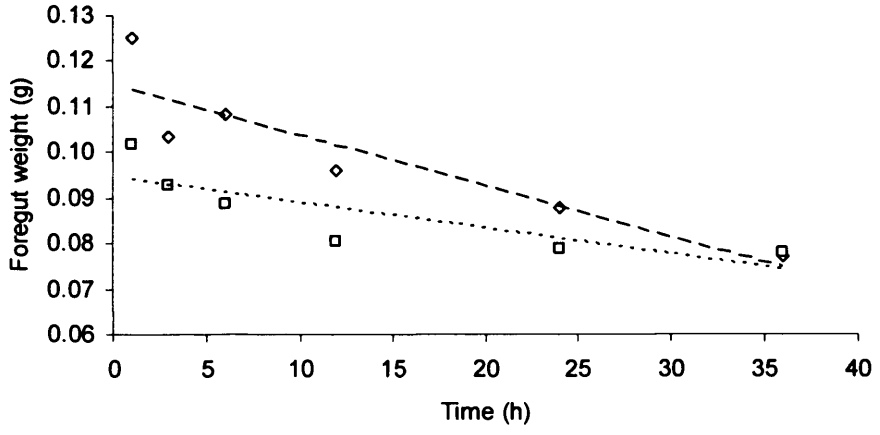


Figure 6.3.10 Weight of the foregut of the beetle *Pterostichus melanarius* against time since feeding on *Galleria mellonella* infected with the nematode *Steinernema feltiae* for 24 h (◊ — —) and 120 h (□ ----). Line represents best fitting linear regression.

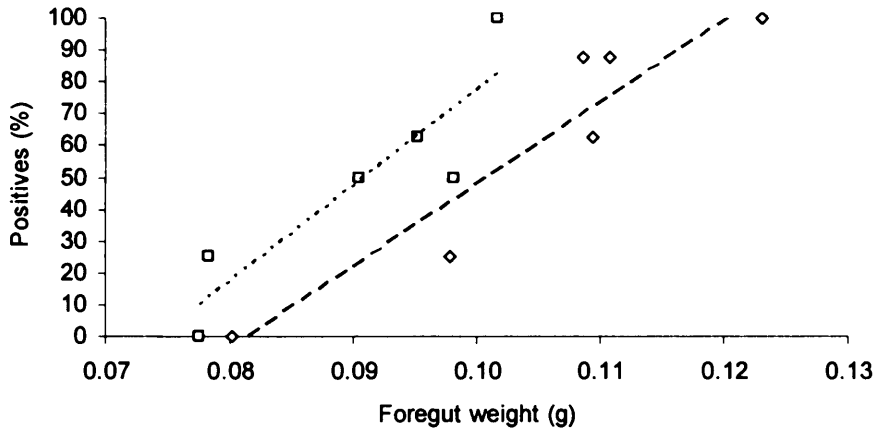


Figure 6.3.11 Percentage of *Pterostichus melanarius* foreguts positives for prey DNA. Detection of *Deroceras reticulatum* against foregut weight after 24 h of infection (◊ — —); detection of *Phasmarhabditis hermaphrodita* DNA against foregut weight after 120 h of infection (□ ----). Lines represent best fitting linear regressions.

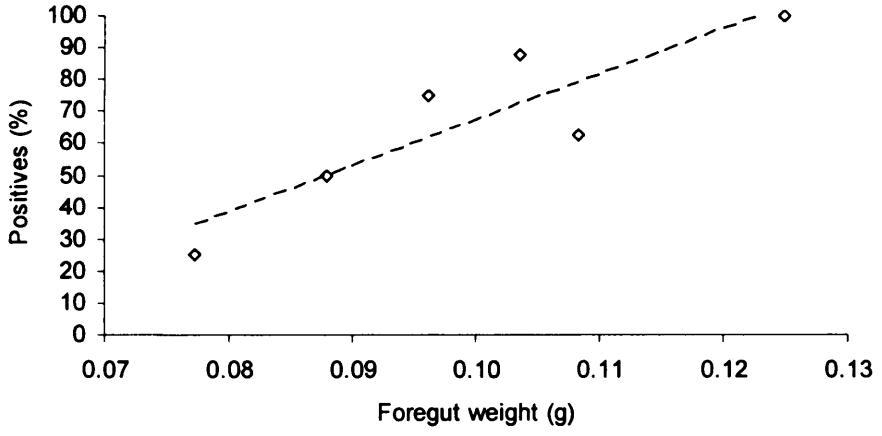


Figure 6.3.12 Percentage of *Pterostichus melanarius* foreguts positives for prey DNA. Detection of *Galleria mellonella* DNA against foregut weight after 24 h of infection by *Steinernema feltiae* (◊ — —). Lines represent best fitting linear regressions.

6.4. Discussion

The use of molecular methods for the detection of parasitism has some similarities to the application of molecular methods for the detection of predation. This is especially true at the early stages of parasitism, where parasite DNA will make up a very small proportion of the total DNA extracted from the host. One aspect that makes the detection of parasitism technically more feasible is that over time the quantity of parasite DNA will increase, either due to an increase in the size of the parasite, or an increase in the numbers of parasites present in the host. The PCR-based methods used in this study were designed to detect DNA from three species of insect- and mollusc-parasitic nematode, in three different nematode-host complexes. In all three complexes, the PCR-based method was able to detect nematode infection in the early stages; between 40 % and 70 % of the hosts were positive for nematode DNA in as little as 1 h after initial nematode exposure (Figure 6.3.1). In contrast, by examining host tissue after pepsin digest, it was not possible to reveal the presence of nematode infection in such a high proportion of individuals at the early stages of infection (0 % of *Deroceras reticulatum* and *Galleria mellonella* individuals, and 30 % *Tenebrio molitor* after 1 h of infection) (Figure 6.3.3). In all cases the number of positives for nematode DNA increased as the time since initial infection increased, reaching an asymptote after approximately 72 h. This increase correlated with time since infection, host mortality (except for *T. molitor* infected hosts) and the average number of nematodes found in the host tissue. The logical explanation for this is that the increasing number of nematodes within the host tissue provide more template DNA, resulting in increased PCR success and quantity of the PCR product.

The ability to detect parasites in the early developmental stages is useful when conducting field surveys to estimate the prevalence of parasite or parasitoid infection (Agusti *et al.* 2005). The majority of previous studies that have attempted to use species-specific primers to detect developing parasites within invertebrate hosts have focussed on Hymenopteran parasitoids (e.g. Suckling *et al.* 2001; Ashfaq *et al.* 2004; Gariepy *et al.* 2005; Chen *et al* 2006) and the detection of malarial causing protozoan parasites associated with mosquitoes (Mharakurwa *et al.* 2006). The detection limits in these systems is also high, and it is possible to detect a single parasitoid egg within the host (Li and Shen 2002). The primers used in this study appear to have a

comparable level of sensitivity, in which it is possible to detect nematode parasites in the early stages of infection.

One limitation when working with large hosts such as beetle larvae, lepidopteran larvae and molluscs is that DNA extraction requires relatively small quantities of tissue (5-20 mg per extraction for the PureGene kit used in this study). Entomopathogenic nematodes often infect relatively large insects (Kaya and Gaugler 1993). Because of this it is not possible to screen the entire host in one DNA extract, as would be possible when working with small hosts. The cost and time consuming nature of DNA extractions makes the division of each host into 5-20 mg sections prohibitive. As a result, the decision was made to extract DNA from the point at which the nematodes were most likely to be found. In the case of the slug-parasitic nematode *Phasmarhabditis hermaphrodita*, the nematodes tend to enter the body cavity through the pneumostome and accumulate in the rear mantle (Tan and Grewal 2001). The entomopathogenic nematode *Steinernema feltiae* and *Heterorhabditis megidis* enter the host through the mouth, spiracles and anus (Kaya and Gaugler 1993). The method of targeting potential nematode entry points proved to be successful in this study, although further investigation might reveal host sites that result in higher detection success.

None of the nematodes tested had any effect on the mortality of the adult *P. melanarius* when compared to the control. Previous research into the non target effects of entomopathogenic nematodes on adult predatory beetles has also found no negative effects. For example, the nematode *Steinernema carpocapsae* did not kill adult carabids *Bembidion properans* and *Pterostichus cupreus* (see Ropek and Jaworska 1994), and applications of *Heterorhabditis bacteriophora, S. feltiae* and *S. carpocapsae* had no effect on the short term abundance of beetles from the families Carabidae, Histeridae and Staphylinidae (Georgis *et al.* 1991). Georgis and Wojcik (1987) conducted a study on the effects of *Heterorhabditis heliothidis* and *S. feltiae* on selected predatory soil insects, including: carabid beetles, staphylinid beetles, cicinelid beetles, and labidurid earwigs. They concluded that the nematode species had little or no effect on various stages of these predatory insects. It has been speculated that adult beetles are largely protected from nematode infection as their bodies do not come in contact with the soil (Bathon 1996) and that the thickness of

the cuticle may offer some level of protection (Georgis *et al.* 1991). In this study, two beetles tested positive for nematode DNA in the foregut, although it is possible that this was due to contamination from nematodes attached to the outside of the beetle during the foregut dissection. The bioassay experiment can be considered a worst case scenario, as the beetles were restricted to soil containing a high number of nematodes for a prolonged period of time. It can therefore be concluded that the potential for direct infection by nematodes on the adult stages of *P. melanarius* is not a significant source of error when screening for predation. Although the nematodes do not cause mortality of adult *P. melanarius*, the effects on the larval stages are currently unknown. Entomopathogenic nematodes are known to infect and kill the larval stages of many species of beetle, including *Phyllopertha horticola* (see Sulistyanto and Ehlers 1996), *Popillia japonica* (see Koppenhofer and Fuzy 2004), *Cyclocephala borealis* (see Simard *et al.* 2001) and *Rhizotrogus majalis* (see Cappaert and Koppenhofer 2003). As a result, further work is needed before any conclusions can be made about the effects of parasitic nematodes on the whole life-cycle of carabid beetles such as *P. melanarius*.

Nematode and host specific primers were used to detect DNA in the foregut of *P. melanarius* after scavenging on nematode infected hosts. In all three nematode-host complexes, at all levels of infection, the ability to detect nematode and host DNA in the foregut of *P. melanarius* declined as time since consumption increased. These results are in agreement with previous studies using species-specific primers to detect prey DNA in the gut of invertebrate predators (Agusti *et al.* 2003; Foltan *et al.* 2005; Harper *et al.* 2005). Detection half-lives for prey DNA in the foregut of *P. melanarius* in this study ranged from 0.79 h to 28.9 h. Comparable results were found by Harper *et al.* (2005) in a previous study using *P. melanarius*, with half-lives of 9.7 h for the aphid *Sitobion avenae*, 31.0 h for the slug *Arion hortensis* and up to 88.5 h for the earthworm, *Allolobophora chlorotica*. Using the same primers, the detection time for *D. reticulatum* in the present study was found to be 8.8 h to 16.11 h, shorter than the 26.3 h found by Harper *et al.* (2005). This may be due to the fluorescent primer and sequencer approach used for fragment analysis by Harper *et al.* (2005), resulting in higher assay sensitivity.

The decline in the ability to detect the prey DNA in the host is likely to be caused by two mechanisms. The first is that the digestive processes in the gut cause the DNA

fragments to be broken up, and no longer act as suitable template for PCR. Evidence for this has been found in the fact that primers amplifying smaller fragments of DNA are able to detect target DNA in the gut for longer (Agusti *et al.* 1999; Zaidi *et al.* 1999; Chen *et al.* 2000; Hoogendoorn and Heimpel 2001). It has also been shown in sea lion faecal samples, using quantitative PCR (qPCR), that smaller fragments of DNA are present in higher numbers than larger fragments (Deagle *et al.* 2006). The second possible reason for the decline prey detection over time is due to the physical expulsion of food from the foregut into the midgut. The weight of the foregut did decline in all of the feeding trials, except for when the beetles were fed *D. reticulatum* infected with *P. hermaphrodita* for 216 h and *G. mellonella* infected with *S. feltiae* for 216 h. The lack of a negative correlation between time and foregut weight in these cases may be due to the lower weight of food that was initially consumed, causing natural variation in foregut weight to mask any trends. If the weight of food in the foregut was responsible for the ability to detect prey DNA, it would be expected that a correlation between foregut weight and the number of positives would be observed. This was the case in three of the prey screenings, but not in the other nine. These results do not provide conclusive evidence for one mechanism over the other. It is possible that this is because both mechanisms are responsible for the decline in DNA detectability, and variations in prey composition and predator physiology will determine their relative importance.

It was hypothesised that the ability to detect parasite DNA in the gut of the predator would increase as time since the initial infection of the consumed host increased. This was the case, as the median detection times of *P. hermaphrodita* (0.8 h to 15.4 h) and *S. feltiae* (1.9 h to 28.9 h) increased as the beetles consumed hosts that had been infected for longer periods of time. The ability to detect nematode DNA directly from the host also increased as time since infection increased, and it is likely that the same reasons for increase detection within the host are also responsible for increased detection in the gut i.e. increased numbers of nematodes within the host organism resulting in more nematodes ingested by the predator, and increased levels of template DNA for PCR.

It was predicted that that the decay of the host DNA during infection and subsequent death would cause a decline in the ability to detect the host DNA in the gut of the

consumer. This pattern was broadly observed with both nematode-host systems in this study, although the reason for the lack of a steady decline in detection success as time since infection increased is unknown. Foltan *et al.* (2005) examined the effects of decay on the ability to amplify *D. reticulatum* DNA both directly from the cadaver and in the gut of an invertebrate predator that had consumed slug cadavers. In this study, decay was found to have a significant effect on detection success from the cadaver and in the gut of the scavenger. In the present study, investigation into the effects of the time of decay for the nematode-infected cadaver is confounded by the fact that the exact time of mortality was not known. *Phasmarhabditis hermaphrodita* typically kills its host within 5-10 days (Glen and Wilson 1997), and during this time slugs undergo a period of torpor in which determination of the exact time of death is difficult. It is likely that the rate of decay is higher in slug cadavers infected with nematodes than uninfected cadavers, due to the introduction of the symbiotic bacteria directly into the slug haemocoel. These factors have consequences for the analysis of field data, as the level of infection (time since initial infection) of the consumed hosts will result in a change in the proportion of beetles testing positive for consumption of infected hosts. Sampling of a suitably large number of predators over a range of time points should prevent such biases from affecting data interpretation.

The persistence of introduced biological control agents is an important factor in determining their effectiveness at controlling pest populations (Koppenhofer and Fuzy 2007). Although a number of abiotic factors have been implicated in reducing the short term persistence of nematodes in the soil (Smits 1996), the ability to reproduce in the host is vital for longer term persistence (Georgis *et al.* 2006). Understanding the factors that influence parasite survival in the host is important for developing strategies for pest control. The molecular methods developed in this study can be used to determine rates of predation on infected hosts in the field, something that is not possible using traditional methods. If intraguild predation on nematode parasitized prey is found to be prevalent in agroecosystems, it may be a significant factor in influencing nematode persistence and hence pest control.

Chapter 7

The role of infection in altering tritrophic interactions between invertebrate predators and nematode infected prey

Abstract

Intraguild predation on parasitized prey is thought to be a potentially important process in determining the population dynamics of many parasite species. The impact of this type of intraguild predation may also have an important role in the persistence and success of introduced biological control agents in the field. However, many parasites have developed methods for avoiding predation whilst in the host. This study describes a set of choice-test feeding trials used to determine the response of a generalist predator, the carabid beetle *Pterostichus melanarius*, to three different species of parasitic nematode in three host species. Strong feeding inhibition was observed when beetles were presented with the entomopathogenic nematode *Heterorhabditis megidis* in its coleopteran host *Tenebrio molitor*, whereas the response was less pronounced in steinernematid and phasmarhabditid infected hosts. The feeding inhibition was found to increase as time since initial infection increased. The use of live, rather than pre-killed phasmarhabditid infected slugs resulted in a change from the previously observed results, where carabids preferred to feed on infected over uninfected hosts. This indicated that host defence may play an important role in prey selection. Finally, a field trial was used to determine the fate of infected cadavers in an agroecosystem which is diverse in predators. Patterns of infected host persistence and damage in the field were in agreement with the earlier laboratory-based choice-test results.

7.1. Introduction

Intraguild predation (IGP) is an important process in determining the abundance of both predators and parasites in ecosystems (Rosenheim 1995). Where predators or parasites preferentially feed on or parasitize members of their trophic level, rather than herbivores, their populations may be reduced, causing a subsequent reduction in the top down suppression of herbivore populations. This may cause an increase in herbivore numbers, and through cascade effects, cause a reduction in crop yield or productivity. This type of IGP has been recorded disrupting the biological control of invertebrate pests in agroecosystems (Croft and MacRae 1992; Colfer and Rosenheim 2001; Powell and Webster 2004; Rosenheim 1999; 2005).

Parasites may cause a range of different responses in predators that feed on the host species, with rates of predation being higher, lower, or showing no change (Brodeur and Boivin 2004). Parasitism is generally assumed to weaken the host in a number of ways, including premature or arrested pupation (Pennacchio and Strand 2006), changes in metabolism (Haspel *et al.* 2005), selection of specific habitats (Brodeur and McNeil 1992; Chow and Mackauer 1999), changes in feeding behaviour (Thompson and Redak 2005), and changes in the period of activity and foraging patterns (Sunderland 1996). All these responses to parasitism may change the nature of the trophic interactions that the host normally engages in, resulting in modified food-web dynamics (Hatcher *et al.* 2006). Many predators will prefer to attack weakened or dead prey due to increased handling efficiencies (Lang and Gsodl 2001) potentially resulting in predation levels on parasitized prey being higher than on unparasitized prey (Coyner *et al.* 2001). A number of studies have investigated the role of IGP on parasite success, for example, Jones (1987) demonstrated that predation levels by ants from the genus *Iridomyrmex* were higher on cabbage butterfly caterpillars, *Pieris rapae*, when parasitized by the brachonid parasite *Apanteles glomeratus*. Despite this example, relatively few studies have specifically examined the role of parasitism on predation choices by invertebrates. In studies with vertebrates, an insectivorous bird, *Agelaius phoeniceus*, showed no feeding preference when given a choice of fall armyworms, *Spodoptera frugiperda*, either unparasitized or parasitized with the ectoparasitoid *Euplectrus plathypenae* (see Jones *et al.* 2005). Arctic charr (*Salvelinus alpinus*), however, have been observed

preferentially feeding on *Gammarus lacustris* infected with the helminth *Cystidicola farionis* (see Knudsen *et al.* 2001), and it has been shown that predators selectively prey on red grouse, *Lagopus lagopus scoticus*, that are infected by a nematode parasite (Hudson *et al.* 1992).

For parasites to persist and recycle in the environment, it is necessary that predation on infected hosts occurs at a level that allows a proportion of the parasite population to survive and complete the life cycle. The evolution of predator avoidance is thought to be a common response to predation risk (Brodie *et al.* 1991) and natural selection will promote the evolution of parasite behaviours that result in reduced predation (Brodeur and Boivin 2004). Potential methods of predator avoidance include reliance on host defense (Slansky 1986), host behaviour manipulation (Poulin 1995) and the production of antifeedant chemicals (Baur *et al.* 1998). Host behavioural changes have been observed in many parasites that use intermediate hosts, as a way of increasing the probability of transmission (Hechtel *et al.* 1993). Changes in behaviour that can reduce rates of predation include changes in activity levels and changes in habitat preference (Brodeur and Boivin 2004). Idiobiont parasites, which have the strategy of quickly immobilizing the host after attack, may exploit natural host camouflage to remain inconspicuous (Hawkins *et al.* 1990). Koinobiont parasites, which have a longer term association with the host, tend to preserve host functioning to exploit natural host defenses (Slansky 1986).

Entomopathogenic and mollusc parasitic nematodes are commonly used as biological control agents to control a wide range of invertebrate pests including winter moths (Tomalak 2003), white grubs (Koppenhofer *et al.* 2004), leatherjackets (Peters and Ehlers 1994), scarabid beetles (Ansari *et al.* 2003), thrips (Chyzik *et al.* 1996) and slugs (Wilson *et al.* 1993). Whilst research has focused the short term abiotic factors that influence nematode persistence (Grant and Villani 2003: Glazer 1996: Hass *et al.* 1999), very few studies have investigated the longer term factors that determine the ability of nematodes to recycle in the soil ecosystem. Once such factor is the ability to reproduce and survive within the host cadaver. Nematodes typically take between five and ten days to reproduce within the host (Kaya and Gaugler 1993), leaving the nematodes potentially vulnerable to predators and scavengers during this time.

Evidence of IGP involving parasitic nematodes is scarce, and most research has focused on the interaction between entomopathogenic nematodes and parasitoids. Lacey *et al.* (2003) found that the nematode *Steinernema feltiae* would infect and kill the early stages of the ichneumonid parasitoids *Mastrus ribundus* and *Liotryphon caudatus*, but not during the later parasitoid stages, when the parasitoids were protected by a tightly woven cocoon. Adult parasitoids avoided ovipositing in nematode-infected codling moth larvae as early as 12 h post-infection, allowing these introduced agents to work synergistically. Similar results were found with the combined use of the nematode *Steinernema carpocapsae* and the eulophid parasitoid wasp *Diglyphus begini* for the control of the leafminer *Liriomyza trifolii* (see Sher *et al.* 2000). However, an antagonistic interaction was observed between the nematodes *S. carpocapsae* and *Heterorhabditis bacteriophora*, where both species were capable of reducing the rates of adult emergence of the aphid predator *Aphidoletes aphidimyza* (see Powell and Webster 2004).

Research into the interactions between nematode infected cadavers and predators have received even less attention. This is surprising, as the role of the host is essential in the life-cycle of parasitic nematodes, and there have been many publications highlighting the need to understand the failures of parasitic nematodes to persist in the environment (Glazer 1996; Wilson and Gaugler 2004; Georgis *et al.* 2006; Stuart *et al.* 2006). Baur *et al.* (1998) examined the role of foraging ants as scavengers of nematode killed insects. They found that significantly more steinernematid-killed hosts (60-85%) were scavenged than heterorhabditid-killed hosts (10-20%) in the field. They also found that the symbiotic bacteria associated with heterorhabditid nematodes, *Photorhabdus luminescens*, were responsible for the protection from scavenging. This research was expanded upon by Zhou *et al.* (2002) who investigated the nature of the 'deterrent factor' produced by the bacteria, and found it to be a small, extracellular and possibly nonproteinaceous compound capable of deterring ants from feeding on a sucrose solution.

Using behavioural experiments, the following hypothesis was tested: that infection by the mollusc- and insect-parasitic nematodes *Phasmarhabditis hermaphrodita* (Rhabditida: Rhabditidae), *Steinernema feltiae* (Rhabditida: Steinernematidae), and *Heterorhabditis megidis* (Rhabditida: Heterorhabditidae) would alter the feeding

choices of a generalist invertebrate predator, the carabid *Pterostichus melanarius* (Coleoptera: Carabidae). Choice-tests were conducted to examine the influence of the time since infection of the host organism, and the effect of prey and nematode species on predator feeding preferences. Further choice tests were conducted to examine the effect of prey health status on feeding choices. No-choice tests were used to examine the palatability of nematode infected host cadavers. Finally, field trials were conducted to study the effects of nematode infection on the persistence of host cadavers in the field.

7.2. Materials and Methods

7.2.1. Feeding choices by carabid beetles on pre-killed nematode-infected prey and non-infected prey

Parasitism by nematodes may not only affect the behaviour of the host; it may influence the behaviour of predators and scavengers of infected hosts. To investigate the effects of nematode infection of insects and molluscs on the scavenging choices of the carabid beetle *Pterostichus melanarius*, the following experiment was carried out. Three feeding choice experiments utilized three different host species, *Deroceras reticulatum*, *Tenebrio molitor* and *Galleria mellonella*. These were infected with three different parasitic nematode species, *Phasmarhabditis hermaphrodita*, *Heterorhabditis megidis* and *Steinernema feltiae*, respectively. Slugs fell within the weight category of 100 - 150 mg, as it is known *P. melanarius* will actively predate on slugs this size (Mckemey *et al.* 2001); mealworms weighed 100-200 mg, and waxworms 100-150 mg. The hosts were infected following the procedures in Chapter 6 (Section 6.2.1). The slugs were infected and maintained at 16 ± 1 °C, and the mealworms and waxworms were infected and maintained at 20 ± 1 °C. Twenty hosts were removed at 24 h, 72 h, 120 h, 168 h, and 216 h post-infection and stored at -80 °C. Equal numbers of control (uninfected) individuals of all three host species were also stored at -80 °C.

Carabid beetles, *P. melanarius*, were collected from the field and maintained as described in Chapter 2, Section 2.1.1. The beetles were maintained in a reversed night-day (16 h light: 8 h dark) temperature controlled (16 ± 1 °C) room. The carabids were all fed one *Calliphora* larvae, and then starved for seven days before the start of the feeding trial, to ensure an approximately equal nutritional state in all individuals. The feeding trial commenced at the start of the night period, as this is when beetle activity is highest (Lövei and Sunderland 1996). Ten male and ten female beetles were placed individually in 90 mm Petri dishes with a layer of damp filter paper (Fisher Scientific, Leicestershire, UK) as substrate. Nematode infected and uninfected control hosts from each of the three species were left for a period of 1 h at room temperature to thaw before the start of the experiment. Each host was weighed to the nearest mg before proceeding. The beetles were then presented simultaneously with

two food options, one at either side of the Petri dish, ca. 1.5 cm from the edge, consisting of either an uninfected host or an infected host (Figure 7.2.1). Every 10 min for a period of 1 h the beetles were examined and the choice of prey for feeding (on either infected or uninfected host), or absence of feeding was recorded. The experiment was conducted using a choice of uninfected prey vs. hosts that had been infected for 24 h, 72 h, 120 h, 168 h, or 216 h, with 20 replicate trials (10 male and 10 female) for each level of infection. A control consisted of a choice of uninfected vs. uninfected hosts.

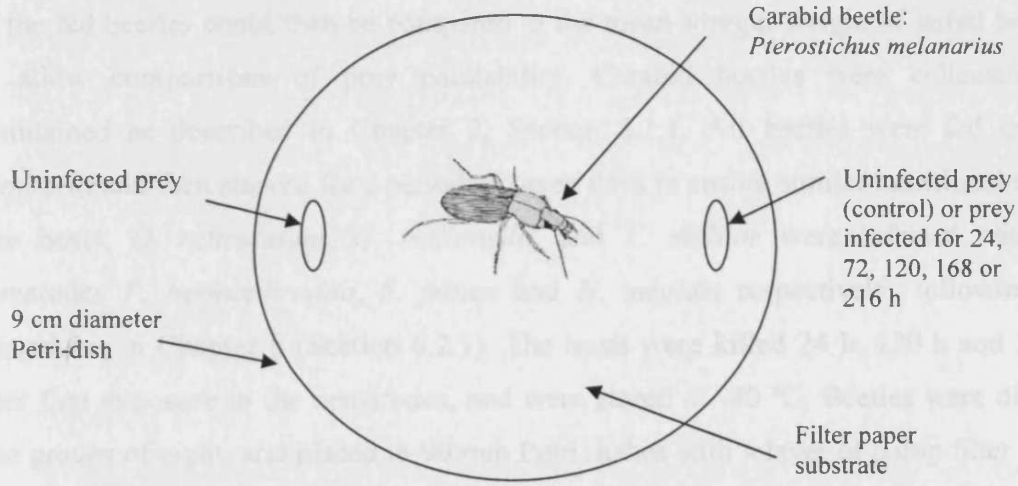


Figure 7.2.1 Experimental set-up used for choice-tests with the carabid *Pterostichus melanarius* and a choice of uninfected prey, or prey infected for 24, 72, 120, 168 or 216 h.

In the second experiment, the methodology was repeated using the *D. reticulatum* and *P. hermaphrodita* host-parasite system, but instead of using pre-killed hosts for the choice tests, the slugs were alive and at different time periods post-infection. Because of this, slugs in the early stages of infection were alive and not noticeably affected by the nematodes, and slugs at the later stages of infection were either moribund and infected by second generation nematodes, or dead. This reflects the natural state of the infected host organisms that occurs in the field, and allows the effect of reduced fitness due to nematode infection to be included as a factor influencing carabid prey choice. As before, prey choice experiments were carried out in 90 mm Petri dishes (Figure 7.2.1). For slug choice experiments, the sides and lids of the Petri-dishes were

painted with Fluon to prevent the slugs escaping from the beetles, and the floor of the containers were divided in half using a 1.5 cm band of Fluon to prevent infected slugs coming in contact with non-infected slugs. Carabid beetles are able to cross this band with no hindrance, whereas slugs are not (see Mckemey *et al.* 2001). The choice experiment was carried out as described in Section 7.2.1.

7.2.2. No-choice feeding trials

To investigate the palatability of nematode infected hosts to the beetle *P. melanarius*, feeding trials were carried out with no choice of prey. The mean weight of the foregut of the fed beetles could then be compared to the mean foregut weight of unfed beetles, to allow comparisons of prey palatability. Carabid beetles were collected and maintained as described in Chapter 2, Section 2.1.1. All beetles were fed one *C. vomitoria* and then starved for a period of seven days to ensure similar nutritional states. The hosts, *D. reticulatum*, *G. mellonella*, and *T. molitor* were infected with the nematodes *P. hermaphrodita*, *S. feltiae* and *H. megidis* respectively, following the procedures in Chapter 6 (Section 6.2.1). The hosts were killed 24 h, 120 h and 216 h after first exposure to the nematodes, and were stored at -80 °C. Beetles were divided into groups of eight, and placed in 90 mm Petri dishes with a layer of damp filter paper as substrate. Each group was fed one of the nematode infected hosts, at one of the levels of infection. The control group consisted of unfed beetles (3 species of host x 4 infection periods = 12 groups: 12 groups x 8 replicates per group = 96 beetles). The beetles were allowed to feed for 2 h, and then killed by freezing at -80 °C, 1 h after the midpoint of the feeding period. The foregut of the beetles was dissected and removed as described in Chapter 6, Section 6.2.4. The foreguts were placed in pre-weighed 1.5 ml centrifuge tubes, which were then re-weighed to determine the weight of the foregut.

7.2.3. Persistence of infected prey in the field

To investigate the influence of parasitic nematode infection on the persistence of arthropod cadavers in the field, the three hosts (*D. reticulatum* 100 - 150 mg, *T. molitor* 100-200 mg and *G, mellonella* 100-150 mg) were infected with their respective nematode parasites (*P. hermaphrodita*, *H. megidis* and *S. feltiae*) as described previously. Hosts were removed and killed at three time points (24 h, 120 h

and 216 h after initial exposure to the nematodes) and stored at – 80 °C. A control group consisted of uninfected hosts, also stored at – 80 °C. The field site was at Burdens Farm, Wenvoe, nr. Cardiff, in a harvested field of wheat on the 21st of September 2006. The trial was carried out at three sites within the field, with 20 m between sites, allowing all three host-nematode cadaver groups to be tested simultaneously. At each site, 40 nematode infected cadavers of one species (i.e. at Site 1; ten *D. reticulatum* at each of the three levels of infection plus ten uninfected *D. reticulatum*) were randomly assigned to points on a grid with 1 m between points. Cadavers were placed in the field in the evening, between 7-9 pm. After 24 h, the cadavers were checked for presence or absence at the placement site. Cadavers that were still present were examined for signs of consumption or damage, and were recorded as either whole, or partially consumed. The experiment was repeated twice, on the 28th Sept, and the 5th Oct. The site at which each trial was carried out was alternated to remove any effects of site on cadaver persistence.

7.2.4. Statistical Analysis

Choice feeding trials

The G-Test for independence, also known as the likelihood ratio test, was used to compare between observed and expected frequencies, based on the multinomial probability distribution.

Where:

$$G = 2 \times \sum \left[Observed \times \ln\left(\frac{Observed}{Expected} \right) \right]$$

The G-Test, conducted in Microsoft Excel, was used to test for significant differences between the feeding choices of the carabid *P. melanarius*. This was initially used to test between all three feeding choices (number of incidents per hour feeding on nematode infected prey, on uninfected prey, and not feeding), where the null hypothesis of no significant difference between feeding choices was used. As the number of 'not feeding' incidents was high in many cases, potentially obscuring any significant differences on the actual prey choice, a second analysis was run excluding these data (number of

incidents per hour feeding on nematode infected prey vs. number of incidents per hour feeding on uninfected prey).

To examine the effect of prey weight on *P. melanarius* feeding choice, the feeding choices in each of the controls (uninfected host vs. uninfected host) in relation to the weight of the prey was analysed. The difference in weight between the two hosts in each feeding arena was plotted against the difference in the number of feeding incidents per hour for each of the 20 control beetles (weight of host 1 – weight of host 2 vs. number of feeding incidents on host 1 – number of feeding incidents on host 2). If weight played a significant factor in prey choice, a trend between weight difference and choice difference would be observed.

To examine whether gender of the carabid predators played a role in prey selection, the number of feeding incidents on infected prey was compared between males and females using two sample T-tests for normally distributed data, and the non-parametric Mann-Whitney U-test for data that could not be normalised after transformation, using MINITAB 14. Analysis of feeding trials using infected *T. molitor* and *G. mellonella* was not carried out at the later stages of infection due to a lack of feeding incidents on infected prey.

The number of feeding incidents on infected *D. reticulatum* was compared between the feeding trial using dead *D. reticulatum*, and the feeding trial using live *D. reticulatum*. As the data were not normally distributed, and could not be normalised after transformation, the non-parametric Mann-Whitney U-test in MINITAB 14 was carried out to compare the number of feeding incidents two feeding trials.

No-choice feeding trials

In the no-choice feeding trials, the weight of the foregut of eight beetles, 1 h after the midpoint of each of the feeding trials was compared using ANOVA on MINITAB 14. Where significant differences were observed, Fishers *a priori* test were used to perform pair-wise comparisons of the mean of the foregut weight for each of the groups. Where the data was not normally distributed, the non-parametric Kruskal-Wallis test was used to compare the medians, and the non-parametric Mann-Whitney test was used to do pair-wise comparisons of the means. The significance level of the Mann-Whitney test

was reduced by using a Bonferroni correction (P = 0.05/n to P = 0.0083), to reduce type I error.

Field trials

Chi-squared goodness of fit tests were used to compare replicate results (i.e. results from the same category (e.g. number of *D. reticulatum* at 24 h of infection that had been damaged) from the three different sites on the three different dates). Data on the persistence of the infected hosts was recorded simultaneously at three sites in the field, and on three different dates. No significant difference was observed between the replicates ($P \geq 0.05$) allowing data from the three days and three sites to be pooled for comparison between treatments.

Chi-squared contingency tables were used to test the null hypothesis that time since initial infection was not associated with the total number of cadavers that were absent, damaged or intact after placement in the field. As the number of incidents in some categories was below five, the G-statistic was adjusted to correct for small sample sizes, using the method in Gotelli and Ellison (2004) proposed by Williams (1976), where $G_{adjusted} = G/q_{min}$. Where:

$$q\min = \frac{1 + \left[N \sum_{i=1}^{n} \frac{1}{\sum_{j=1}^{m} Y_{i,j}} - 1 \right] x \left[N \sum_{j=1}^{m} \frac{1}{\sum_{i-1}^{n} Y_{i,j}} - 1 \right]}{6vN}$$

Where: m = the number of columns, n = the number of rows, N = the total sample size, and v = the degrees of freedom. $Y_{i,j}$ represents the frequency of observations in (row i, column j) of the contingency table. This creates a more conservative P-value, reducing potential error from the use of small sample sizes and expected values. Where significant associations were observed, simple correspondence analysis (CA) was used to look for associations between time since infection and the fate of the infected cadaver in the field.

Chi-squared contingency tables, run in Microsoft Excel, were used to test the null hypothesis that species of nematode and host were not associated with the fate of the cadaver in the field. The G-statistic was again adjusted to correct for small sample sizes, using the method proposed by Williams (1976), where $G_{adjusted} = G/q_{min}$. Chi-squared contingency tables tests were conducted for each of the four levels of infection that were presented in the field (control, 24 h, 120 h and 216 h). Where significant associations were recorded, simple correspondence analysis (CA) was used to check visually for an association between species of nematode and host, and the total number of cadavers that were removed, damaged or intact in the field.

7.3. Results

7.3.1 Feeding choices by carabid beetles on pre-killed nematode-infected prey and non-infected prey

The feeding choices of *Pterostichus melanarius* on *Deroceras reticulatum*, either uninfected, or infected with *Phasmarhabditis hermaphrodita* was initially conducted including all three feeding choices. This resulted in only two of the feeding trials, those containing slugs infected for 72 h and 216 h, having a significant difference between feeding choices (*G-Test*: 72 h: $G = 8.60$, $df = 2$, $P < 0.05$. 216 h: $G = 11.94$, $df = 2$, $P < 0.05$). In all other feeding trials no significant difference was observed in feeding choice (*G-Test*: $P \geq 0.05$). (Figure 7.3.1)

In a subsequent analysis excluding the 'not feeding' data, infection by *P. hermaphrodita* on *D. reticulatum* had a significant effect on the scavenging behaviour of *P. melanarius*. At the early stages of infection (uninfected vs. 24 h post infection) and the control (uninfected vs. uninfected), no effect was observed on the choice of feeding on uninfected or infected prey (*G-Test*: $P \geq 0.05$). In three of the feeding trials, those containing *D. reticulatum* infected for 72 h, 120 h and 216 h as the alternative choice to uninfected slugs, there was a significant preference for feeding on uninfected slugs rather than nematode infected *D. reticulatum* (*G-Test*: 72 h: $G = 4.231843$, $df = 1$, $P < 0.05$. 120 h: $G = 4.498587$, $df = 1$, $P < 0.05$. 216 h: $G = 9.604396$, $df = 1$, $P < 0.05$). The total number of feeding incidents per hour on infected prey was compared against the level of the infected prey (= hours since initial infection) by using linear regression analysis. No significant correlation was observed ($P \geq 0.05$).

The feeding choices of *P. melanarius* on *Tenebrio molitor*, either uninfected, or infected with *Heterorhabditis megidis* was initially conducted including all three feeding choices. All trials showed highly significant differences in the number of feeding incidents per hour (*G-Test*: $P < 0.001$) (Figure 7.3.2).

A subsequent analysis, excluding the 'not feeding' showed no significant differences between feeding incidents on infected or uninfected *T. molitor* in the control

(uninfected vs. uninfected) and the feeding trial with the first level of infection (uninfected vs. 24 h) (*G-Test*: $P \geq 0.05$). In the next four feeding trials, with *T. molitor* infected for 72 h, 120 h, 168 h and 216 h, the carabids showed a highly significant preference for feeding on uninfected, rather than nematode infected *T. molitor* (*G-Test*: 72 h: G = 44.27347, df = 1, $P < 0.001$; 120 h: G = 99.82886, df = 1, $P < 0.001$; 168 = G = 135.1995, df = 1, P = $P < 0.001$; 216 h: G = 45.71752, df = 1, $P < 0.001$). A significant negative correlation ($F_{1,4} = 7.82$, $P < 0.05$) between level of infection (= hours since initial infection) and the total number of feeding incidents per hour on infected prey was found using linear regression analysis ($y = 49.6 - 0.239 x$, $R^2 = 0.66$).

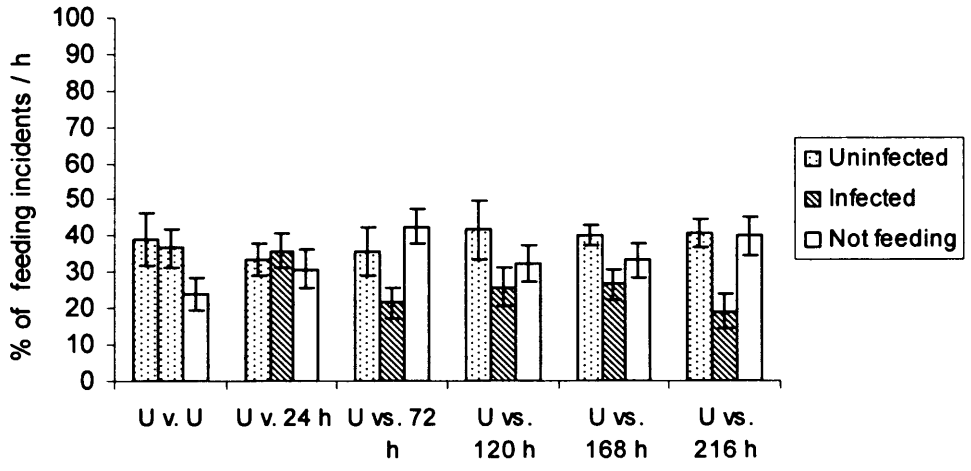


Figure 7.3.1 Percentage of feeding incidents per hour by *Pterostichus melanarius* in feeding trials with a choice of either pre-killed uninfected *Deroceras reticulatum* or pre-killed *D. reticulatum* infected with the nematode *Phasmarhabditis hermaphrodita*. Graph shows the results of six feeding trials with six different levels in infection. (U = uninfected).

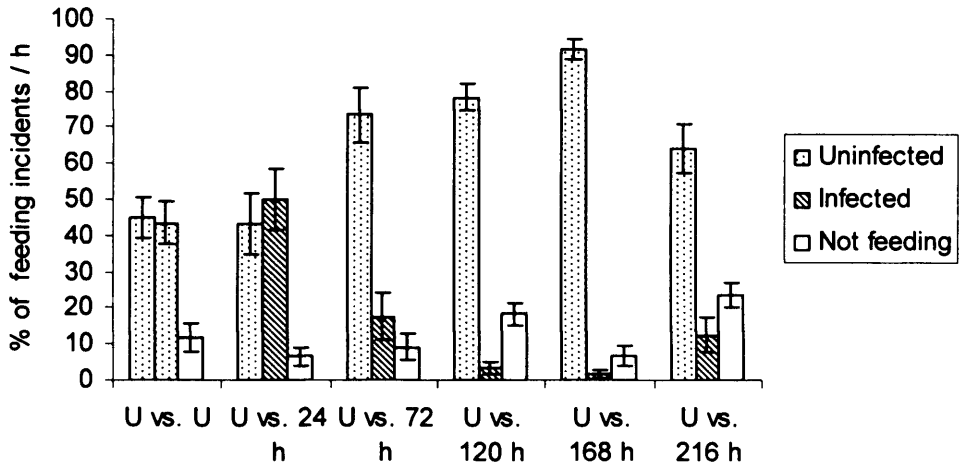


Figure 7.3.2 Percentage of feeding incidents per hour by *Pterostichus melanarius* in feeding trials with a choice of either pre-killed uninfected *Tenebrio molitor* or pre-killed *T. molitor* infected with the nematode *Heterorhabditis megidis*. Graph shows the results of six feeding trials with six different levels in infection. (U = uninfected).

The feeding choices of *P. melanarius* on *Galleria mellonella*, either infected with the nematode *S. feltiae* or uninfected, was initially conducted including all three feeding choices . All trials showed highly significant differences in feeding incidents per hour (*G-Test*: $P < 0.001$) (Figure 7.3.3).

Subsequent tests excluding the 'not feeding' data were conducted for each feeding trial. The control (uninfected vs. uninfected) and the feeding trial including the first level of infection (uninfected vs. 24 h) resulted in no significant difference in feeding choices (*G-Test*: $P \geq 0.05$). In the next four feeding trials with *G. mellonella* infected for 72 h, 120 h, 168 h and 216 h, the beetles showed a significant preference for feeding on uninfected, rather than nematode-infected, *G. mellonella* (*G-Test*: 72 h: $G = 61.24896$, $df = 1$, $P < 0.001$; 120 h: $G = 80.97413$, $df = 1$, $P < 0.001$; 168 = $G = 74.37206$, $df = 1$, $P < 0.001$; 216 h: $G = 86.55179$, $df = 1$, $P < 0.001$). A significant negative correlation ($F_{1,4} = 9.67$, $P < 0.05$) between level of infection (hours since initial infection) and the total number of feeding incidents per hour on infected prey was found using linear regression analysis ($y = 46.5 - 0.211\ x$, $R^2 = 0.71$).

Feeding trials were conducted involving the carabid *P. melanarius* and a choice of live *D. reticulatum* either uninfected, or infected with the nematode *P. hermaphrodita* are shown (Figure 7.3.4). in the analysis using all three feeding choices, all trials showed highly significant differences in feeding incidents per hour (*G-Test*: $P < 0.001$).

Subsequent tests excluding the 'not feeding' data were also carried out for each feeding trial. In the control (uninfected vs. uninfected) and feeding trials including *D. reticulatum* infected for 24 h and 72 h there was no significant difference in feeding choices (*G-Test*: $P \geq 0.05$). In trials with *D. reticulatum* infected for 120 h, 168 h and 216 h, the beetles showed a highly significant preference for feeding on infected *D. reticulatum* rather than uninfected *D. reticulatum* (*G-Test*: 120 h: $G = 14.48594$, $df = 1$, $P < 0.001$; 168 = $G = 85.57543$, $df = 1$, $P = P < 0.001$; 216 h: $G = 57.31195$, $df = 1$, $P < 0.001$). A significant positive correlation between level of infection (hours since initial infection) and mean feeding incidents per hour on infected prey was found using linear regression analysis ($y = 14.3 + 0.259\ x$, $R^2 = 0.72$, $P < 0.05$).

The number of feeding incidents on nematode infected *D. reticulatum* was compared between the feeding trial using dead *D. reticulatum*, and the feeding trial using live *D. reticulatum*. In some of the feeding trial using live slugs, the beetles spent significantly more of their time not feeding, than in the feeding trials using dead slugs (feeding trials using slugs infected for 24 h, 72 h and 120 h (Mann Whitney: $P < 0.001$)). In some of the live-slug feeding trials, the beetles spent significantly more time feeding on infected *D. reticulatum* than the dead-slug feeding trials (those using slugs infected for 168 h and 216 h (Mann Whitney: $P < 0.001$)).

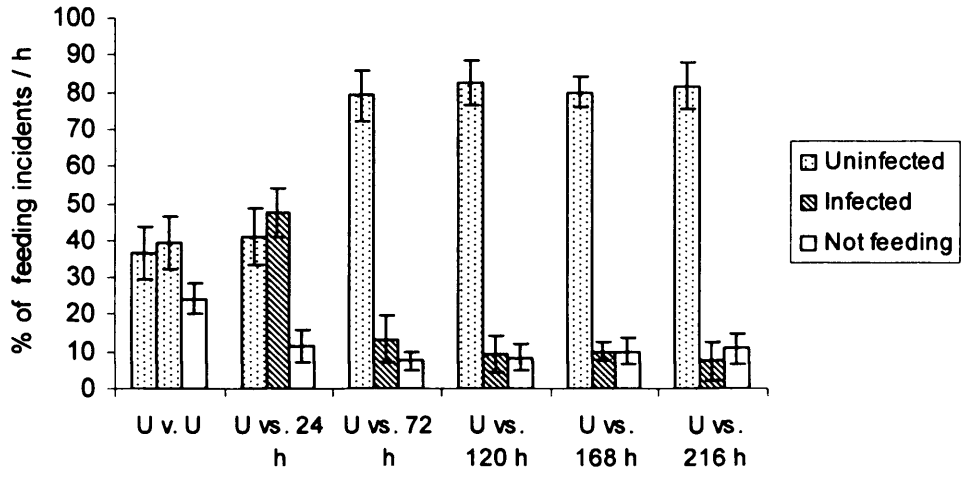


Figure 7.3.3 Percentage of feeding incidents per hour by *Pterostichus melanarius* in feeding trials with a choice of either pre-killed uninfected *Galleria mellonella* or pre-killed *G. mellonella* infected with the nematode *Steinernema feltiae*. Graph shows the results of six feeding trials with six different levels in infection. (U = uninfected).

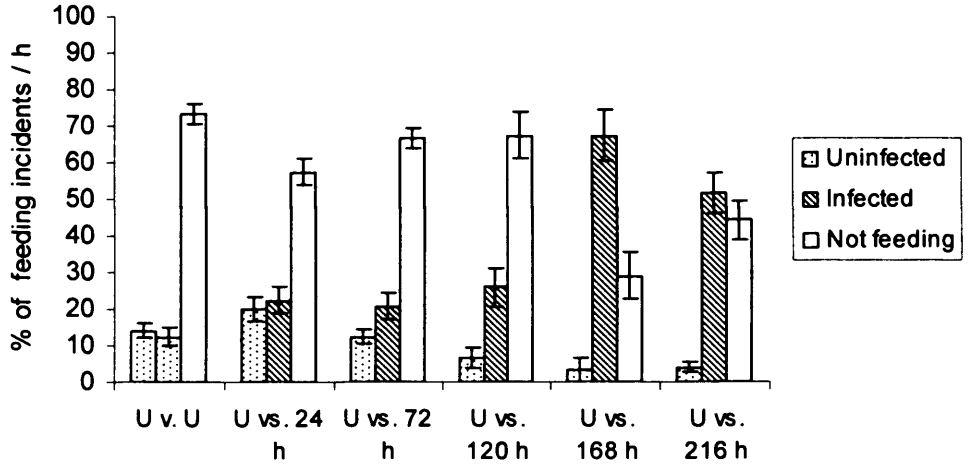


Figure 7.3.4 Percentage of feeding incidents per hour by *Pterostichus melanarius* in feeding trials with a choice of either live uninfected *Deroceras reticulatum* or *D. reticulatum* following the natural progression of infection with the nematode *Phasmarhabditis hermaphrodita*. Graph shows the results of six feeding trials with six different levels in infection. (U = uninfected).

No significant correlations were observed between the weight of the prey in the control feeding trials, and the number of feeding incidents on the prey (Linear regression: $P \geq 0.05$). This indicated that prey weight did not play a significant role in prey choice in the choice arenas used in this study.

Only in one feeding trial, a choice of uninfected live *D. reticulatum* vs. uninfected live *D. reticulatum*, did gender play a significant role in feeding choice (Mann-Whitney: $W = 70.5$, $P < 0.05$) (Table 7.3.1). In all other feeding trials, no effect of gender on feeding choice was detected (Mann-Whitney and T-Test: $P \geq 0.05$).

Table 7.3.1 Students T-Test (TT) and Mann-Whitney (MW) tests to examine the role of beetle gender in cadaver/prey choice. Un = uninfected. N/A indicates a lack of sufficient data for analysis. Highlighted row indicates significant correlation between gender and feeding choice.

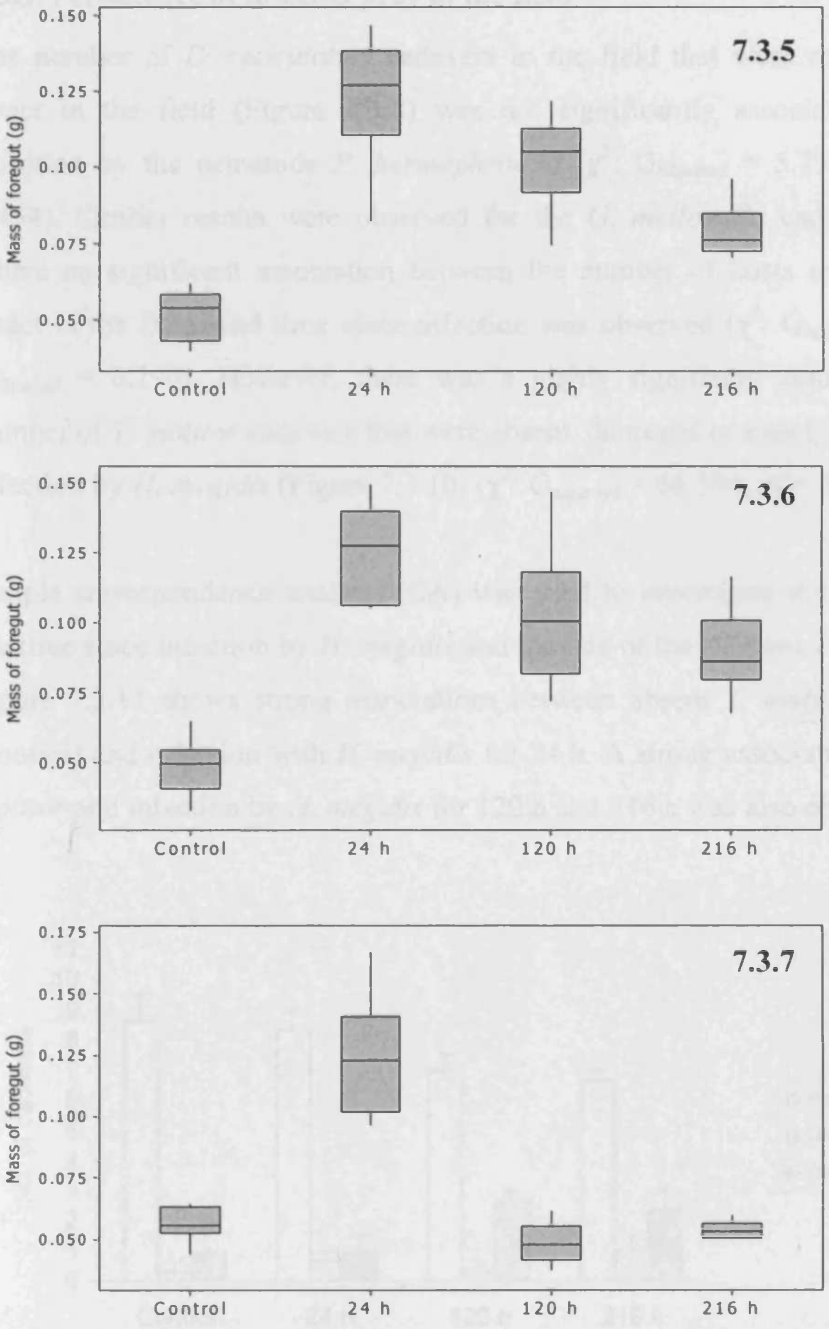
Feeding trial	Feeding trial	Test	T-Value/ W	df	P-value	P-value
Dead *D. reticulatum* infected with *P. hermaphrodita*	Un vs. Un	TT	-1.26	18	0.222	$P \geq 0.05$
	Un vs. 24 h	MW	100.5		0.7550	$P \geq 0.05$
	Un vs. 72 h	MW	96.5		0.5169	$P \geq 0.05$
	Un vs. 120 h	MW	85.5		0.1400	$P \geq 0.05$
	Un vs. 168 h	MW	106.0		0.9675	$P \geq 0.05$
	Un vs. 216 h	MW	117.5		0.3272	$P \geq 0.05$
Dead *T. molitor* infected with *H. megidis*	Un vs. Un	TT	0.00	18	1.000	$P \geq 0.05$
	Un vs. 24 h	MW	99.5		0.6989	$P \geq 0.05$
	Un vs. 72 h	MW	117.0		0.3251	$P \geq 0.05$
	Un vs. 120 h	N/A	N/A	N/A	N/A	N/A
	Un vs. 168 h	N/A	N/A	N/A	N/A	N/A
	Un vs. 216 h	N/A	N/A	N/A	N/A	N/A
Dead *G. mellonella* infected with *S. feltiae*	Un vs. Un	TT	0.34	18	0.734	$P \geq 0.05$
	Un vs. 24 h	TT	1.12	18	0.279	$P \geq 0.05$
	Un vs. 72 h	N/A	N/A	N/A	N/A	N/A
	Un vs. 120 h	N/A	N/A	N/A	N/A	N/A
	Un vs. 168 h	N/A	N/A	N/A	N/A	N/A
	Un vs. 216 h	N/A	N/A	N/A	N/A	N/A
Live *D. reticulatum* infected with *P. hermaphrodita*	Un vs. Un	MW	70.5		0.0051	$P < 0.05$
	Un vs. 24 h	TT	0.21	18	0.836	$P \geq 0.05$
	Un vs. 72 h	MW	119.0		0.2851	$P \geq 0.05$
	Un vs. 120 h	MW	97.5		0.5835	$P \geq 0.05$
	Un vs. 168 h	MW	129.5		0.0621	$P \geq 0.05$
	Un vs. 216 h	MW	97.5		0.5767	$P \geq 0.05$

7.3.2. No-choice feeding trials

In the *D. reticulatum – P. hermaphrodita* feeding trial (Figure 7.3.5) there was a highly significant difference between the means of four beetle groups (ANOVA: $F_{3,28} = 37.86$, $P < 0.001$). All the groups were significantly different from each other at the 95% level (LSD = 0.01431). There was a clear trend in the mean weight of the beetle foreguts, as the length of time *D. reticulatum* was infected for increased, the weight of the beetle foreguts decreased. The lightest foreguts were found in the control (unfed) beetles.

In the *G. mellonella – S. feltiae* feeding trial (Figure 7.3.6), there was a highly significant difference between the means of the foregut weight for each of the four beetle groups (ANOVA: $F_{3,28} = 27.12$, $P < 0.001$). All groups had significantly different means at the 95% level, except when the feeding trial on *G. mellonella* infected for 120 h was compared to the feeding trial on *G. mellonella* infected for 216 h (LSD = 0.01787). The trend in foregut weight was the same as for the previous feeding trial; as the length of time *G. mellonella* was infected for increased, the weight of the beetle foreguts decreased. Again, the lightest foreguts were found in the control (unfed) beetles.

In the *T. molitor – H. megidis* feeding trial (Figure 7.3.7) there was a highly significant difference between the median foregut weight in the four feeding trials (Kruskal-Wallis: $H = 19.56$, $df = 3$, $P < 0.001$). The median foregut weight of the beetles that had consumed mealworms infected for 24 h was significantly larger than those at the other three feeding treatments (Mann-Whitney: $P < 0.001$). The median foregut weight in the remaining feeding treatments (control, 120 h and 216 h) was not significantly different when compared to each other (Mann-Whitney: $P < 0.05$).



Figures 7.3.5 7.3.6 and 7.3.7 Box-plot showing the mean weight of the foregut of eight *Pterostichus melanarius* after feeding for 2 h on: **Figure 7.3.5**: *Deroceras reticulatum* infected with *Phasmarhabditis hermaphrodita*; **Figure 7.3.6**: *Galleria mellonella* infected with *Steinernema feltiae*; **Figure 7.3.7**: *Tenebrio molitor* infected with *Heterorhabditis megidis*. Hosts had been infected for 24, 120 or 216 h. The control consisted of eight beetles that had not been fed.

7.3.3. Persistence of infected prey in the field

The number of *D. reticulatum* cadavers in the field that were removed, damaged or intact in the field (Figure 7.3.8) was not significantly associated with time since infection by the nematode *P. hermaphrodita* (χ^2: $G_{adjusted}$ = 5.732, df = 6, $P_{adjusted}$ = 0.454). Similar results were observed for the *G. mellonella* cadavers (Figure 7.3.9), where no significant association between the number of hosts removed, damaged or intact in the field, and time since infection was observed (χ^2: $G_{adjusted}$ = 7.347, df = 6, $P_{adjusted}$ = 0.290). However, there was a highly significant association between the number of *T. molitor* cadavers that were absent, damaged or intact, and time since initial infection by *H. megidis* (Figure 7.3.10) (χ^2: $G_{adjusted}$ = 66.594, df = 6, $P_{adjusted}$ < 0.001).

Simple correspondence analysis (CA) was used to investigate the association between the time since infection by *H. megidis* and the fate of the infected *T. molitor* in the field. Figure 7.3.11 shows strong associations between absent *T. molitor*, and no infection (control) and infection with *H. megidis* for 24 h. A strong association between intact *T. molitor* and infection by *H. megidis* for 120 h and 216 h was also observed.

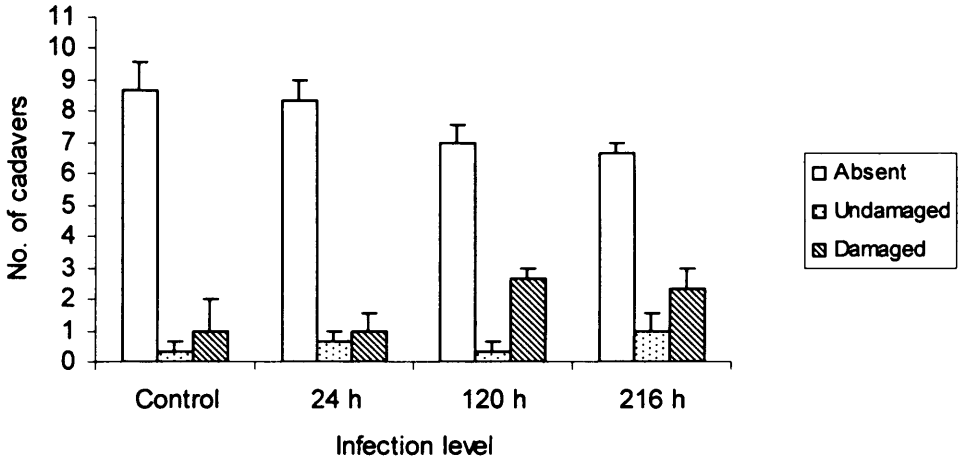


Figure 7.3.8 Mean number of *Deroceras reticulatum* individuals infected with *Phasmarhabditis hermaphrodita*, pooled from three sites and three sampling days, that were either absent, intact or damaged after 24 h in the field. Bars represent + S.E.

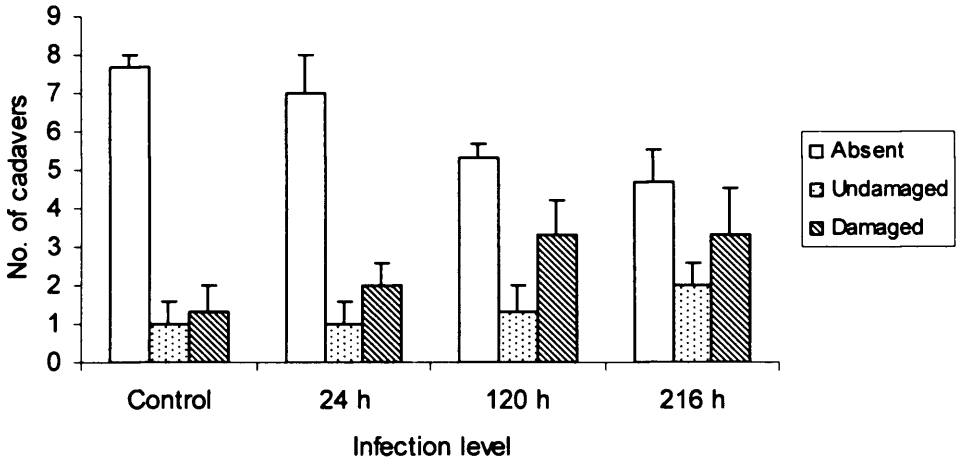


Figure 7.3.9 Mean number of individuals of *Galleria mellonella* infected with *Steinernema feltiae*, pooled from three sites and three sampling days, that were either absent, intact or damaged after 24 h in the field. Bars represent + S.E.

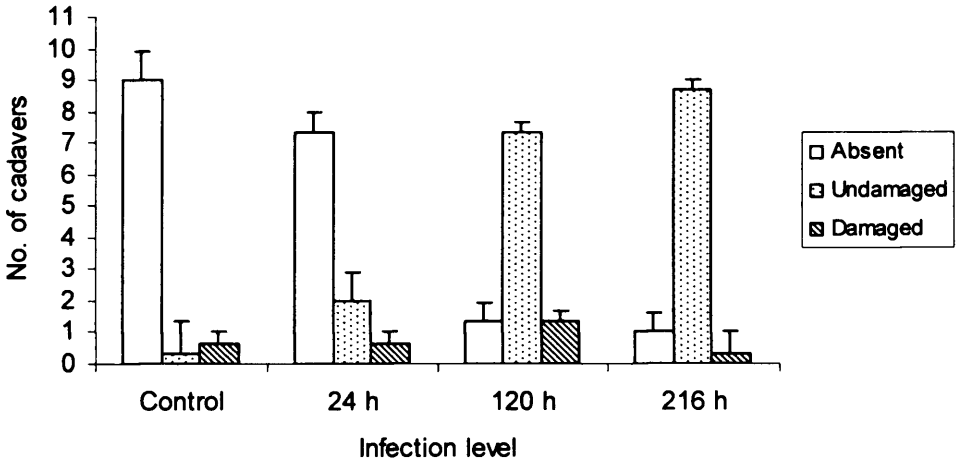


Figure 7.3.10 Mean number of individuals of *Tenebrio molitor* infected with *Heterorhabditis megidis*, pooled from three sites and three sampling days, that were either absent, intact or damaged after 24 h in the field. Bars represent + S.E.

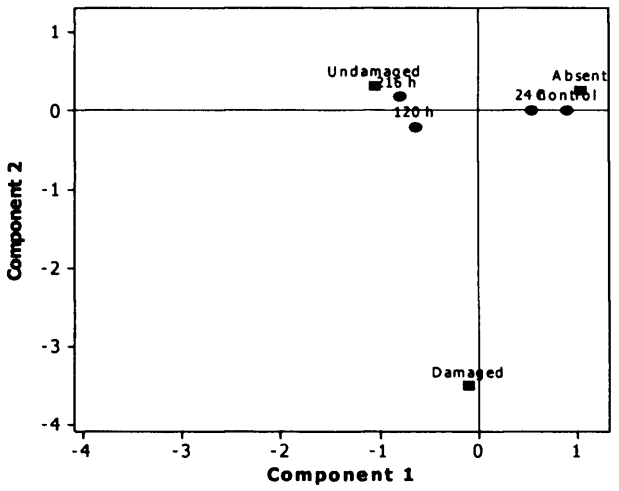


Figure 7.3.11 Simple correspondence analysis of the relationship between the fate of *Tenebrio molitor* cadavers in the field, and the time since initial infection by the nematode *Heterorhabditis megidis*. Squares represent the fate of the cadaver in the field. Circles represent level of infection.

In both the control and with hosts infected for 24 h, there was no association between the species of nematode and host, and the total number of hosts that were removed, damaged or intact after 24 h in the field (χ^2: Control: $G_{adjusted} = 2.290$, df = 4, $P_{adjusted} = 0.683$; 24 h: $G_{adjusted} = 4.721$, df = 4, $P_{adjusted} = 0.317$). However, for cadavers infected for 120 h and 216 h with their respective nematodes, there was a highly significant association between the species of nematode and host and the total number of cadavers that were removed, damaged or intact in the field (χ^2: 120 h: $G_{adjusted} = 40.653$, df = 4, $P_{adjusted} < 0.001$; 216 h: $G_{adjusted} = 43.90$, df = 4, $P_{adjusted} < 0.001$).

To examine the nature of this association with cadavers infected for 120 h and 216 h, simple correspondence analysis (CA) was conducted. Figure 7.3.12 shows the ordination of species of host and nematode after infection for 120 h, with respect to the fate of the cadavers in the field. An association with undamaged cadavers and the *T. molitor* cadavers infected with *H. megidis* is apparent. Figure 7.3.13 shows a similarly strong association with intact cadavers and the *T. molitor-H. megidis* complex after infection for 216 h.

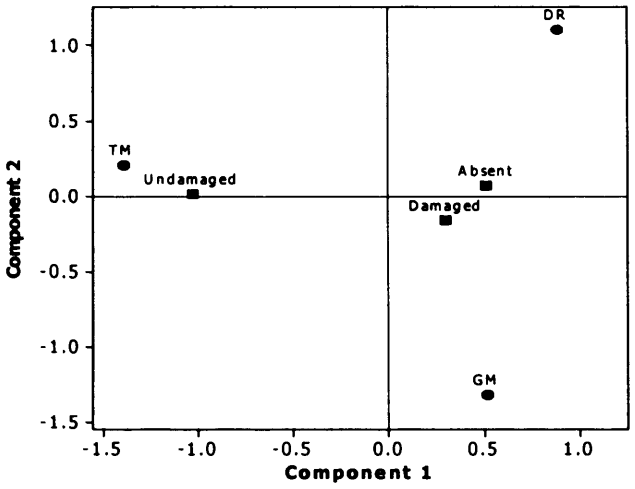


Figure 7.3.12 Simple correspondence analysis of the relationship between the number of host cadavers that were absent, damaged or intact in the field, and the type of host-nematode complex after 120 h of infection (DR = *Deroceras reticulatum* and *Phasmarhabditis hermaphrodita*, TM = *Tenebrio molitor* and *Heterorhabditis megidis*, GM = *Galleria mellonella* and *Steinernema feltiae*). Squares represent the fate of the cadaver in the field. Circles represent species of cadaver.

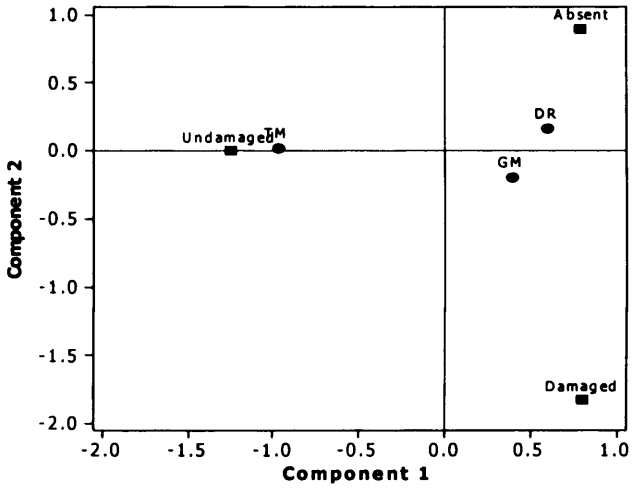


Figure 7.3.13 Simple correspondence analysis of the relationship between the number of host cadavers that were absent, damaged or intact in the field, and the type of host-nematode complex after 216 h of infection (DR = *Deroceras reticulatum* and *Phasmarhabditis hermaphrodita*, TM = *Tenebrio molitor* and *Heterorhabditis megidis*, GM = *Galleria mellonella* and *Steinernema feltiae*). Squares represent the fate of the cadaver in the field. Circles represent species of cadaver.

7.4. Discussion

The effect of parasite infection on the behavioural aspects of predator-prey dynamics is a potentially important factor influencing predator, parasite and prey populations. Infection by the mollusc parasitic nematode *Phasmarhabditis hermaphrodita* on the slug *Deroceras reticulatum* had a significant effect on the scavenging behaviour of the carabid beetle *Pterostichus melanarius* in the laboratory choice tests. In the trials using pre-killed prey, no effect of infection was seen on prey choice at the early stages of infection (24 h post-infection). At the later stages of nematode infection (72 h, 120 h and 216 h) there was a significant preference for feeding on uninfected slugs rather than nematode infected slugs. Despite the preference for feeding on uninfected prey, the presence of nematode infection did not result in complete cessation of feeding on nematode-infected slugs; even in feeding trials containing slugs infected for 216 h over 20% of the feeding incidents were on infected slugs. There was no correlation between the number of feeding incidents on infected prey and time since *D. reticulatum* was initially infected with *P. hermaphrodita*, indicating that time since infection was not the most important factor influencing prey choice.

Similar patterns were observed in the *Heterorhabditis megidis* - *Tenebrio molitor* and *Steinernema feltiae* - *Galleria mellonella* nematode-host systems, where no change in feeding choice was observed at the early stages of infection (24 h post-infection), but in feeding choices involving hosts at the later stages of infection, there was a significant preference for feeding on uninfected prey. However, unlike the *P. hermaphrodita* feeding trial, the significant negative correlation between time since infection and the number of feeding incidents on infected prey indicated that time since infection by *H. megidis* and *S. feltiae* did play an important role in determining feeding choice.

Further evidence for the repellent effects of the *H. megidis*-infected cadavers can been seen in the analysis of the weight of the beetle foreguts in the no-choice feeding trial. Interestingly, the strong repellent effect of infection by *S. feltiae* which occurred when the beetles were given a choice of food items was no longer as prevalent. When offered no choice of food, the beetles consumed *G. mellonella* cadavers even after 216 h of infection by *S. feltiae*. In the feeding trials using *P. hermaphrodita* and *S.*

feltiae infected cadavers there is a clear hierarchy of feeding preference, with the largest quantity of the host consumed at the earlier stages of infection and the least at the later stages. In contrast, when *P. melanarius* were offered *H. megidis* infected cadavers there was a complete cessation of feeding on mealworms infected for more than 24 h, as average foregut weights in the 120 h and 216 h feeding trials were no higher than in the unfed control group. These results seem to indicate that there is a hierarchy of food preference by *P. melanarius* for the three nematode-host complexes that were tested, with *D. reticulatum – P. hermaphrodita* being the most palatable, *G. mellonella – S. feltiae* second and *T. molitor – H. megidis* the least. It is unclear which experiment, the choice or no-choice, more accurately reflects the situation in the field. Whether or not the beetles have a choice of food items in the field will depend on the prey abundance and density. Hunger levels, which were not examined in this study, may also play an important role in determining the feeding choices when presented with an unpalatable prey item.

The health status of prey, and hence the effects of parasitism, can have a significant effect on trophic interactions between predator and prey (Langan *et al.* 2004). When using a choice of live, dying or dead *D. reticulatum* infected with *P. hermaphrodita*, the carabids spent significantly more time not feeding when compared to the equivalent feeding trials using pre-killed prey. This is likely to be a result of the defensive abilities of the live slugs, resulting in reduced feeding rates when compared to the feeding trials using dead slugs (and therefore with no active defensive abilities). More importantly, using live slugs for the choice test instead of pre-killed slugs resulted in a reverse of the previously observed *P. melanarius* feeding behaviour, as it resulted in significantly more feeding incidents on nematode-infected slugs.

There have been several studies investigating the influence of prey health on feeding choices by carabid beetles. In feeding choice experiments with the carabid *Pterostichus madidus*, slugs exposed to lethal doses of metaldehyde are preferentially attacked over healthy individuals (Langan *et al.* 2001); metaldehyde poisoning of slugs increased the duration of feeding bouts by *P. melanarius* (see Langan *et al.* 2004), and dead slugs are preferred to live ones (Mair and Port 2001). These results indicate that the defensive abilities of slugs may play an important role in prey choice. Further evidence comes from the fact that smaller slugs are preferred to larger

ones (Mckemey *et al.* 2001), and that the production of mucus plays an important role in deterring predatory beetles (Mair and Port 2002). The defensive abilities of slugs have also been shown to be the deciding factor influencing species-specific prey choice by *P. melanarius* (see Foltan 2005). Lang and Gsodl (2001) investigated the effects of prey vulnerability on prey choice by the carabid *Pterostichus cupreus*. They found that the order of prey preference for live prey was for aphids (*Rhopalosiphum padi*) over fruit flies (*Drosophila melanogaster*) over house crickets (*Acheta domestica*). This preference was reversed when presented with dead prey. It was concluded that prey vulnerability plays a larger role in prey choice than choice based on prey palatability. This appears to confirm the results observed in this study, where the increased vulnerability of nematode infected slugs results in preferential feeding on them, despite a preference for uninfected slugs in the dead slug choice tests. As well as the production of defensive mucus, many limacid slugs (including *D. reticulatum*) respond to predators by a burst of speed and tail wagging (Foltan 2005). As nematode infection typically kills the host within 72 h, it is likely that infected slugs are weakened by infection, and as a result are less able to exhibit this escape behaviour, thus increasing predation risk.

The weight of the host was not found to have an influence on the prey choice, either with dead prey or using live, nematode-infected *D. reticulatum*. The lack of a correlation between prey weight and choice in the live slug feeding trials is unexpected, as previous studies have found a correlation between these two factors (Mckemey *et al.* 2001). However, the slugs in this trial were selected to cover a fairly narrow weight range (100-200 mg), so it is possible that weight variations within such a range do not influence feeding choices in this type of feeding trial.

The results from the field trials confirmed the previously observed results from the laboratory based choice and no-choice feeding trials. Infection by *H. megidis* significantly affected cadaver fate, with a strong association between increasing infection time and the number of cadavers being left intact in the field. The length of infection by *P. hermaphrodita* had no effect of the cadaver fate in the field. This was expected, as any feeding deterrents had already found to be weak in the choice and no-choice laboratory feeding trials. No effect of time since infection by *S. feltiae* on cadaver fate was observed in the field trials. The laboratory trials had confirmed some

feeding deterrent in this species, although it appears this was not strong enough to significantly affect the fate of infected cadavers in the field.

The results from the field trials also confirmed the results from the choice and no-choice feeding trials concerning the palatability of each of the three species of nematode infected cadavers. Comparisons of the species of nematode and host with the fate of the cadaver in the field indicated that at the earlier stages of infection, species of nematode and host had no effect on cadaver fate. At the later stages of infection (120 h and 216 h), again confirming the laboratory feeding trials, infection by *H. megidis* had a significant effect on cadaver fate, with *H. megidis* infected cadavers being associated with being intact in the field.

Differences in the level of palatability of heterorhabditid- and steinernematid-killed hosts has also been investigated by Baur *et al.* (1998), in which they observed that steinernematid-killed hosts were more likely to be scavenged that heterorhabditid-killed hosts. They also discovered that the symbiotic bacteria present in the gut of nematode IJs are responsible for the deterrent factor towards ants, with infection by the bacterial symbiont of heterorhabditid nematodes, *Photorhabdus luminescens*, offering a higher level of protection from scavenging ants that the symbiont of steinernematid nematodes, *Xenorhabdus nematophilus*. Further work investigating the repellent effects of cadavers infected with these bacterial symbionts has indicated that there are differences in the strength of the antifeedant effect amongst different strains and forms of the same species of bacteria (Zhou *et al.* 2002). The bacterial symbiont associated with *P. hermaphrodita* when reared for pest control is *Moraxella osloensis* (see Wilson *et al.* 1995), although there has been no research into the potential production of antifeedant compounds by this species.

In this study, the antifeedant effect produced by *H. megidis* and/or its bacterial symbiont, and to a lesser extent *S. feltiae*, is effective in deterring feeding by the carabid beetle *P. melanarius*. The fact that the antifeedant effect appears to increase with time corresponds with the findings of Zhou *et al.* (2002), who found that the length of time of bacterial growth in culture affected the number of visitations by ants to a treated sucrose solution. They speculate that the antifeedant is an extracellular substance that accumulates as the bacterial population increases. In the early stages of

nematode (and bacterial) infection of the host the antifeedant substance is probably not at a high enough concentration to deter feeding by the beetles, whilst at the later stages enough antifeedant accumulates to cause the observed reduction in feeding on infected hosts. Decreasing palatability with time since infection is a general trend that has been observed amongst idiobiont parasites developing in sessile hosts, where newly infected hosts are readily eaten, and those at the later stages are often ignored (Brodeur and Boivin 2004). For example, a predator of whiteflies, the coccinellid *Delphastus pusillus*, has been shown to readily feed on *Bemisia tabaci* infected with the first instar of the parasitoid *Encarsia transvena*, but avoids feeding on those containing third instar and pupal parasitoids (Hoelmer *et al.* 1994).

The results in this study indicate that any antifeedant produced in *P. hermaphrodita*-infected hosts is either weak or completely absent. The choice not to feed on infected slugs and the reduction in the quantity of *P. hermaphrodita* infected slugs consumed at the later stages of infection may be a result of reduced palatability, caused by the decay of the slug host, rather than a specific antifeedant compound. The lack of a strong antifeedant effect caused by *P. hermaphrodita* infection is surprising, as this would appear to expose the developing nematodes to a high risk of predation and scavenging whilst within the cadaver.

Scavenging by invertebrates is thought to be widespread, and has been recorded amongst a range of different invertebrates, including Formicidae, Chrysopidae, Coccinellidae, Miridae and Carabidae (Bestelmeyer and Wiens, 2003; Wheeler 1974, 1977; Lang and Gsodl 2001). Sunderland *et al.* (1987) recorded 48 different species of predator that would scavenge on dead aphids, illustrating how widespread this trait may be amongst invertebrate 'predators'. Few studies have measured the persistence of invertebrate cadavers in the field. In semi-arid grassland, ants removed 53% to 70.3% of insect carcasses within 20 min of placement (Bestelmeyer and Wiens, 2003). Foltan *et al.* (2005) found that the mean length of time before the removal of *D. reticulatum* cadavers in wheat and grass pasture was between 10.5 and 11.7 h in two consecutive experiments. *Pterostichus melanarius* is one of the most abundant ground dwelling invertebrate predators found in agroecosystems (Lovei and Sunderland 1996), and it can be expected that scavenging by this species would be a cause of the high removal rate of cadavers in the field. These high levels of

scavenging indicate that either *P. hermaphrodita* has developed alternative methods for avoiding predation, or that the nematodes are subject to high losses whilst in the cadaver. One method for minimising predation on the cadaver would be to modify the host behaviour in such a way that it reduces the scavenging risk (Brodeur and Boivin 2004). The parasitoid *Eucelatoria bryani* causes its larval lepidopteran host, *Helicoverpa zea*, to burrow into the ground 0.7 to 3.4 days earlier than unparasitized larvae, as a way of reducing predation on pupating larvae (Reitz and Nettles 1993). It is possible that infection by *P. hermaphrodita* causes slugs to die out of reach of invertebrate scavengers, either within the soil matrix or on plant surfaces above the ground, although this has not been confirmed experimentally. Most research into the modification of host behaviour by parasites has focussed on parasites that increase predation risk, as a method of gaining access to intermediate hosts (Hechtel *et al.* 1993).

Although steinernematid, heterorhabditid and phasmarharhabditid nematodes are not closely related, their life-cycles are very similar due to convergent evolution (Poinar 1993). Therefore it is surprising that *P. hermaphrodita* has not evolved a method of predator and scavenger deterrence. Wilson *et al.* (1995) identified nine bacterial isolates from *P. hermaphrodita* collected from the field. Only one of these isolates, *Moraxella osloensis*, was pathogenic to the slug *D. reticulatum*, resulting in this species of bacteria being used commercially for the culturing of *P. hermaphrodita* as a biological control agent. It is possible that one of the other species of bacteria present in the gut of naturally occurring *P. hermaphrodita* is responsible for the deterrent factor. Another possible explanation comes from the life-cycle of *P. hermaphrodita*. Rae *et al.* (2006) speculate that *P. hermaphrodita* is a facultative parasite that is capable of reproducing on decaying animal and plant material in the soil, as well as host molluscs. In contrast, entomopathogenic nematodes from the families Heterorhabditidae and Steinernematidae are obligate parasites of insects. This difference may be responsible for the low levels or the absence of the production of an antifeedant by *P. hermaphrodita* infection, as the life-cycle plasticity may allow it to recycle, despite high levels of predation and scavenging on infected hosts.

The entomopathogenic and mollusc parasitic nematodes used in this study produce thousands to tens of thousands of offspring within a single cadaver (Kaya and

Gaugler 1993). This may be a strategy for coping with high levels of predation on host cadavers. Only a few hosts need to survive predation or scavenging for the nematode population to successfully recycle, and produce thousands of next generation nematodes, each capable of infecting a new host.

As stated previously, the persistence and recycling of pathogenic nematodes has often been poor, and the reasons for this are not fully understood (Georgis *et al.* 2006). These results give an insight into the potential for interference caused by invertebrate scavengers in agroecosystems. Biological control can often involve an integrative approach, with multiple predator and parasite species to successfully control a single pest species (Ebassa *et al.* 2006). For this approach to be a success, these species must work synergistically to control the pest, and not interfere through IGP. The results in this study indicate that predation by *P. melanarius* may be an important factor in limiting the recycling of the mollusc parasitic nematode *P. hermaphrodita*, whilst the feeding deterrents produced by *H. megidis*, and to a lesser extent *S. feltiae* would reduce the impact of this form of IGP.

Chapter 8

General discussion and conclusions

8.1. Summary

Research into below-ground interactions is a topic that has lagged behind other areas of ecology, mostly due to the inaccessible and concealed nature of these ecosystems, and the interactions that occur within them. Understanding the dynamics of below-ground food webs will benefit research into the control of herbivorous insect pests; it has been estimated that more than 90 % of insect species have life history stages in the soil (Klein 1990).

The primary aim of this research was to investigate the interactions of introduced parasitic nematodes within the soil food-web, in the context of the biological control of soil-dwelling agricultural pests. Potentially, one of the primary factors influencing the successful control of soil-dwelling pests with parasitic nematodes is the persistence of the nematode populations after application. Previous research has highlighted and quantified some of the abiotic factors that determine nematode persistence (Smits 1996). However, there is a significant lack of knowledge on how parasitic nematodes interact with other soil-dwelling biota. The interactions of introduced mollusc and entomopathogenic nematodes can be divided into four main categories: (1) Interactions with the host, (2) Interactions with predators of the infected hosts, (3) Interactions with predators of the free-living nematodes, and (4) Interactions with other free-living nematodes.

Interactions in the first category have gained the most interest, as this link is essential for the biological control of agricultural pests. However, the interactions of predators and scavengers, both with free-living nematodes and nematodes in their hosts, and interactions of parasitic nematodes with other nematodes in the soil, have received relatively little attention due to the difficulties in determining trophic links in below-ground food-webs. In response to these problems, this research has developed molecular methods for determining the fate of introduced nematodes within the soil food-web.

The experiments conducted in this study were designed to address the seven objectives outlined in Chapter 1, Section 1.9.

8.1.1 "Develop molecular methods for the detection of nematode biopesticides within their hosts, within potential predators of the free-living nematode stages, and within predators of the infected hosts"

The molecular probes designed and tested in this study can be considered a success, in that they were species-specific after extensive non-target testing and had sufficient sensitivity to detect predation by microarthropods on nematodes (Chapter 2, Section 2.2.2, and Chapter 3, Section 3.3.3). The current primers were tested against 74 common invertebrate species and 48 unidentified nematode individuals, compared to most previous studies in which 5-10 non-targets organisms were screened. This allows these primers to be used with a degree of confidence. However, the number of species found even in agroecosystems is likely to be much higher, and primer specificity can never be completely guaranteed. The use of general primers, and a cloning and sequencing approach, for the analysis of a subset of the gut contents of field collected predators may be a more efficient way of identifying non-target prey sequences.

8.1.2 "Test the newly developed molecular tools to determine accuracy and potential error when utilizing these methods for determining predation on nematode IJs."

Previous studies and reviews have identified a number of potential errors associated with the molecular detection of predation. These errors can be divided into two groups, those that cause false positives or negatives, and those that cause variation in prey detection times in predator guts. This study covered, in the author's opinion, the most important factors that may influence reliability and detection of predation in this system, including those that cause false positives (infection of predator, phoresy and scavenging) and changes in detection times (predator size and ambient temperature). The size of the amplified DNA fragment has also been shown to have a significant effect on detection times, although in the present study, no significant differences were observed. From the results, it would appear that scavenging is the most serious potential error, where DNA from dead nematodes in sterile soil was detectable in 50 % of the samples after approximately 95 h. The diverse diet of most soil-dwelling organisms means that dead nematodes are just as likely to be eaten as live ones, potentially causing false positives and overestimation of trophic links. This potential error cannot be ruled out and extreme care needs to be taken interpreting molecular data in isolation.

However, in this study, additional evidence from the laboratory based feeding trials indicate that feeding on live nematodes by both mites and collembola does occur (Chapter 3, Section 3.3.7). As a result, it can be assumed that field-based positives in this study indicate predation (although scavenging still cannot be ruled out). Detection of scavenging still may be useful, as it monitors energy flow to consumers within the food-web, but not the impact of predation.

There are a number of other factors that may influence prey detection times that were not considered in this study, including predator health, nutritional state, and activity, the presence of alternative prey and the sequence composition of the amplified DNA fragment. This list is far from exhaustive, and shows that it is probably impossible to obtain an exact model of prey detection times, due to the number of interacting and conflicting influencing factors. Fortunately this is not necessary for most studies, as the screening of a sufficiently large number of predators should reduce the effect of variation in detection time on the overall results.

8.1.3 "Through laboratory based feeding trials, investigate the potential for soil-dwelling microarthropods to limit the persistence of introduced nematode IJs."

Although the molecular methods used in this study are able to reveal the occurrence of predation events, they do not reveal quantities of prey eaten, or distinguish predation from scavenging. As a result, feeding-trials were used to examine how a model microarthropod (*Folsomia candida*) could affect the persistence and infectivity of an entomopathogenic nematode. The experiments in Chapter 3, Section 3.3.7 showed that the collembolan *F. candida* was capable of reducing nematode numbers on plaster-of-Paris (ca. 46 % in 10 h) and soil (49 % reduction in 120 h). More importantly, it was shown that this translated into a reduction in the number of hosts that could be infected in these soil arenas. An argument could be made that other collembolan species may have different feeding behaviours, and that these results are not applicable to the field. Unfortunately it was not possible to culture the most common species from the field site (*Isotoma viridis* and *Isotomurus palustris*) in the laboratory, despite numerous attempts. However, *F. candida* has been chosen as a 'typical' collembolan for toxicology experiments (Fountain and Hopkin 2005), and it is unlikely that its feeding behaviour

(i.e. quantity of food eaten and frequency of feeding) differs from other similar sized epiedaphic species.

8.1.4 "Investigate the impact of the application of nematode IJs in agroecosystems, and identify potential predators of the introduced nematode to identify potential nematode antagonists."

The experiments contained in Chapter 4 were conducted in an attempt to quantify the impact of nematode inundation on the component organisms of the soil food-web, and to identify the predators of the introduced nematode on or near the soil surface. The initial aim was to use the developed molecular probes to identify the full range of potential nematode predators, both on and within the soil matrix. However, a number of technical difficulties made this unrealistic and beyond the scope of this project. The foremost technical issue is the extraction of soil biota whilst preserving the gut contents that would be found naturally in the field. The conventional, cheaper, extraction methods used in this study, such as Tullgren funnels for microarthropods, and Baermann funnels for nematodes, generally take 24-72 h to extract soil biota. This is clearly not satisfactory for predation analysis, as the stomach contents will have changed or emptied during this time. Faster extraction methods are available for microarthropods, such as Heptane floatation (Walter 1987), which has the added advantage of being four times more efficient than Tullgren funnel type extractions (Andre *et al.* 2002). Heptane floatation also works with dead microarthropods, so it may be possible to rapidly freeze soil samples to preserve the gut contents of the soil-dwelling microarthropods, for processing at a later date. Unfortunately the cost of solvent based extraction was prohibitive for this study. Alternative extraction methods for soil microfauna, such as sugar centrifugation (Southey 1970), may also allow rapid extraction of nematodes and tardigrades from the soil. However, a major drawback is that these methods have been found to extract fewer omnivorous and predatory nematodes than the Baermann funnel (McSorley and Frederick 2004). Additionally, a number of technical difficulties need to be overcome to ensure the gut contents of such small organisms are preserved during handling and extraction.

These technical problems limited the study, in that only the surface-dwelling microarthropods, that could be quickly collected and preserved, were screened for

predation on the introduced nematode. As a result, a true soil food-web encompassing a wider range of soil biota could not be obtained. However, the screening for predation by surface-dwelling microarthropods revealed that feeding on the introduced nematode, *Phasmarhabditis hermaphrodita*, by the most common species of collembola was frequent in the early stages post-nematode application, and this declined over time. Knowledge of rates of predation on the soil surface is valuable, as nematodes are generally applied to the soil surface, and despite advice to wash the nematodes into the soil with a second application of water, it is likely a large proportion will stay on the surface. Microarthropods on the surface are more likely to fit into the definitions supplied by Lavelle and Spain (2001) in which epiedaphic species are larger, have a higher metabolism and consume more food than the smaller euedaphic species. As a result, the predation impact by the epiedaphic species is likely to be proportionally higher.

The application method (soil surface) and the inherent differences between epiedaphic and euedaphic microarthropod species may explain the pronounced differences in the response of the above- and below-ground microarthropods. Only the epiedaphic microarthropods and the euedaphic mites responded to nematode application. Further screening needs to be conducted to see if this could be explained by a lack of nematophagy in the majority of euedaphic microarthropods.

8.1.5 "Develop methods for assessing nematode diversity in the field, and use these methods for studying the impact of introducing nematode IJs on existing nematode communities."

The use of T-RFLP as a method shows great promise for fast and cheap assessment of nematode community composition in soils. The results in Chapter 5 showed that T-RFLP, with this selection of primers and enzyme, was capable of separating some (but not all) nematode species in a mixed sample. As a result, a nematode 'community profile' could also be obtained from a mixed sample of nematodes. However, as not all species were separated when multiple sources were mixed together, further work is needed to quantify the accuracy and limitations of this technique before it can be used to make meaningful measurements of nematode diversity in the field. The work carried out in this section could have been improved significantly by sequencing the unidentified

nematodes, to allow a comparison of T-RFLP profiles with the actual genetic diversity in the sample. Unfortunately attempts at sequencing failed with this set of primers, making this unachievable for this project.

Once calibrated, T-RFLP has the potential to measure not only differences in nematode species composition from soil samples, but also differences in the relative biomass of species or groups of nematodes between samples. This is important, as changes in some soils may be reflected in the abundance of particular nematode species, not overall species composition. The use of primers that are specific to different nematode groups (i.e. families or genera) would allow for the separation and comparison of relevant groups of nematodes, rather than the whole community. For example, it may be possible to design primers that amplify just the Steinernematidae, allowing for the rapid identification of species from this group of entomopathogenic nematode from soil samples. It may even be possible to apply this technique to the analysis of predator gut samples, although the need for relatively short fragments of DNA may reduce the taxonomic division that can be achieved.

T-RFLP was originally developed for assessing microbial diversity in a variety of sediments (Marsh 1999) and it is increasingly being used for comparing fungal diversity (Anderson and Cairney 2004). The use of T-RFLP for nematode community analysis is a logical progression, considering the value of these organisms as biological indicators (Neher 2001; Yeates 2003). Future applications of T-RFLP may include the assessment of the diversity of other taxonomically difficult soil biota, including enchytraeids, tardigrades, protozoans, mites and collembola.

8.1.6 "Test the molecular tools for use in investigating tri-trophic interactions between potential predators of nematode infected hosts" and "Investigate the potential for nematode infection to alter the prey choice of invertebrate predators."

Although the use of PCR for the detection of predation and parasitism is widespread (reviews in Symondson 2002; Sheppard and Harwood 2005; Greenstone 2006), this is the first time that these techniques have successfully been used to detect predation on parasitized prey (Chapter 6) (but see Traugott and Symondson, submitted, for predation

by carabids on parasitized aphids). The influence of predation on parasite populations, whilst widely acknowledged as a potentially important factor (Rosenheim *et al.* 1995; Hall *et al.* 2005), has only been studied in a relatively small number of predator-prey and parasite systems. These techniques open up the potential to detect predation on parasitized prey in the field, to examine how prevalent it is, and to determine what impact predation may have on parasite populations. Moreover, this technique has applications both in the areas of the persistence of biological control agents and in the study of natural parasite populations.

The feeding choice tests that were conducted in Chapter 7 hint at potentially complex species-specific interactions between parasitized hosts and their predators. The varying degrees of feeding inhibition in the three nematode species tested was interesting, and further work with more heterorhabditid and steinernematid species is necessary to see if the observed trends in feeding deterrence are family specific. All feeding trials were conduced with the carabid *Pterostichus melanarius* as the scavenger, due to its high abundance in UK agroecosystems. However, a wider range of species also need to be tested to see if the feeding inhibition patterns occur in a wider range of potential predators and scavengers.

Unfortunately, other than the introduction of infected cadavers to the field (Chapter 7, Section 7.3.3), it was not possible to conduct field scale investigations on the effects of nematode biopesticides and intraguild predation. One method for doing this would be to create a number of treatment plots (nematode sprayed) and control plots (no nematodes) in a field crop. Invertebrate scavengers and predators could be sampled at regular time points before and after nematode application, and these could be screened for predation on both the host and parasite DNA. This would provide information about the rates of intraguild predation on parasitized prey, rates of predation on uninfected prey and the effect of nematode application on food supply for these predators. Screening the predators for other non-pest prey may highlight prey switching, in response to declining pest numbers or nematode infection and subsequent feeding inhibition. Repeating this experiment with different nematode and bacterial species, with different levels of feeding inhibition, may allow for the identification of nematode strains that persist in the environment for longer. However, due to the high degree of spatial clustering of predators and prey in the field (Bohan *et al.* 2000; Symondson *et al.* 2002; Winder *et al.*

2005), a large number of plots are needed to account for predator and host population variation. As *P. melanarius* are highly mobile (Fournier and Loreau 2001) and present in relatively low densities in the field (Kromp 1999), each of the treatment and control plots would need to be relatively large, and ideally surrounded by barriers to prevent movement from treatment into control plots and vice versa. These difficulties were insurmountable for the present study, but this experiment may make for interesting future research.

8.2. Implications of study and future work

The use of molecular tools can generate information on the nature and impact of interactions within the food-web directly in the field. This can be used to help design nematode application methods that will reduce levels of antagonistic food-web interactions, and increase the persistence and effectiveness of introduced biological control agents. For example, the use of molecular tools to identify direct predators of free-living nematode IJs can be used to make decisions about application timings and methods, in an attempt to reduce levels of predation and increase nematode persistence. It may be possible to identify soils that, due to the high numbers of nematophagous organisms, are not suitable for nematode application, allowing research to focus on other methods of pest control.

A similar case for the advantages of identifying intraguild predators of nematodes within their hosts can be made. With this information, a number of changes could be made to nematode-based biological control programs, including timing of application to avoid conflict with IG predators, and management practices to reduce IG predator populations. Understanding the antifeedant-effect produced by some nematode-bacterium complexes may prove to be useful for the development of nematode and bacterial strains that reduce the rates of predation and scavenging on nematode infected cadavers, and hence increases the long-term recycling and persistence. Knowledge of potential conflict between biological control agents may allow for the release of multiple agents when a lack of IGP between these species has been recorded.

A number of studies have investigated the application of nematode-infected cadavers directly to the field, as an alternative to spraying nematode IJs onto the soil surface (Shapiro-Ilan *et al.* 2001; 2003). These studies have indicated that application of nematode-infected cadavers to the soil provide superior efficacy when compared to an aqueous solution, by minimising the exposure to stress in the form of desiccation, UV light and extremes of temperature. This study has indicated that whilst application of heterorhabditid-infected cadavers may be a viable method for the application of nematodes to the field, steinernematid- and phasmarhabditid-infected cadavers would soon be consumed by invertebrate scavengers. The development and testing of molecular tools for the detection of intraguild predation has laid the groundwork for future studies. These can investigate the potential of antagonistic interactions between generalist predators and nematode parasites in the field. Although it was not possible to conduct fieldwork investigating intraguild predation in this study, future studies using the molecular techniques can quantify the strength of such interactions.

Mathematical models of pest populations have been used to help understand the timing of pest increases, the factors that influence population size and predict crop damage. For example, a number of studies have sought to model the population dynamics of the pest slug *Deroceras reticulatum* (see Mair *et al.* 2001; Schley and Bees 2006; Willis *et al.* 2006). Models have also been used to predict the dose rates and timing of *P. hermaphrodita* to control slug populations (Wilson *et al.* 2004). These models have largely ignored biotic interactions of nematodes other than with their hosts. Without a full understanding of the interactions of parasitic nematodes within the soil food web, both in the soil and the host, these models will be incomplete. Knowledge of the factors that influence nematode persistence, and hence infectivity, may allow for more accurate predictions of slug populations and nematode persistence after application. This can be used to formulate more effective pest control programmes

The implications of this research are not limited to the biological control of insect and mollusc pests. Identifying nematophagous organisms within the soil food-web is useful for the biological control of plant-parasitic nematodes. Plant-parasitic nematodes can take up to one quarter of the net primary production in grassland (Stanton 1998) and are thought to do damage costing somewhere in the region of $125 billion per annum

worldwide (Chitwood 2003). A number of reviews have identified organisms that may have a suppressive effect on plant-parasitic nematode populations (Mankau 1980; Jatala 1986; Sayre and Walter 1991; Sikora 1992; Khan and Kim 2007), but it has been difficult to test these assumptions in the field. Using species-specific primers designed for plant-parasitic nematodes will enable biotic antagonists to be identified. This may be used to increase plant-parasitic nematode control through soil management techniques or predator augmentation.

The role of top-down control of nematodes is not just relevant for pest control. Soil-dwelling nematodes are thought to play an important role in many soil processes, including nutrient cycling and mineralization (Anderson *et al.* 1983), grazing on bacteria and fungi (De Mesel *et al.* 2004; Bakhtiar *et al.* 2001), decomposition of organic material (Ingham *et al.* 1985) and plant growth (Ferris *et al.* 2004). Therefore, understanding the factors that control nematode numbers and diversity is important for many other soil processes that ultimately affect crop productivity and food production. Soil-food webs are also ideal models for testing ecological theory about trophic cascades, intraguild predation, carbon and nitrogen cycling and the role of species diversity in ecosystem functioning.

References

Abadon M, Grenier E, Laumond C, Abad P (1998) A species-specific satellite DNA from the entomopathogenic nematode *Heterorhabditis indicus*. *Genome* **41**, 148-153.

Adams BJ, Burnell AM, Powers TO (1998) A phylogenetic analysis of *Heterorhabditis* (Nematoda : Rhabditidae) based on internal transcribed spacer 1 DNA sequence data. *Journal of Nematology* **30**, 22-39.

Adams BJ, Fodor A, Koppenhofer HS, *et al.* (2006) Biodiversity and systematics of nematode-bacterium entomopathogens (vol 37, pg 32-49, 2006). *Biological Control* **38**, 4-21.

Adams BJ, Nguyen KB (2002) Taxonomy and Systematics. In: *Entomopathogenic Nematology* (ed. Gaugler R), pp. 1-33. CABI International, Wallingford, Oxon.

Addison JA, Trofymow JA, Marshall VG (2003) Functional role of Collembola in successional coastal temperate forests on Vancouver Island, Canada. *Applied Soil Ecology* **24**, 247-261.

Admassu B, Juen A, Traugott M (2006) Earthworm primers for DNA-based gut content analysis and their cross-reactivity in a multi-species system. *Soil Biology and Biochemistry* **38**, 1308-1315.

Agustí N, Bourguet D, Spataro T, *et al.* (2005) Detection, identification and geographical distribution of European corn borer larval parasitoids using molecular markers. *Molecular Ecology* **14**, 3267-3274.

Agustí N, De Vicente MC, Gabarra R (1999) Development of sequence amplified characterized region (SCAR) markers of *Helicoverpa armigera*: a new polymerase chain reaction-based technique for predator gut analysis. *Molecular Ecology* **8**, 1467-1474.

Agustí N, de Vicente MC, Gabarra R (2000) Developing SCAR markers to study predation on Trialeurodes vaporariorum. *Insect Molecular Biology* **9**, 263-268.

Agustí N, Shayler SP, Harwood JD, *et al.* (2003a) Collembola as alternative prey sustaining spiders in arable ecosystems: prey detection within predators using molecular markers. *Molecular Ecology* **12**, 3467-3475.

Agustí N, Unruh TR, Welter SC (2003b) Detecting *Cacopsylla pyricola* (Hemiptera : Psyllidae) in predator guts using COI mitochondrial markers. *Bulletin of Entomological Research* **93**, 179-185.

Aitchison CW (1983) Low temperature and preferred feeding by winter-active collembola (Insecta, Apterygota). *Pedobiologia* **25**, 27-36.

Akhurst RJ, Boemare NE (1990) Biology and Taxonomy of *Xenorhabdus*. In: *Entomopathogenic Nematodes in Biological Control* (eds. Gaugler R, Kaya HK), pp. 75-90. CRC Press, Boca Raton, Florida.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic Local Alignment Search Tool. *Journal of Molecular Biology* **215**, 403-410.

Anderson JM, Healey IN (1972) Seasonal and interspecific variation in major components of gut contents of some woodland Collembola. *Journal of Animal Ecology* **41**, 359-368

Anderson RV, Gould WD, Woods LE, *et al.* (1983) Organic and inorganic nitrogenous losses by microbivorous nematodes in soil. *Oikos* **40**, 75-80.

Anderson IC, Cairney JWG (2004) Diversity and ecology of soil fungal communities: increased understanding through the application of molecular techniques. *Environmental Microbiology* **6**, 769-779.

Ansari MA, Tirry L, Moens M (2003) Entomopathogenic nematodes and their symbiotic bacteria for the biological control of *Hoplia philanthus* (Coleoptera : Scarabaeidae). *Biological Control* **28**, 111-117.

Armendariz I, Downes MJ, Griffin CT (2002) Effect of timber condition on parasitization of pine weevil (*Hylobius abietis* L.) larvae by entomopathogenic nematodes under laboratory conditions. *Biocontrol Science and Technology* **12**, 225-233.

Asahida T, Yamashita Y, Kobayashi T (1998) Identification of consumed stone flounder, *Kareius bicoloratus* (Basilewsky), from the stomach contents of sand shrimp, *Crangon affinis* (De Haan) using mitochondrial DNA analysis (vol 217, pg 153, 1997). *Journal of Experimental Marine Biology and Ecology* **225**, 169-169.

Ashfaq M, Braun L, Hegedus D, Erlandson M (2004) Estimating parasitism levels in *Lygus* spp. (Hemiptera : Miridae) field populations using standard and molecular techniques. *Biocontrol Science and Technology* **14**, 731-735.

Atkins SD, Manzanilla-Lopez RH, Franco J, Peteira B, Kerry BR (2005) A molecular diagnostic method for detecting *Nacobbus* in soil and in potato tubers. *Nematology* **7**, 193-202.

Bajaj HK, Walia KK (2005) Studies on a *Pasteuria* isolate from an entomopathogenic nematode, *Steinernema pakistanense* (Nematoda : Steinernematidae). *Nematology* **7**, 637-640.

Bakhtiar Y, Miller D, Cavagnaro T, Smith S (2001) Interactions between two arbuscular mycorrhizal fungi and fungivorous nematodes and control of the nematode with Fenamifos. *Applied Soil Ecology* **17**, 107-117.

Ballard JWO (2000) Comparative genomics of mitochondrial DNA in members of the *Drosophila melanogaster* subgroup. *Journal of Molecular Evolution*, **51**, 48-63.

Barbara KA, Buss EA (2005) Integration of insect parasitic nematodes (Rhabditida Steinernematidae) with insecticides for control of pest mole crickets (Orthoptera : Gryllotalpidae : *Scapteriscus* spp.). *Journal of Economic Entomology* **98**, 689-693.

Bathon H (1996) Impact of entomopathogenic nematodes on non-target hosts. *Biocontrol Science and Technology* **6**, 421-434.

Baur ME, Kaya HK (2001) Persistence of entomopathogenic nematodes. In: *Environmental persistence of entomopathogens and nematodes* (eds. Baur ME, Fuxa J), p. 47. Southern Cooperative Series Bulletin 398, Stillwater, Oklahoma.

Baur ME, Kaya HK, Strong DR (1998) Foraging ants as scavengers on entomopathogenic nematode-killed insects. *Biological Control* **12**, 231-236.

Beardsley S (2005) Bending to bar codes - Is a one-gene method to define species truly effective? *Scientific American* **292**, 26.

Beebee TJC, Rowe G (2004) *An Introduction to Molecular Ecology.* Oxford University Press, New York.

Beier S, Bolley M, Traunspurger W (2004) Predator-prey interactions between *Dugesia gonocephala* and free-living nematodes. *Freshwater Biology* **49**, 77-86.

Belay D, Ebssa L, Borgemeister C (2005) Time and frequency of applications of entomopathogenic nematodes and their persistence for control of western flower thrips *Frankliniella occidentalis*. *Nematology* **7**, 611-622.

Benevolenskaya EV, Kogan GL, Tulin AV, Philipp D, Gvozdev VA (1997) Segmented gene conversion as a mechanism of correction of 18S rRNA pseudogene located outside of rDNA cluster in *D. melanogaster*. *Journal of Molecular Evolution* **44**, 646-651.

Bestelmeyer BT, Wiens JA (2003) Scavenging ant foraging behavior and variation in the scale of nutrient redistribution among semi-arid grasslands. *Journal of Arid Environments* **53**, 373-386.

Blackwood CB, Marsh T, Kim SH, Paul EA (2003) Terminal restriction fragment length polymorphism data analysis for quantitative comparison of microbial communities. *Applied and Environmental Microbiology* **69**, 926-932.

Blanc C, Sy M, Djigal D, *et al.* (2006) Nutrition on bacteria by bacterial-feeding nematodes and consequences on the structure of soil bacterial community. *European Journal of Soil Biology* **42**, S70-S78.

Blankenship LE, Yayanos AA (2005) Universal primers and PCR of gut contents to study marine invertebrate diets. *Molecular Ecology* **14**, 891-899.

Blaxter ML, De Ley P, Garey JR, *et al.* (1998) A molecular evolutionary framework for the phylum Nematoda. *Nature* **392**, 71-75.

Blaxter M (2003) Counting angels with DNA. *Nature*, **421**, 122-124.

Blouin MS (1998) Mitochondrial DNA diversity in nematodes. *Journal of Helminthology* **72**, 285-289.

Blouin MS (2002) Molecular prospecting for cryptic species of nematodes: mitochondrial DNA versus internal transcribed spacer. *International Journal for Parasitology* **32**, 527-531.

Blouin MS, Liu J, Berry RE (1999) Life cycle variation and the genetic structure of nematode populations. *Heredity* **83**, 253-259.

Boff MIC, Wiegers GL, Smits PH (2001) Influence of insect larvae and plant roots on the host-finding behaviour of *Heterorhabditis megidis*. *Biocontrol Science and Technology* **11**, 493-504.

Bohan DA (2000) Spatial structuring and frequency distribution of the nematode *Steinernema feltiae* Filipjev. *Parasitology* **121**, 417-425.

Bongers T (1999) The maturity index, the evolution of nematode life history traits, adaptive radiation and cp-scaling. *Plant and Soil* **212**, 13-22.

Bongers T, Ferris H (1999) Nematode community structure as a bioindicator in environmental monitoring. *Trends in Ecology and Evolution* **14**, 224-228.

Brito EM, Guyoneaud R, Goni-Urriza M, *et al.* (2006) Characterization of hydrocarbonoclastic bacterial communities from mangrove sediments in Guanabara bay, brazil. *Research in Microbiology* **157**, 752-762.

Brodeur J, Boivin G (2004) Functional ecology of immature parasitoids. *Annual Review of Entomology* **49**, 27-49.

Brodeur J, McNeil JN (1992) Host behavior-modification by the endoparasitoid *Aphidius nigripes* - a strategy to reduce hyperparasitism. *Ecological Entomology* **17**, 97-104.

Brodeur J, Rosenheim JA (2000) Intraguild interactions in aphid parasitoids. *Entomologia Experimentalis Et Applicata* **97**, 93-108.

Brodie ED, Formanowicz DR, Brodie ED (1991) Predator avoidance and antipredator mechanisms - distinct pathways to survival. *Ethology Ecology and Evolution* **3**, 73-77.

Brooke MM, Proske HO (1946) Precipitin test for determining natural insect predators of immature mosquitoes. *Journal of the National Malaria Society* **5**, 45-56.

Brown W (1954) Collembola feeding upon nematodes. *Ecology Letters* **35**, 421.

Brown WM, Prager EM, Wang A, Wilson AC (1982) Mitochondrial-DNA sequences of primates - tempo and mode of evolution. *Journal of Molecular Evolution* **18**, 225-239.

Brust GE, House GJ (1988) A Study of *Tyrophagus putrescentiae* (Acari, Acaridae) as a Facultative Predator of Southern Corn-Rootworm Eggs. *Experimental and Applied Acarology* **4**, 335-344.

Buling A, Criado-Fornelio A, Asenzo G, *et al.* (2007) A quantitative PCR assay for the detection and quantification of *Babesia bovis* and *B. bigemina*. *Veterinary Parasitology* **147**, 16-25.

Campbell JF, Gaugler R (1993) Nictation behaviour and its ecological implications in the host search strategies of entomopathogenic nematodes (Heterorhabditidae and Steinernematidae). *Behaviour* **126**, 155-169.

Campbell JF, Gaugler RR (1997) Inter-specific variation in entomopathogenic nematode foraging strategy: Dichotomy or variation along a continuum? *Fundamental and Applied Nematology* **20**, 393-398.

Campbell JF, Koppenhofer AM, Kaya HK, Chinnasri B (1999) Are there temporarily non-infectious dauer stages in entomopathogenic nematode populations: A test of the phased infectivity hypothesis. *Parasitology* **118**, 499-508.

Campbell JF, Lewis E, Yoder F, Gaugler R (1995) Entomopathogenic nematode (Heterorhabditidae and Steinernematidae) seasonal population-dynamics and impact on insect populations in turfgrass. *Biological Control* **5**, 598-606.

Campbell JF, Lewis EE, Stock SP, Nadler S, Kaya HK (2003) Evolution of host search strategies in entomopathogenic nematodes. *Journal of Nematology* **35**, 142-145.

Campos-Herrera R, Trigo D, Gutierrez C (2006) Phoresy of the entomopathogenic nematode *Steinernema feltiae* by the earthworm *Eisenia fetida*. *Journal of Invertebrate Pathology* **92**, 50-54.

Cao AX, Liu XZ, Zhu SF, Lu BS (2005) Detection of the pinewood nematode, *Bursaphelenchus xylophilus*, using a real-time polymerase chain reaction assay. *Phytopathology* **95**, 566-571.

Cappaert DL, Koppenhofer AM (2003) *Steinernema scarabaei*, an entomopathogenic nematode for control of the European chafer. *Biological Control* **28**, 379-386.

Chahartaghi M, Langel R, Scheu S, Ruess L (2005) Feeding guilds in Collembola based on nitrogen stable isotope ratios. *Soil Biology and Biochemistry* **37**, 1718-1725.

Chamberlain PM, Bull ID, Black HIJ, Ineson P, Evershed RP (2005) Fatty acid composition and change in Collembola fed differing diets: identification of trophic biomarkers. *Soil Biology and Biochemistry* **37**, 1608-1624.

Chamberlain PM, Bull ID, Black HIJ, Ineson P, Evershed RP (2006a) Collembolan trophic preferences determined using fatty acid distributions and compound-specific stable carbon isotope values. *Soil Biology and Biochemistry* **38**, 1275-1281.

Chamberlain PM, Bull ID, Black HIJ, Ineson P, Evershed RP (2006b) The effect of diet on isotopic turnover in Collembola examined using the stable carbon isotopic compositions of lipids. *Soil Biology and Biochemistry* **38**, 1146-1157.

Chan OC, Yang XD, Fu Y, *et al.* (2006) 16s rRNA gene analyses of bacterial community structures in the soils of evergreen broad-leaved forests in south-west china. *Fems Microbiology Ecology* **58**, 247-259.

Chen Y, Giles KL, Payton ME, Greenstone MH (2000) Identifying key cereal aphid predators by molecular gut analysis. *Molecular Ecology* **9**, 1887-1898.

Chen Y, Pike KS, Greenstone MH, Shufran KA (2006) Molecular markers for identification of the hyperparasitoids *Dendrocerus carpenteri* and *Alloxysta xanthopsis* in *Lysiphlebus testaceipes* parasitizing cereal aphids. *Biocontrol* **51**, 183-194.

Chevalier R, Webster JM (2006) Temporary changes in populations of soil organisms after field application of entomopathogenic nematodes. *Journal of Nematology* **38**, 466-473.

Chitwood DJ (2003) Research on plant-parasitic nematode biology conducted by the United States Department of Agriculture - Agricultural Research Service. *Pest Management Science* **59**, 748-753.

Chikoski JM, Ferguson SH, Meyer L (2006) Effects of water addition on soil arthropods and soil characteristics in a precipitation-limited environment. *Acta Oecologica-International Journal of Ecology* **30**, 203-211.

Chow A, MacKauer M (1999) Altered dispersal behaviour in parasitised aphids: Parasitoid-mediated or pathology? *Ecological Entomology* **24**, 276-283.

Chyzik R, Glazer O, Klein M (1996) Virulence and efficacy of different entomopathogenic nematode species against western flower thrips (*Frankliniella occidentalis*). *Phytoparasitica* **24**, 103-110.

Clarke K.R. 1993. Non-parametric multivariate analyses of changes in community structure. Australian Journal of Ecology. 18:117–143

Colfer RG, Rosenheim JA (2001) Predation on immature parasitoids and its impact on aphid suppression. *Oecologia* **126**, 292-304.

Coyner DF, Schaack SR, Spalding MG, Forrester DJ (2001) Altered predation susceptibility of mosquitofish infected with *Eustrongylides ignotus*. *Journal of Wildlife Diseases* **37**, 556-560.

Croft BA, Macrae IV (1992) Persistence of *Typhlodromus pyri* and *Metaseiulus occidentalis* (Acari, Phytoseiidae) on apple after inoculative release and competition with *Zetzellia mali* (Acari, Stigmaeidae). *Environmental Entomology* **21**, 1168-1177.

Curcic BPM, Sudhaus W, Dimitrijevic RN, Tomic VT, Curcic SB (2004) Phoresy of *Rhabditophanes schneideri* (Butschli) (Rhabditida : Alloionematidae) on pseudoscorpions (Arachnida : Pseudoscorpiones). *Nematology* **6**, 313-317.

Curran J, Heng J (1992) Comparison of 3 methods for estimating the number of entomopathogenic nematodes present in soil samples. *Journal of Nematology* **24**, 170-176.

Curry J (1994) *Grassland invertebrates. Ecology, influences on soil invertebrates and effects on plant growth* Chapman and Hall, New York.

Cuthbertson AGS, Fleming CC, Murchie AK (2003) Detection of *Rhopalosiphum insertum* (apple-grass aphid) predation by the predatory mite *Anystis baccarum* using molecular gut analysis. *Agricultural and Forest Entomology* **5**, 219-225.

Czarnetzki AB, Tebbe CC (2004) Detection and phylogenetic analysis of *Wolbachia* in Collembola. *Environmental Microbiology* **6**, 35-44.

Dabire RK, Ndiaye S, Mounport D, Mateille T (2007) Relationships between abiotic soil factors and epidemiology of the biocontrol bacterium *Pasteuria penetrans* in a root-knot nematode *Meloidogyne javanica*-infested field. *Biological Control* **40**, 22-29.

Dauch AL, Watson AK, Seguin P, Jabaji-Hare SH (2006) Real-time PCR quantification of *Colletotrichum coccodes* DNA in soils from bioherbicide field-release assays, with normalization for PCR inhibition. *Canadian Journal of Plant Pathology-Revue Canadienne De Phytopathologie* **28**, 42-51.

Daugherty MP, Briggs CJ (2007) Multiple sources of isotopic variation in a terrestrial arthropod community: Challenges for disentangling food webs. *Environmental Entomology* **36**, 776-791.

de Doucet MMA, Bertolotti MA, Giayetto AL, Miranda MB (1999) Host range, specificity, and virulence of *Steinernema feltiae*, *Steinernema rarum*, and *Heterorhabditis bacteriophora* (Steinernematidae and Heterorhabditidae) from Argentina. *Journal of Invertebrate Pathology* **73**, 237-242.

de Leon JH, Fournier V, Hagler JR, Daane KM (2006) Development of molecular diagnostic markers for sharpshooters *Homalodisca coagulata* and *Homalodisca liturata* for use in predator gut content examinations. *Entomologia Experimentalis Et Applicata* **119**, 109-119.

de Mesel I, Derycke S, Moens T, *et al.* (2004) Top-down impact of bacterivorous nematodes on the bacterial community structure: a microcosm study. *Environmental Microbiology* **6**, 733-744.

de Mesel I, Derycke S, Moens T, *et al.* (2004) Top-down impact of bacterivorous nematodes on the bacterial community structure: a microcosm study. *Environmental Microbiology* **6**, 733-744.

de Nardo EAB, Grewal PS, McCartney D, Stinner BR (2006) Non-target effects of entomopathogenic nematodes on soil microbial community and nutrient cycling processes: A microcosm study. *Applied Soil Ecology* **34**, 250-257.

de Nardo EAB, Sindermann AB, Grewal SK, Grewal PS (2004) Non-susceptibility of earthworm *Eisenia fetida* to the rhabditid nematode *Phasmarhabditis hermaphrodita*, a biocontrol agent of slugs. *Biocontrol Science and Technology* **14**, 93-98.

de Ruiter PC, Moore JC, Zwart KB, *et al.* (1993) Simulation of nitrogen mineralization in the below-ground food webs of two winter wheat fields. *Journal of Applied Ecology* **30**, 95-106.

Deagle BE, Tollit DJ (2006) Quantitative analysis of prey DNA in pinniped faeces: Potential to estimate diet composition? *Conservation Genetics*.

Desquesnes M, Davila AMR (2002) Applications of PCR-based tools for detection and identification of animal trypanosomes: a review and perspectives. *Veterinary Parasitology* **109**, 213-231.

Dodd CS (2004) *Development and optimization of PCR based techniques in predator gut analysis*, Unpublished PhD Thesis, Cardiff University.

Donn S, Daniell TJ, Griffiths BS, Neilson R (2007) T-RFLP approaches to nematode assemblage analysis. *Journal of Nematology* **39**, 81-81.

Dowds BCA, Peters A (2002) Virulence Mechanisms. In: *Entomopathogenic Nematology* (ed. Gaugler R), pp. 225-240. CABI International, Wallingford, Oxon.

Ebssa L, Borgemeister C, Poehling HM (2006) Simultaneous application of entomopathogenic nematodes and predatory mites to control western flower thrips *Frankliniella occidentalis*. *Biological Control* **39**, 66-74.

Egert M, Marhan S, Wagner B, Scheu S, Friedrich MW (2004) Molecular profiling of 16S rRNA genes reveals diet-related differences of microbial communities in soil, gut, and casts of *Lumbricus terrestris* l. (oligochaetes : Lumbricidae). *Fems Microbiology Ecology* **48**, 187-197.

Ekschmitt K, Bakonyi G, Bongers M, *et al.* (2001) Nematode community structure as indicator of soil functioning in European grassland soils. *European Journal of Soil Biology* **37**, 263-268.

Eng MS, Preisser EL, Strong DR (2005) Phoresy of the entomopathogenic nematode *Heterorhabditis marelatus* by a non-host organism, the isopod *Porcellio scaber*. *Journal of Invertebrate Pathology* **88**, 173-176.

Epsky ND, Walter DE, Capinera JL (1988) Potential role of nematophagous microarthropods as biotic mortality factors of entomogenous nematodes (Rhabditida, Steinernematidae, Heterorhabditidae). *Journal of Economic Entomology* **81**, 821-825.

Erbilgin N, Dahlsten DL, Chen PY (2004) Intraguild interactions between generalist predators and an introduced parasitoid of *Glycaspis brimblecombei* (Homoptera : Psylloidea). *Biological Control* **31**, 329-337.

Ester A, van Rozen K, Molendijk LPG (2003) Field experiments using the rhabditid nematode *Phasmarhabditis hermaphrodita* or salt as control measures against slugs in green asparagus. *Crop Protection* **22**, 689-695.

Fenton A, Gwynn RL, Gupta A, *et al.* (2002) Optimal application strategies for entomopathogenic nematodes: integrating theoretical and empirical approaches. *Journal of Applied Ecology* **39**, 481-492.

Ferguson SH (2004) Does predation or moisture explain distance to edge distribution of soil arthropods? *American Midland Naturalist* **152**, 75-87.

Ferris H, Bongers T (2006) Nematode indicators of organic enrichment. *Journal of Nematology* **38**, 3-12.

Ferris H, Venette RC, Scow KM (2004) Soil management to enhance bacterivore and fungivore nematode populations and their nitrogen mineralisation function. *Applied Soil Ecology* **25**, 19-35.

Ffrench-Constant RH, Dowling A, Waterfield NR (2007) Insecticidal toxins from *Photorhabdus* bacteria and their potential use in agriculture. *Toxicon* **49**, 436-451.

Filser J (2002) The role of collembola in carbon and nitrogen cycling in soil. *Pedobiologia* **46**, 234-245.

Finch S (1993) Integrated pest-management of the cabbage root fly and the carrot fly. *Crop Protection* **12**, 423-430.

Fischer P, Erickson SM, Fischer K, *et al.* (2007) Persistence of *Brugia malayi* DNA in vector and non-vector mosquitoes: Implications for xenomonitoring and transmission monitoring of lymphatic filariasis. *American Journal of Tropical Medicine and Hygiene* **76**, 502-507.

Flegg JJ, Hooper DJ (1970) Extraction of free-living stages from the soil. In: Laboratory Methods for work with Plant and Soil nematodes. Ministry of Agriculture, Fisheries and Food. London. Pp 5-22

Floyd R, Abebe E, Papert A, Blaxter M (2002) Molecular barcodes for soil nematode identification. *Molecular Ecology* **11**, 839-850.

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for the amplification of mitochondrial cytochrome c oxidase subunit I from diverse

metazoan invertebrates. *Molecular Marine Biology and Biotechnology*, **3**, 294-299.

Foltan P, Sheppard S, Konvicka M, Symondson WOC (2005) The significance of facultative scavenging in generalist predator nutrition: Detecting decayed prey in the guts of predators using PCR. *Molecular Ecology* **14**, 4147-4158.

Foucher A, Bongers T, Noble LR, Wilson MJ (2004) Assessment of nematode biodiversity using DGGE of 18S rDNA following extraction of nematodes from soil. *Soil Biology and Biochemistry* **36**, 2027-2032.

Foucher A, Wilson M (2002) Development of a polymerase chain reaction-based denaturing gradient gel electrophoresis technique to study nematode species biodiversity using the 18s rDNA gene. *Molecular Ecology Notes* **2**, 45-48.

Fountain MT, Hopkin SP (2005) *Folsomia candida* (Collembola): A "standard" soil arthropod. *Annual Review of Entomology* **50**, 201-222.

Fournier E, Loreau M (2001) Activity and satiation state in *Pterostichus melanarius*: an experiment in different agricultural habitats. *Ecological Entomology* **26**, 235-244.

Frank JH, McCoy ED (2007) The risk of classical biological control in Florida. *Biological Control* **41**, 151-174.

Frank SD, Wratten SD, Sandhu HS, Shrewsbury PM (2007) Video analysis to determine how habitat strata affects predator diversity and predation of *Epiphyas postvittana* (Lepidoptera : Tortricidae) in a vineyard. *Biological Control* **41**, 230-236.

Frazier MR, Huey RB, Berrigan D (2006) Thermodynamics constrains the evolution of insect population growth rates: "Warmer is better". *American Naturalist* **168**, 512-520.

Gardner SL, Stock SP, Kaya HK (1994) A New Species of *Heterorhabditis* from the Hawaiian-Islands. *Journal of Parasitology* **80**, 100-106.

Gariepy TD, Kuhlmann U, Gillott C, Erlandson M (2007) Parasitoids, predators and PCR: The use of diagnostic molecular markers in biological control of arthropods. *Journal of Applied Entomology* **131**, 225-240.

Gariepy TD, Kuhlmann U, Haye T, Gillott C, Erlandson M (2005) A single-step multiplex PCR assay for the detection of European *Peristenus* spp., parasitoids of *Lygus* spp. *Biocontrol Science and Technology* **15**, 481-495.

Gaugler R, Lewis E, Stuart RJ (1997) Ecology in the service of biological control: The case of entomopathogenic nematodes. *Oecologia* **109**, 483-489.

Georgis R, Kaya HK, Gaugler R (1991) Effect of Steinernematid and Heterorhabditid nematodes (Rhabditida, Steinernematidae and Heterorhabditidae) on non-target arthropods. *Environmental Entomology* **20**, 815-822.

Georgis R, Koppenhofer AM, Lacey LA, *et al.* (2006) Successes and failures in the use of parasitic nematodes for pest control. *Biological Control* **38**, 103-123.

Georgis R, Wojcik W (1987) The effect of entomogenous nematodes *Heterorhabditis heliothidis* and *Steinernema feltiae* on selected predatory soil insects. *Journal of Nematology* **19**, 523-524.

Giblin-Davis RM, Hazir S, Center BJ, *et al.* (2005) *Bursaphelenchus anatolius* n. sp (Nematoda : Parasitaphelenchidae), an associate of bees in the genus *Halictus*. *Journal of Nematology* **37**, 336-342.

Giller PS (1986) The natural diet of the Notonectidae - field trials using electrophoresis. *Ecological Entomology* **11**, 163-172.

Gillet S, Ponge JF (2003) Changes in species assemblages and diets of Collembola along a gradient of metal pollution. *Applied Soil Ecology* **22**, 127-138.

Gillet S, Ponge JF (2005) Species assemblages and diets of Collembola in the organic matter accumulated over an old tar deposit. *European Journal of Soil Biology* **41**, 39-44.

Gilmore SK (1970) Collembola predation on nematodes. *Search: Agriculture* **1**, 1-12.

Gilmore SK, Potter DA (1993) Potential Role of collembola as biotic mortality agents for entomopathogenic nematodes. *Pedobiologia* **37**, 30-38.

Gilmore SK, Raffen EM (1970) Foods ingested by *Tomocerus* Spp Collembola, Entomobryidae in relation to habitat. *Pedobiologia* **10**, 135

Glazer I (1996) Survival mechanisms of entomopathogenic nematodes. *Biocontrol Science and Technology* **6**, 373-378.

Glen DM, Wilson MJ (1997) Slug-parasitic nematodes as biocontrol agents for slugs. *Agro Food Industry Hi-Tech* **8**, 23-27.

Glen DM, Wilson MJ, Brain P, Stroud G (2000) Feeding activity and survival of slugs, *Deroceras reticulatum*, exposed to the rhabditid nematode, *Phasmarhabditis hermaphrodita*: A model of dose response. *Biological Control* **17**, 73-81.

Goralczyk K (1998) Nematodes in a coastal dune succession: Indicators of soil properties? *Applied Soil Ecology* **9**, 465-469.

Gotelli N, Ellison A (2004) *A primer of ecological statistics* Sinauer Associates, Massachusetts, USA.

Grant JA, Villani MG (2003) Soil moisture effects on entomopathogenic nematodes. *Environmental Entomology* **32**, 80-87.

Greenslade P, Simpson JA, Grgurinovic CA (2002) Collembola associated with fungal fruit-bodies in Australia. *Pedobiologia* **46**, 345-352.

Greenstone MH (2006) Molecular methods for assessing insect parasitism. *Bulletin of Entomological Research* **96**, 1-13.

Greenstone MH, Shufran KA (2003) Spider predation: Species-specific identification of gut contents by polymerase chain reaction. *Journal of Arachnology* **31**, 131-134.

Grenier E, Bonifassi E, Abad P, Laumond C (1996) Use of species-specific satellite DNA's as diagnostic probes in the identification of Steinernematidae and Heterorhabditidae entomopathogenic nematodes. *Parasitology* **113**, 483-489.

Grenier E, Laumond C, Abad P (1996) Molecular characterization of two species-specific tandemly repeated DNA from entomopathogenic nematodes *Steinernema* and *Heterorhabditis* (Nematoda: Rhabditida). *Molecular and Biochemical Parasitology* **83**, 47-56.

Gress J, Stock S (2007) Nematodes against nematodes: Assessing entomopathogenic nematodes (Steinernematidae, Heterorhabditidae) for the control of the citrus nematode *Tylenchulus semipenetrans* (Tylenchulidae) in Arizona. *Journal of Nematology* **39**, 78-79.

Grewal PS, Grewal SK, Tan L, Adams BJ (2003) Parasitism of molluscs by nematodes: Types of associations and evolutionary trends. *Journal of Nematology* **35**, 146-156.

Grewal PS, Grewal SK, Taylor RAJ, Hammond RB (2001) Application of molluscicidal nematodes to slug shelters: A novel approach to economic biological control of slugs. *Biological Control* **22**, 72-80.

Grewal PS, Lewis EE, Gaugler R, Campbell JF (1994) Host finding behavior as a predictor of foraging strategy in entomopathogenic nematodes. *Parasitology* **108**, 207-215.

Grewal PS, Lewis EE, Venkatachari S (1999) Allelopathy: A possible mechanism of suppression of plant-parasitic nematodes by entomopathogenic nematodes. *Nematology* **1**, 735-743.

Grewal PS, Martin WR, Miller RW, Lewis EE (1997) Suppression of plant-parasitic nematode populations in turfgrass by application of entomopathogenic nematodes. *Biocontrol Science and Technology* **7**, 393-399.

Grewal PS, Power KT, Grewal SK, Suggars A, Haupricht S (2004) Enhanced consistency in biological control of white grubs (Coleoptera : Scarabaeidae) with new strains of entomopathogenic nematodes. *Biological Control* **30**, 73-82.

Grewal SK, Grewal PS (2003) Survival of earthworms exposed to the slug-parasitic nematode *Phasmarhabditis hermaphrodita*. *Journal of Invertebrate Pathology* **82**, 72-74.

Grewal SK, Grewal PS, Hammond RB (2003) Susceptibility of North American native and non-native slugs (Mollusca : Gastropoda) to *Phasmarhabditis hermaphrodita* (Nematoda : Rhabditidae). *Biocontrol Science and Technology* **13**, 119-125.

Griffiths BS, Donn S, Neilson R, Daniell TJ (2006) Molecular sequencing and morphological analysis of a nematode community. *Applied Soil Ecology* **32**, 325-337.

Grimm B (2002) Effect of the nematode *Phasmarhabditis hermaphrodita* on young stages of the pest slug *Arion lusitanicus*. *Journal of Molluscan Studies* **68**, 25-28.

Hack MA (1997) The effects of mass and age on standard metabolic rate in house crickets. *Physiological Entomology* **22**, 325-331.

Hagler JR, Cohen AC (1990) Effects of time and temperature on digestion of purified antigen by *Geocoris punctipes* (Hemiptera, Lygaeidae) reared on artificial diet. *Annals of the Entomological Society of America* **83**, 1177-1180.

Hagler JR, Naranjo SE (1997) Measuring the sensitivity of an indirect predator gut content ELISA: Detectability of prey remains in relation to predator species, temperature, time, and meal size. *Biological Control* **9**, 112-119.

Hajek AE, McManus ML, Delalibera I (2007) A review of introductions of pathogens and nematodes for classical biological control of insects and mites. *Biological Control* **41**, 1-13.

Hall TA (1999) BioEdit: A user friendly biological sequence alignment editor and analysis program for Win 95/98/NT. *Nucleic Acids Symposium Series* **41**, 95-98.

Hall SR, Duffy MA, Caceres CE (2005) Selective predation and productivity jointly drive complex behavior in host-parasite systems. *American Naturalist* **165**, 70-81.

Hammond PM, Hawksworth DL, Kalin-Arroyo MT (1995) Magnitude and distribution of diversity. In: *Global Biodiversity Assessment* (ed. Heywood V), pp. 113-138. Cambridge University Press, Cambridge.

Harmon AF, Zarlenga DS, Hildreth MB (2006) Improved methods for isolating DNA from *Ostertagia ostertagi* eggs in cattle feces. *Veterinary Parasitology* **135**, 297-302.

Harper GL, King RA, Dodd CS, *et al.* (2005) Rapid screening of invertebrate predators for multiple prey DNA targets. *Molecular Ecology* **14**, 819-827.

Harper GL, Sheppard SK, Harwood JD, *et al.* (2006) Evaluation of temperature gradient gel electrophoresis for the analysis of prey DNA within the guts of invertebrate predators. *Bulletin of Entomological Research* **96**, 295-304.

Harvey CT, Eubanks MD (2005) Intraguild predation of parasitoids by *Solenopsis invicta*: A non-disruptive interaction. *Entomologia Experimentalis et Applicata* **114**, 127-135.

Hashmi G, Gaugler R (1998) Genetic diversity in insect-parasitic nematodes (Rhabditida : Heterorhabditidae). *Journal of Invertebrate Pathology* **72**, 185-189.

Haspel G, Gefen E, Ar A, Glusman JG, Libersat F (2005) Parasitoid wasp affects metabolism of cockroach host to favor food preservation for its offspring. *Journal of Comparative Physiology a-Neuroethology Sensory Neural and Behavioral Physiology* **191**, 529-534.

Hass B, Downes MJ, Griffin CT (2002) Persistence of four *Heterorhabditis* spp. Isolates in soil: Role of lipid reserves. *Journal of Nematology* **34**, 151-158.

Hass B, Glen DM, Brain P, Hughes LA (1999) Targeting biocontrol with the slug-parasitic nematode *Phasmarhabditis hermaphrodita* in slug feeding areas: A model study. *Biocontrol Science and Technology* **9**, 587-598.

Hass B, Griffin CT, Downes MJ (1999) Persistence of *Heterorhabditis* infective juveniles in soil: Comparison of extraction and infectivity measurements. *Journal of Nematology* **31**, 508-516.

Hass B, Hughes LA, Glen DM (1999) Overall versus band application of the nematode *Phasmarhabditis hermaphrodita* with and without incorporation into soil, for

biological control of slugs in winter wheat. *Biocontrol Science and Technology* **9**, 579-586.

Hatcher MJ, Dick JTA, Dunn AM (2006) How parasites affect interactions between competitors and predators. *Ecology Letters* **9**, 1253-1271.

Haubert D, Haggblom MM, Langel R, Scheu S, Ruess L (2006) Trophic shift of stable isotopes and fatty acids in Collembola on bacterial diets. *Soil Biology and Biochemistry* **38**, 2004-2007.

Haubert D, Langel R, Scheu S, Ruess L (2005) Effects of food quality, starvation and life stage on stable isotope fractionation in Collembola. *Pedobiologia* **49**, 229-237.

Hawkins BA, Askew RR, Shaw MR (1990) Influences of host feeding-niche and food plant type on generalist and specialist parasitoids. *Ecological Entomology* **15**, 275-280.

Hechtel LJ, Johnson CL, Juliano SA (1993) Modification of antipredator behavior of *Caecidotea intermedius* by its parasite *Acanthocephalus dirus*. *Ecology* **74**, 710-713.

Heininger P, Hoss S, Claus E, Pelzer J, Traunspurger W (2007) Nematode communities in contaminated river sediments. *Environmental Pollution* **146**, 64-76.

Heusner AA (1985) Body Size and Energy-Metabolism. *Annual Review of Nutrition* **5**, 267-295.

Hodda M, Wanless FR (1994) Nematodes from an English chalk grassland - species distributions. *Nematologica* **40**, 116-132.

Hoddle MS (2003) The effect of prey species and environmental complexity on the functional response of *Franklinothrips orizabensis*: a test of the fractal foraging model. *Ecological Entomology* **28**, 309-318.

Hodgkinson JE (2006) Molecular diagnosis and equine Parasitology. *Veterinary Parasitology* **136**, 109-116.

Hoelmer KA, Osborne LS, Yokomi RK (1994) Interactions of the whitefly predator *Delphastus pusillus* (Coleoptera, Coccinellidae) with parasitized sweet-potato whitefly (Homoptera, Aleyrodidae). *Environmental Entomology* **23**, 136-139.

Hoffmann A, Thimm T, Tebbe CC (1999) Fate of plasmid-bearing, luciferase marker gene tagged bacteria after feeding to the soil microarthropod *Onychiurus fimatus* (Collembola). *Fems Microbiology Ecology* **30**, 125-135.

Hohberg K (2006) Tardigrade species composition in young soils and some aspects on life history of *Macrobiotus richtersi* J. Murray, 1911. *Pedobiologia* **50**, 267-274.

Hohberg K, Traunspurger W (2005) Predator-prey interaction in soil food web: Functional response, size-dependent foraging efficiency, and the influence of soil texture. *Biology and Fertility of Soils* **41**, 419-427.

Holeva R, Phillips MS, Neilson R, *et al.* (2006) Real-time PCR detection and quantification of vector Trichodorid nematodes and tobacco rattle virus. *Molecular and Cellular Probes* **20**, 203-211.

Holland JM, Perry JN, and Winder L (1999) The within-field spatial and temporal distribution of arthropods in winter wheat. *Bulletin of Entomological Research*, **89**, 499-513.

Holt RD, Polis GA (1997) A theoretical framework for intraguild predation. *American Naturalist* **149**, 745-764.

Hominick WM, Briscoe BR, del Pino FG, *et al.* (1997) Biosystematics of entomopathogenic nematodes: current status, protocols and definitions. *Journal of Helminthology* **71**, 271-298.

Hominick WM, Reid AP, Bohan DA, Briscoe BR (1996) Entomopathogenic nematodes: Biodiversity, geographical distribution and the convention on biological diversity. *Biocontrol Science and Technology* **6**, 317-331.

Hood-Nowotny R, Knols BGJ (2007) Stable isotope methods in biological and ecological studies of arthropods. *Entomologia Experimentalis et Applicata* **124**, 3-16.

Hoogendoorn M, Heimpel GE (2001) PCR-based gut content analysis of insect predators: Using ribosomal its-1 fragments from prey to estimate predation frequency. *Molecular Ecology* **10**, 2059-2067.

Hooper DJ, Wilson MJ, Rowe JA, Glen DM (1999) Some observations on the morphology and protein profiles of the slug-parasitic nematodes *Phasmarhabditis hermaphrodita* and *P. neopapillosa* (Nematoda : Rhabditidae). *Nematology* **1**, 173-182.

Hooper DU, Bignell DE, Brown VK, *et al.* (2000) Interactions between aboveground and belowground biodiversity in terrestrial ecosystems: Patterns, mechanisms, and feedbacks. *Bioscience* **50**, 1049-1061.

Hopkin SP (1997) *The biology of springtails. Insecta: Collembola* Oxford. University Press, Oxford.

Hopkin SP (2007) *A key to the Collembola (Springtails) of Britain and Ireland.* FSC Publications, Shrewsbury.

Hori T, Haruta S, Ueno Y, Ishii M, Igarashi Y (2006) Direct comparison of single-strand conformation polymorphism (SSCP) and denaturing gradient gel electrophoresis (DGGE) to characterize a microbial community on the basis of 16s rRNA gene fragments. *Journal of Microbiological Methods* **66**, 165-169.

Hoy MA (1994) *Insect Molecular Genetics: an Introduction to Principals and Applications*. Academic Press, San Diego.

Hu M, Chilton NB, El-Osta YGA, Gasser RB (2003) Comparative analysis of mitochondrial genome data for *Necator americanus* from two endemic regions reveals substantial genetic variation. *International Journal for Parasitology* **33**, 955-963.

Hu M, Chilton NB, Zhu XQ, Gasser RB (2002) Single-strand conformation polymorphism-based analysis of mitochondrial cytochrome c oxidase subunit 1 reveals significant substructuring in hookworm populations. *Electrophoresis* **23**, 27-34.

Hu M, Gasser RB (2006) Mitochondrial genomes of parasitic nematodes - progress and perspectives. *Trends in Parasitology* **22**, 78-84.

Hudson PJ, Dobson AP, Newborn D (1992) Do parasites make prey vulnerable to predation - red grouse and parasites. *Journal of Animal Ecology* **61**, 681-692.

Huhta V, Sulkava P, Viberg K (1998) Interactions between enchytraeid (*Cognettia sphagnetorum*), microarthropod and nematode populations in forest soil at different moistures. *Applied Soil Ecology* **9**, 53-58.

Hutchinson MT, Streu HT (1960) Tardigrades attacking nematodes. *Nematologica* **5**, 149.

Hyvonen R, Persson T (1996) Effects of fungivorous and predatory arthropods on nematodes and tardigrades in microcosms with coniferous forest soil. *Biology and Fertility of Soils* **21**, 121-127.

Iglesias J, Castellejo J, Castro R (2001) Field test using the nematode *Phasmarhabditis hermaphrodita* for biocontrol of slugs in Spain. *Biocontrol Science and Technology* **11**, 93-98.

Iglesias J, Castillejo J, Castro R (2001) Mini-plot field experiments on slug control using biological and chemical control agents. *Annals of Applied Biology* **139**, 285-292.

Imaz A, Hernandez MA, Arino AH, Armendariz I, Jordana R (2002) Diversity of soil nematodes across a Mediterranean ecotone. *Applied Soil Ecology* **20**, 191-198.

Imbriani JL, Mankau R (1983) Studies on *Lasioseius scapulatus*, a mesostigmatid mite predaceous on nematodes. *Journal of Nematology* **15**, 523-528.

Ingerson-Mahar J (2002) Relating diet and morphology in adult carabid beetles. In: *The Agroecology of Carabid Beetles* (ed. Holland J), pp. 111-136. Intercept, Andover, UK.

Ingham RE, Trofymow JA, Ingham ER, Coleman DC (1985) Interactions of bacteria, fungi, and their nematode grazers - effects on nutrient cycling and plant-growth. *Ecological Monographs* **55**, 119-140.

Inserra RN, Davis DW (1983) *Hypoaspis* nr *aculeifer* - a mite predacious on root-knot and cyst nematodes. *Journal of Nematology* **15**, 324-325.

Jaffee BA (1992) Population Biology and Biological-Control of Nematodes. *Canadian Journal of Microbiology* **38**, 359-364.

Jaffee BA, Strong DR (2005) Strong bottom-up and weak top-down effects in soil: nematode-parasitized insects and nematode-trapping fungi. *Soil Biology and Biochemistry* **37**, 1011-1021.

Jagdale GB, Somasekhar N, Grewal PS, Klein MG (2002) Suppression of plant-parasitic nematodes by application of live and dead infective juveniles of an entomopathogenic nematode, *Steinernema carpocapsae*, on boxwood (*Buxus* spp.). *Biological Control* **24**, 42-49.

Jarman SN (2004a) Amplicon: Software for designing PCR primers on aligned DNA sequences. *Bioinformatics* **20**, 1644-1645.

Jarman SN, Deagle BE, Gales NJ (2004b) Group-specific polymerase chain reaction for DNA-based analysis of species diversity and identity in dietary samples. *Molecular Ecology* **13**, 1313-1322.

Jatala P (1986) Biological-control of plant-parasitic nematodes. *Annual Review of Phytopathology* **24**, 453-489.

Jess S, Bingham JFW (2004) Biological control of sciarid and phorid pests of mushroom with predatory mites from the genus *Hypoaspis* (Acari: Hypoaspidae) and the entomopathogenic nematode *Steinernema feltiae*. *Bulletin of Entomological Research* **94**, 159-167.

Jiang LQ, Zheng JW, Waeyenberge L, Subbotin SA, Moens M (2005) Duplex PCR based identification of *Bursaphelenchus xylophilus* (Steiner and Buhrer, 1934) Nickle, 1970. *Russian Journal of Nematology* **13**, 115-121.

Johnson DM, Akre BG, Crowley PH (1975) Modelling arthropod predation - wasteful killing by damselfly naiads. *Ecology* **56**, 1081-1093.

Jones GA, Sieving KE, Avery ML, Meagher RL (2005) Parasitized and non-parasitized prey selectivity by an insectivorous bird. *Crop Protection* **24**, 185-189.

Jones KL, Todd TC, Herman MA (2006a) Development of taxon-specific markers for high-throughput screening of microbial-feeding nematodes. *Molecular Ecology Notes* **6**, 712-714.

Jones KL, Todd TC, Wall-Beam JL, *et al.* (2006b) Molecular approach for assessing responses of microbial-feeding nematodes to burning and chronic nitrogen enrichment in a native grassland. *Molecular Ecology* **15**, 2601-2609.

Jones RE (1987) Ants, parasitoids, and the cabbage butterfly *Pieris rapae*. *Journal of Animal Ecology* **56**, 739-749.

Jorgensen HB, Elmholt S, Petersen H (2003) Collembolan dietary specialisation on soil grown fungi. *Biology and Fertility of Soils* **39**, 9-15.

Joyce SA, Burnell AM, Powers TO (1994) Characterization of *Heterorhabditis* Isolates by PCR Amplification of Segments of mtDNA and rDNA Genes. *Journal of Nematology* **26**, 260-270.

Juen A, Traugott M (2005) Detecting predation and scavenging by DNA gut-content analysis: a case study using a soil insect predator-prey system. *Oecologia* **142**, 344-352.

Juen A, Traugott M (2006) Amplification facilitators and multiplex PCR: Tools to overcome PCR-inhibition in DNA-gut-content analysis of soil-living invertebrates. *Soil Biology and Biochemistry* **38**, 1872-1879.

Kariuki GM, Dickson DW (2007) Transfer and development of *Pasteuria penetrans*. *Journal of Nematology* **39**, 55-61.

Kaya HK, Gaugler R (1993) Entomopathogenic nematodes. *Annual Review of Entomology* **38**, 181-206.

Kaya HK, Koppenhofer AM (1996) Effects of microbial and other antagonistic organism and competition on entomopathogenic nematodes. *Biocontrol Science and Technology* **6**, 357-371.

Khan Z, Kim YH (2007) A review on the role of predatory soil nematodes in the biological control of plant parasitic nematodes. *Applied Soil Ecology* **35**, 370-379.

Kim HH, Choo HY, Kaya HK, *et al.* (2004) *Steinernema carpocapsae* (Rhabditida : Steinernematidae) as a biological control agent against the fungus gnat *Bradysia agrestis* (Diptera : Sciaridae) in propagation houses. *Biocontrol Science and Technology* **14**, 171-183.

Klein MG (1990) Efficacy against soil-inhabiting insect pests. In: *Entomopathogenic nematodes in biological control* (eds. Gaugler R, Kaya HK), pp. 195-210. CRC Press, Boca Raton, Florida.

Knudsen R, Gabler HM, Kuris AM, Amundsen PA (2001) Selective predation on parasitized prey - a comparison between two helminth species with different life-history strategies. *Journal of Parasitology* **87**, 941-945.

Koehler HH (1999) Predatory mites (Gamasina, Mesostigmata). *Agriculture Ecosystems and Environment* **74**, 395-410.

Koppenhofer AM, Baur ME, Stock SP, *et al.* (1997) Survival of entomopathogenic nematodes within host cadavers in dry soil. *Applied Soil Ecology* **6**, 231-240.

Koppenhofer AM, Fuzy EM (2004) Effect of white grub developmental stage on susceptibility to entomopathogenic nematodes. *Journal of Economic Entomology* **97**, 1842-1849.

Koppenhofer AM, Fuzy EM (2006) Effect of soil type on infectivity and persistence of the entomopathogenic nematodes *Steinernema scarabaei*, *Steinernema glaseri*, *Heterorhabditis zealandica*, and *Heterorhabditis bacteriophora*. *Journal of Invertebrate Pathology* **92**, 11-22.

Koppenhofer AM, Fuzy EM (2007) Soil moisture effects on infectivity and persistence of the entomopathogenic nematodes *Steinernema scarabaei*, *S. glaseri*, *Heterorhabditis zealandica*, and *H. bacteriophora*. *Applied Soil Ecology* **35**, 128-139.

Koppenhofer AM, Jaffee BA, Muldoon AE, Strong DR, Kaya HK (1996) Effect of nematode-trapping fungi on an entomopathogenic nematode originating from the same field site in California. *Journal of Invertebrate Pathology* **68**, 246-252.

Koppenhofer AM, Kaya HK (2001) Entomopathogenic nematodes and insect pest management. In: *Advances in Biopesticide research* (ed. Koul O), pp. 277-305. Harwood Academic Publishers, Amsterdam, The Netherlands.

Krieger J, Fuerst PA (2002) Evidence of multiple alleles of the nuclear 18S ribosomal RNA gene in sturgeon (Family: Acipenseridae). *Journal of Applied Ichthyology* **18**, 290-297.

Kromp B (1999) Carabid beetles in sustainable agriculture: a review on pest control efficacy, cultivation impacts and enhancement. *Agriculture Ecosystems and Environment* **74**, 187-228.

Laakso J, Setala H (1999) Population- and ecosystem-level effects of predation on microbial-feeding nematodes. *Oecologia* **120**, 279-286.

Lacey LA (2006) Entomopathogenic nematodes for control of codling moth in apples and pears: Overcoming obstacles of environment and attitude. *Journal of Nematology* **38**, 277-277.

Lacey LA, Kaya HK, Bettencourt R (1995) Dispersal of *Steinernema glaseri* (Nematoda, Steinernematidae) in Adult Japanese-Beetles, *Popillia japonica* (Coleoptera, Scarabaeidae). *Biocontrol Science and Technology* **5**, 121-130.

Lacey LA, Unruh TR, Headrick HL (2003) Interactions of two idiobiont parasitoids (Hymenoptera: Ichneumonidae) of codling moth (Lepidoptera : Tortricidae) with the entomopathogenic nematode *Steinernema carpocapsae* (Rhabditida : Steinernematidae). *Journal of Invertebrate Pathology* **83**, 230-239.

Lang A, Gsodl S (2001) Prey vulnerability and active predator choice as determinants of prey selection: A carabid beetle and its aphid prey. *Journal of Applied Entomology-Zeitschrift fur Angewandte Entomologie* **125**, 53-61.

Lang A, Gsodl S (2003) "Superfluous killing" of aphids: a potentially beneficial behaviour of the predator *Poecilus cupreus* (L.) (Coleoptera: Carabidae)? *Zeitschrift fur Pflanzenkrankheiten und Pflanzenschutz-Journal of Plant Diseases and Protection* **110**, 583-590.

Langan AM, Pilkington G, Wheater CP (2001) Feeding preferences of a predatory beetle (*Pterostichus madidus*) for slugs exposed to lethal and sub-lethal dosages of metaldehyde. *Entomologia Experimentalis et Applicata* **98**, 245-248.

Langan AM, Taylor A, Wheater CP (2004) Effects of metaldehyde and methiocarb on feeding preferences and survival of a slug predator (*Pterostichus melanarius* (f.): Carabidae, Pterostichini). *Journal of Applied Entomology* **128**, 51-55.

Lavelle P, Spain AV (2001) *Soil ecology* Kluwer Academic Publishers, Dordrecht.

Leal I, Green M, Allen E, Humble L, Rott M (2005) An effective PCR-based diagnostic method for the detection of *Bursaphelenchus xylophilus* (Nematoda :

Aphelenchoididae) in wood samples from lodgepole pine. *Nematology* **7**, 833-842.

Lee Q, Widden P (1996) *Folsomia candida*, a "fungivorous" collembolan, feeds preferentially on nematodes rather than soil fungi. *Soil Biology and Biochemistry* **28**, 689-690.

Lensing JR, Todd S, Wise DH (2005) The impact of altered precipitation on spatial stratification and activity-densities of springtails (collembola) and spiders (Araneae). *Ecological Entomology* **30**, 194-200.

Lewis EE, Gaugler R, Harrison R (1992) Entomopathogenic nematode host finding - response to host contact cues by cruise and ambush foragers. *Parasitology* **105**, 309-315.

Lewis EE, Grewal PS, Sardanelli S (2001) Interactions between the *Steinernema feltiae Xenorhabdus bovienii* insect pathogen complex and the root-knot nematode *Meloidogyne incognita*. *Biological Control* **21**, 55-62.

Lewis EE, Shapiro-Ilan DI (2002) Host cadavers protect entomopathogenic nematodes during freezing. *Journal of Invertebrate Pathology* **81**, 25-32.

Li Y-F, Shen Z-R (2002) PCR-based technique for identification and detection of *Trichogramma* spp. (Hymenoptera: Trichogrammatidae) with specific primers. *Entomologia Sinica* **9**, 9-16.

Lister A, Usher MB, Block W (1987) Description and quantification of field attack rates by predatory mites - an example using an electrophoresis method with a species of Antarctic mite. *Oecologia* **72**, 185-191.

Liu J, Berry RE, Moldenke AF (1997) Phylogenetic relationships of entomopathogenic nematodes (Heterorhabditidae and Steinernematidae) inferred from partial 18S rRNA gene sequences. *Journal of Invertebrate Pathology* **69**, 246-252.

Liu J, Poinar GO, Berry RE (2000) Control of insect pests with entomopathogenic nematodes: The impact of molecular biology and phylogenetic reconstruction. *Annual Review of Entomology* **45**, 287-306.

Loder PMJ, Hargrove JW, Randolph SE (1998) A model for blood meal digestion and fat metabolism in male tsetse flies (Glossinidae). *Physiological Entomology* **23**, 43-52.

Logan JD, Joern A, Wolesensky W (2002) Location, time, and temperature dependence of digestion in simple animal tracts. *Journal of Theoretical Biology* **216**, 5-18.

Lola-Luz T, Downes M (2007) Biological control of black vine weevil *Otiorhynchus sulcatus* in Ireland using *Heterorhabditis megidis*. *Biological Control* **40**, 314-319.

Lovei GL, Sunderland KD (1996) Ecology and behavior of ground beetles (Coleoptera: Carabidae). *Annual Review of Entomology* **41**, 231-256.

Ma J, Li D, Keller M, Schmidt O, Feng X (2005) A DNA marker to identify predation of *Plutella xylostella* (Lep., Plutellidae) by *Nabis kinbergii* (Hem., Nabidae) and Lycosa sp (Aranaea, Lycosidae). *Journal of Applied Entomology* **129**, 330-335.

MacMillan K, Blok V, Young I, Crawford J, Wilson MJ (2006) Quantification of the slug parasitic nematode *Phasmarhabditis hermaphrodita* from soil samples using real time qPCR. *International Journal for Parasitology* **36**, 1453-1461.

Madani M, Subbotin SA, Moens M (2005) Quantitative detection of the potato cyst nematode, *Globodera pallida*, and the beet cyst nematode, *Heterodera schachtii*, using Real-Time PCR with SYBR green I dye. *Molecular and Cellular Probes* **19**, 81-86.

Mair J, Shirley MDF, Rushton SP, Port GR (2001a) Spatial dynamics of predation by carabid beetles on slugs. *Journal of Animal Ecology* **70**, 875-876.

Mair J, Port G (2001b) Predation on the slug *Deroceras reticulatum* by the carabid beetles *Pterostichus madidus* and *Nebria brevicollis* in the presence of alternative prey. *Agricultural and Forest Entomology* **3**, 169-174.

Mair J, Port GR (2002) The influence of mucus production by the slug, *Deroceras reticulatum*, on predation by *Pterostichus madidus* and *Nebria brevicollis* (Coleoptera : Carabidae). *Biocontrol Science and Technology* **12**, 325-335.

Mankau R (1980) Biological-Control of Nematode Pests by Natural Enemies. *Annual Review of Phytopathology* **18**, 415-440.

Mannion CM, McLane W, Klein MG, *et al.* (2001) Management of early-instar Japanese beetle (Coleoptera : Scarabaeidae) in field-grown nursery crops. *Journal of Economic Entomology* **94**, 1151-1161.

Marsh TL (1999) Terminal restriction fragment length polymorphism (T-RFLP): An emerging method for characterizing diversity among homologous populations of amplification products. *Current Opinion in Microbiology* **2**, 323-327.

Maupin JL, Riechert SE (2001) Superfluous killing in spiders: A consequence of adaptation to food-limited environments? *Behavioral Ecology* **12**, 569-576.

McKemey AR, Symondson WOC, Glen DM (2003) Predation and prey size choice by the carabid beetle *Pterostichus melanarius* (Coleoptera : Carabidae): the dangers of extrapolating from laboratory to field. *Bulletin of Entomological Research* **93**, 227-234.

McKemey AR, Symondson WOC, Glen DM, Brain P (2001) Effects of slug size on predation by *Pterostichus melanarius* (Coleoptera : Carabidae). *Biocontrol Science and Technology* **11**, 81-91.

McSorley R, Frederick JJ (2004) Effect of extraction method on perceived composition of the soil nematode community. *Applied Soil Ecology* **27**, 55-63.

Mehrabani D, Motazedian MH, Oryan A, *et al.* (2007) A search for the rodent hosts of *Leishmania major* in the Larestan region of southern Iran: demonstration of the parasite in *Tatera indica* and *Gerbillus* sp., by microscopy, culture and PCR. *Annals of Tropical Medicine and Parasitology* **101**, 315-322.

Meier P, Zettel J (1997) Cold hardiness in *Entomobrya nivalis* (Collembola, Entomobryidae): Annual cycle of polyols and antifreeze proteins, and antifreeze triggering by temperature and photoperiod. *Journal of Comparative Physiology B-Biochemical Systemic and Environmental Physiology* **167**, 297-304.

Metz JAJ, Sabelis MW, Kuchlein JH (1988) Sources of variation in predation rates at high prey densities - an analytic model and a mite example. *Experimental and Applied Acarology* **5**, 187-205.

Mharakurwa S, Simoloka C, Thuma PE, Shiff CJ, Sullivan DJ (2006) PCR detection of *Plasmodium falciparum* in human urine and saliva samples. *Malaria Journal* **5**, 103.

Miles PM (1971) Nematodes associated with *Orchesella villosa* (Geoff.) (Collembola, Onchiuridae). *Entomologist's Gazette* **25**, 31-34.

Mochizuki R, Endoh D, Onuma M, Fukumoto S (2006) PCR-based species-specific amplification of ITS of *Mecistocirrus digitatus* and its application in identification of GI nematode eggs in bovine faeces. *Journal of Veterinary Medical Science* **68**, 345-351.

Mohanty A, Kar P, Mishra K, *et al.* (2007) Multiplex PCR assay for the detection of *Anopheles fluviatilis* species complex, human host preference, and *Plasmodium falciparum* sporozoite presence, using a unique mosquito processing method. *American Journal of Tropical Medicine and Hygiene* **76**, 837-843.

Moore JE, Millar BC, Kenny F, *et al.* (2007) Detection of *Cryptosporidium parvum* in lettuce. *International Journal of Food Science and Technology* **42**, 385-393.

Morales ME, Wesson DM, Sutherland IW, *et al.* (2003) Determination of *Anopheles gambiae* larval DNA in the gut of insectivorous dragonfly (Libellulidae) nymphs by polymerase chain reaction. *Journal of the American Mosquito Control Association* **19**, 163-165.

Morales SE, Mouser PJ, Ward N, *et al.* (2006) Comparison of bacterial communities in New England sphagnum bogs using terminal restriction fragment length polymorphism (T-RFLP). *Microbial Ecology* **52**, 34-44.

Moreno M, Cano J, Nzambo S, *et al.* (2004) Malaria Panel Assay versus PCR: detection of naturally infected *Anopheles melas* in a coastal village of Equatorial Guinea. *Malaria Journal* **3**.

Moriyama EN, Powell JR (1997) Synonymous substitution rates in *Drosophila*: Mitochondrial versus nuclear genes. *Journal of Molecular Evolution* **45**, 378-391.

Mracek Z, Becvar S, Kindlmann P, Webster JM (1999) Factors influencing the infectivity of a Canadian isolate of *Steinernema kraussei* (Nematoda : Steinernematidae) at low temperature. *Journal of Invertebrate Pathology* **73**, 243-247.

Muaoka M, Ishibashi (1976) Nematode feeding mites and their feeding behavior. *Applied Entomology and Zoology,* **11**, 1-7.

Mueller RL (2006) Evolutionary rates, divergence dates and the performance of mitochondrial genes in Bayesian phylogenetic analysis. *Systematic Biology*, **55**, 289-300.

Muller CB, Brodeur J (2002) Intraguild predation in biological control and conservation biology. *Biological Control* **25**, 216-223.

Muraoka M, Ishibashi N (1976) Nematode feeding mites and their feeding behaviour. *Applied Entomology and Zoology* **11**, 1-7.

Nadler SA, Bolotin E, Stock SP (2006) Phylogenetic relationships of *Steinernema* Travassos, 1927 (Nematoda : Cephalobina : Steinernematidae) based on nuclear, mitochondrial and morphological data. *Systematic Parasitology* **63**, 161-181.

Neher DA (1999) Nematode communities in organically and conventionally managed agricultural soils. *Journal of Nematology* **31**, 142-154.

Neher DA (2001) Role of nematodes in soil health and their use as indicators. *Journal of Nematology* **33**, 161-168.

Nguyen KB, Maruniak J, Adams BJ (2001) Diagnostic and phylogenetic utility of the rDNA internal transcribed spacer sequences of *Steinernema*. *Journal of Nematology* **33**, 73-82.

Nguyen KB, Smart GC (1994) *Neosteinernema longicurvicauda* N-Gen, N-Sp (Rhabditida, Steinernematidae), a Parasite of the Termite *Reticulitermes flavipes* (Koller). *Journal of Nematology* **26**, 162-174.

Nickle WR, Shapiro M (1994) Effects of 8 Brighteners as Solar-Radiation Protectants for *Steinernema carpocapsae*, All Strain. *Journal of Nematology* **26**, 782-784.

Nielsen O (2003) Susceptibility of *Delia radicum* to steinernematid nematodes. *Biocontrol* **48**, 431-446.

Nuchprayoon S, Junpee A, Poovorawan Y, Scott AL (2005) Detection and differentiation of filarial parasites by universal primers and polymerase chain reaction-restriction fragment length polymorphism analysis. *American Journal of Tropical Medicine and Hygiene* **73**, 895-900.

O'Halloran DM, Burnell AM (2003) An investigation of chemotaxis in the insect parasitic nematode *Heterorhabditis bacteriophora*. *Parasitology* **127**, 375-385.

Okada H, Harada H (2007) Effects of tillage and fertilizer on nematode communities in a japanese soybean field. *Applied Soil Ecology* **35**, 582-598.

Oliveira AR, de Moraes GJ, Ferraz L (2007) Consumption rate of phytonematodes by *Pergalumna* sp (Acari : Oribatida : Galumnidae) under laboratory conditions determined by a new method. *Experimental and Applied Acarology* **41**, 183-189.

Osborne CA, Rees GN, Bernstein Y, Janssen PH (2006) New threshold and confidence estimates for terminal restriction fragment length polymorphism analysis of complex bacterial communities. *Applied and Environmental Microbiology* **72**, 1270-1278.

Osman GY, Zaki AM, Salem FM, Darwish ETE (1988) Biological-control study on *Tylenchulus semipenetrans* (Cobb) (Nematodes) by certain soil mesofauna. *Anzeiger für Schadlingskunde Pflanzenschutz Umweltschutz* **61**, 116-118.

Pamjav H, Triga D, Buzas Z, *et al.* (1999) Novel application of PhastSystem polyacrylamide gel electrophoresis using restriction fragment length polymorphism - internal transcribed spacer patterns of individuals for molecular identification of entomopathogenic nematodes. *Electrophoresis* **20**, 1266-1273.

Park S, Ku YK, Seo MJ, *et al.* (2006) The characterization of bacterial community structure in the rhizosphere of watermelon (*Citrullus vulgaris* Schard.) using culture-based approaches and terminal fragment length polymorphism (T-RFLP). *Applied Soil Ecology* **33**, 79-86.

Parkman JP, Smart GC (1996) Entomopathogenic nematodes, a case study: Introduction of *Steinernema scapterisci* in Florida. *Biocontrol Science and Technology* **6**, 413-419.

Partsch S, Milcu A, Scheu S (2006) Decomposers (Lumbricidae, Collembola) affect plant performance in model grasslands of different diversity. *Ecology* **87**, 2548-2558.

Payton ME, Greenstone MH, Schenker N (2004) Overlapping confidence intervals or standard error intervals: what do they mean in terms of statistical significance? *Journal of Insect Science*. **3**, 1-6.

Pennacchio F, Strand MR (2006) Evolution of developmental strategies in parasitic hymenoptera. *Annual Review of Entomology* **51**, 233-258.

Pereira MG, Latchford JW, Mudge SM (2006) The use of terminal restriction fragment length polymorphism (T-RFLP) for the characterisation of microbial communities in marine sediments. *Geomicrobiology Journal* **23**, 247-251.

Perez EE, Lewis EE, Shapiro-Ilan DI (2003) Impact of the host cadaver on survival and infectivity of entomopathogenic nematodes (Rhabditida : Steinernematidae and Heterorhabditidae) under desiccating conditions. *Journal of Invertebrate Pathology* **82**, 111-118.

Perry RN, Hominick WM, Beane J, Briscoe B (1998) Effect of the entomopathogenic nematodes, *Steinernema feltiae* and *S. carpocapsae*, on the potato cyst nematode, *Globodera rostochiensis*, in pot trials. *Biocontrol Science and Technology* **8**, 175-180.

Peters A, Ehlers RU (1994) Susceptibility of leatherjackets (*Tipula paludosa* and *Tipula oleracea,* Tipulidae, Nematocera) to the entomopathogenic nematode *Steinernema feltiae*. *Journal of Invertebrate Pathology* **63**, 163-171.

Poinar GO (1976) Taxonomy and biology of a new insect parasitic rhabditoid, *Heterorhabditis bacteriophora* n. gen. n. sp. (Rhabditida: Heterorhabditidae n. fam.). *Nematologica* **21**, 463-470.

Poinar GO (1979) *Nematodes for Biological Control of Insects* CRC Press, Boca Raton, Florida.

Poinar GO (1988) Protozoan diseases. In: *Diseases of Nematodes* (eds. Poinar GO, Jansson HB), pp. 103-131. CRC Press, Boca Raton, Florida.

Poinar GO (1993) Origins and phylogenetic-relationships of the entomophilic *Rhabditis*, *Heterorhabditis* and *Steinernema*. *Fundamental and Applied Nematology* **16**, 333-338.

Poinar GO, Jansson HB (1988) *Diseases of Nematodes* CRC Press, Boca Raton, Florida.

Polis GA, Myers CA, Holt RD (1989) The ecology and evolution of intraguild predation - potential competitors that eat each other. *Annual Review of Ecology and Systematics* **20**, 297-330.

Ponge JF (1991) Food resources and diets of soil animals in a small area of Scots pine litter. *Geoderma* **49**, 33-62.

Ponge JF (2000) Vertical distribution of Collembola (Hexapoda) and their food resources in organic horizons of beech forests. *Biology and Fertility of Soils* **32**, 508-522.

Poulin R (1995) "Adaptive" changes in the behaviour of parasitized animals: A critical review. *International Journal for Parasitology* **25**, 1371-1383.

Powell JR, Webster JM (2004) Interguild antagonism between biological controls: Impact of entomopathogenic nematode application on an aphid predator, *Aphidoletes aphidimyza* (Diptera : Cecidomyiidae). *Biological Control* **30**, 110-118.

Purves WK, Orians GH, Heller HC, *et al.* (1998) *Life: The Science of Biology*, pp 641. Sinauer Associates, Inc. Massachusetts.

Qiu JJ, Westerdahl BB, Anderson C, Williamson VM (2006) Sensitive PCR detection of *Meloidogyne arenaria, M. incognita, and M. javanica* extracted from soil. *Journal of Nematology* **38**, 434-441.

Qiu L, Bedding RA (2000) Energy metabolism and survival of the infective juveniles of *Steinernema carpocapsae* under oxygen-deficient conditions. *Journal of Nematology* **32**, 271-280.

Qvarnstrom Y, Sullivan JJ, Bishop HS, Hollingsworth R, da Silva AJ (2007) PCR-based detection of *Angiostrongylus cantonensis* in tissue and mucus secretions from molluscan hosts. *Applied and Environmental Microbiology* **73**, 1415-1419.

Rae RG, Robertson J, Wilson MJ (2005) Susceptibility of indigenous UK earthworms and an invasive pest flatworm to the slug parasitic nematode *Phasmarhabditis hermaphrodita*. *Biocontrol Science and Technology* **15**, 623-626.

Rae RG, Robertson JF, Wilson MJ (2006) The chemotactic response of *Phasmarhabditis hermaphrodita* (Nematoda : Rhabditida) to cues of *Deroceras reticulatum* (Mollusca : Gastropoda). *Nematology* **8**, 197-200.

Ramos-Rodriguez O, Campbell JF, Ramaswamy SB (2007) Efficacy of the entomopathogenic nematode *Steinernema riobrave* against the stored-product insect pests *Tribolium castaneum* and *Plodia interpunctella*. *Biological Control* **40**, 15-21.

Ratnasingham S, Hebert PDN (2007) BOLD: The Barcode of Life Data System (www.barcodinglife.org). *Molecular Ecology Notes* **7**, 355-364.

Read DS, Sheppard SK, Bruford MW, Glen DM, Symondson WOC (2006) Molecular detection of predation by soil microarthropods on nematodes. *Molecular Ecology* **15**, 1963-1972.

Rees GN, Baldwin DS, Watson GO, Perryman S, Nielsen DL (2004) Ordination and significance testing of microbial community composition derived from terminal restriction fragment length polymorphisms: Application of multivariate statistics. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* **86**, 339-347.

Reid AP, Hominick WM (1992) Restriction-Fragment-Length-Polymorphisms within the ribosomal DNA repeat unit of British entomopathogenic nematodes (Rhabditida, Steinernematidae). *Parasitology* **105**, 317-323.

Reid AP, Hominick WM, Briscoe BR (1997) Molecular taxonomy and phylogeny of entomopathogenic nematode species (Rhabditida: Steinernematidae) by RFLP analysis of the ITS region of the ribosomal DNA repeat unit. *Systematic Parasitology* **37**, 187-193.

Reitz SR, Nettles WC (1994) Regulation of *Helicoverpa zea* larval behavior by the parasitoid *Eucelatoria bryani*. *Entomologia Experimentalis et Applicata* **71**, 33-39.

Riga E, Lacey LA, Guerra N, Headrick HL (2006) Control of the oriental fruit moth, *Grapholita molesta*, using entomopathogenic nematodes in laboratory and fruit bin assays. *Journal of Nematology* **38**, 288-288.

Ropek D, Jaworska M (1994) Effect of an entomopathogenic nematode, *Steinernema carpocapsae* Weiser (Nematoda, Steinernematidae), on carabid beetles in-field trials with annual legumes. *Anzeiger für Schadlingskunde Pflanzenschutz Umweltschutz* **67**, 97-100.

Rosenheim JA (2005) Intraguild predation of *Orius tristicolor* by *Geocoris* spp. and the paradox of irruptive spider mite dynamics in California cotton. *Biological Control* **32**, 172-179.

Rosenheim JA, Kaya HK, Ehler LE, Marois JJ, Jaffee BA (1995) Intraguild predation among biological-control agents - theory and evidence. *Biological Control* **5**, 303-335.

Rosenheim JA, Limburg DD, Colfer RG (1999) Impact of generalist predators on a biological control agent, *Chrysoperla carnea*: Direct observations. *Ecological Applications* **9**, 409-417.

Rozen S, Saletsky HJ (1996-1994) Primer3 on the WWW for general users and for biologist programmers. In: *Bioinformatics Methods and Protocols: Methods in Molecular Biology.* (ed. Krawetz S MS), pp 365-386. Humana Press, Totowa, NJ.

Ruess L, Haggblom MM, Langel R, Scheu S (2004) Nitrogen isotope ratios and fatty acid composition as indicators of animal diets in belowground systems. *Oecologia* **139**, 336-346.

Ruess L, Schutz K, Haubert D, *et al.* (2005a) Application of lipid analysis to understand trophic interactions in soil. *Ecology* **86**, 2075-2082.

Ruess L, Tiunov A, Haubert D, *et al.* (2005b) Carbon stable isotope fractionation and trophic transfer of fatty acids in fungal based soil food chains. *Soil Biology and Biochemistry* **37**, 945-953.

Ryder JJ, Griffin CT (2003) Phased infectivity in *Heterorhabditis megidis*: The effects of infection density in the parental host and filial generation. *International Journal for Parasitology* **33**, 1013-1018.

Saeki Y, Kawano E, Yamashita C, Akao S, Nagatomo Y (2003) Detection of plant parasitic nematodes, *Meloidogyne incognita* and *Pratylenchus coffeae* by multiplex PCR using specific primers. *Soil Science and Plant Nutrition* **49**, 291-295.

Salamon JA, Schaefer M, Alphei J, Schmid B, Scheu S (2004) Effects of plant diversity on collembola in an experimental grassland ecosystem. *Oikos* **106**, 51-60.

Samu F, Biro Z (1993) Functional-response, multiple feeding and wasteful killing in a wolf spider (Araneae, Lycosidae). *European Journal of Entomology* **90**, 471-476.

Samways MJ (2007) Insect conservation: A synthetic management approach. *Annual Review of Entomology* **52**, 465-487.

Sandoval N, Siles-Lucas M, Perez-Arellano JL, *et al.* (2006) A new PCR-based approach for the specific amplification of DNA from different *Schistosoma* species applicable to human urine samples. *Parasitology* **133**, 581-587.

Santos PF, Whitford WG (1981) The effects of microarthropods on litter decomposition in a Chihuahuan desert ecosystem. *Ecology* **62**, 654-663.

Saunders JE, Webster JM (1999) Temperature effects on *Heterorhabditis megidis* and *Steinernema carpocapsae* infectivity to *Galleria mellonella*. *Journal of Nematology* **31**, 299-304.

Sayre RM (1969) A method of culturing the predacious tardigrade on the nematode *Panagrellus redivivus*. *Transactions of the American Microscopical Society* **88**, 266-274.

Sayre RM, Walter DE (1991) Factors affecting the efficacy of natural enemies of nematodes. *Annual Review of Phytopathology* **29**, 149-166.

Schelvis J, Siepel H (1988) Larval Food Spectra of *Pterostichus oblongopunctatus* and *Pterostichus rhaeticus* in the Field (Coleoptera, Carabidae). *Entomologia Generalis* **13**, 61-66.

Scheu S, Falca M (2000) The soil food web of two beech forests (*Fagus sylvatica*) of contrasting humus type: stable isotope analysis of a macro- and a mesofauna-dominated community. *Oecologia* **123**, 285-296.

Schley D, Bees MA (2006) The role of time delays in a non-autonomous host-parasitoid model of slug biocontrol with nematodes. *Ecological Modelling* **193**, 543-559.

Schmidt MH, Thewes U, Thies C, Tscharntke T (2004) Aphid suppression by natural enemies in mulched cereals. *Entomologia Experimentalis Et Applicata* **113**, 87-93.

Shapiro DI, Tylka GL, Berry EC, Lewis LC (1995) Effects of Earthworms on the Dispersal of *Steinernema* spp. *Journal of Nematology* **27**, 21-28.

Shapiro-Ilan DI, Lewis EE, Behle RW, McGuire MR (2001) Formulation of entomopathogenic nematode-infected cadavers. *Journal of Invertebrate Pathology* **78**, 17-23.

Shapiro-Ilan DI, Lewis EE, Tedders WL (2003) Superior efficacy observed in entomopathogenic nematodes applied in infected-host cadavers compared with application in aqueous suspension. *Journal of Invertebrate Pathology* **83**, 270-272.

Sheppard SK, Bell J, Sunderland KD, *et al.* (2005) Detection of secondary predation by PCR analyses of the gut contents of invertebrate generalist predators. *Molecular Ecology* **14**, 4461-4468.

Sheppard SK, Harwood JD (2005) Advances in molecular ecology: Tracking trophic links through predator-prey food-webs. *Functional Ecology* **19**, 751-762.

Sheppard SK, Henneman ML, Memmott J, Symondson WOC (2004) Infiltration by alien predators into invertebrate food webs in Hawaii: a molecular approach. *Molecular Ecology* **13**, 2077-2088.

Sher RB, Parrella MP, Kaya HK (2000) Biological control of the leafminer *Liriomyza trifolii* (Burgess): Implications for intraguild predation between *Diglyphus begini* (Ashmead) and *Steinernema carpocapsae* (Weiser). *Biological Control* **17**, 155-163.

Simard L, Belair G, Brodeur J (2001) Susceptibility of the European chafer (Coleoptera : Scarabaeidae) to entomopathogenic nematodes (Rhabditida : Steinernematidae, Heterorhabditidae). *Journal of Nematology* **33**, 297-301.

Simard L, Belair G, Gosselin ME, Dionne J (2006) Virulence of entomopathogenic nematodes (Rhabditida : Steinernematidae, Heterorhabditidae) against *Tipula paludosa* (Diptera : Tipulidae), a turfgrass pest on golf courses. *Biocontrol Science and Technology* **16**, 789-801.

Simon C, Frati F, Beckenbach A, *et al.* (1994) Evolution, Weighting, and Phylogenetic Utility of Mitochondrial Gene-Sequences and a Compilation of Conserved Polymerase Chain- Reaction Primers. *Annals of the Entomological Society of America,* **87**, 651-701.

Singh BK, Nazaries L, Munro S, Anderson IC, Campbell CD (2006) Use of multiplex terminal restriction fragment length polymorphism for rapid and simultaneous analysis of different components of the soil microbial community. *Applied and Environmental Microbiology* **72**, 7278-7285.

Singh SB (1969) Studies on the gut contents of a suctorial Collembolan *Friesea miribalis* (Tullberg) (Insecta). *Entomologist* **102**, 180-184.

Slansky F (1978) Utilization of energy and nitrogen by larvae of imported cabbageworm, *Pieris rapae* (Lepidoptera : Pieridae) as affected by parasitism by *Apanteles glomeratus* (Hymenoptera : Braconidae). *Environmental Entomology* **7**, 179-185.

Small HJ, Neil DM, Taylor AC, Atkinson RJA, Coombs GH (2006) Molecular detection of *Hematodinium* spp. in Norway lobster *Nephrops norvegicus* and other crustaceans. *Diseases of Aquatic Organisms* **69**, 185-195.

Small HJ, Shields JD, Hudson KL, Reece KS (2007) Molecular detection of *Hematodinium* sp infecting the blue crab, *Callinectes sapidus*. *Journal of Shellfish Research* **26**, 131-139.

Smart GC (1995) Entomopathogenic nematodes for the biological control of insects. *Journal of Nematology* **27**, 529-534.

Smits PH (1996) Post-application persistence of entomopathogenic nematodes. *Biocontrol Science and Technology* **6**, 379-387.

Snowden KF, Logan KS, Vinson SB (2002) Simple, filter-based PCR detection of *Thelohania solenopsae* (Microspora) in fire ants (*Solenopsis invicta*). *Journal of Eukaryotic Microbiology* **49**, 447-448.

Solow AR (1993) A simple test for change in community structure. *Journal of Animal Ecology* **62**, 191-193.

Somasekhar N, Grewal PS, De Nardo EAB, Stinner BR (2002) Non-target effects of entomopathogenic nematodes on the soil nematode community. *Journal of Applied Ecology* **39**, 735-744.

Southey JF (ed.) (1970) *Laboratory Methods for Work with Plant and Soil Nematodes*. Technical Bulletin No. 2, 5th edition. London: Her Majesty's Stationery Office.

Speiser B, Zaller JG, Neudecker A (2001) Size-specific susceptibility of the pest slugs *Deroceras reticulatum* and *Arion lusitanicus* to the nematode biocontrol agent *Phasmarhabditis hermaphrodita*. *Biocontrol* **46**, 311-320.

Spiller DA, Schoener TW (1990) A terrestrial field experiment showing the impact of eliminating top predators on foliage damage. *Nature* **347**, 469-472.

Stock SP, Campbell JF, Nadler SA (2001) Phylogeny of *Steinernema* Travassos, 1927 (Cephalobina : Steinernematidae) inferred from ribosomal DNA sequences and morphological characters. *Journal of Parasitology* **87**, 877-889.

Stock SP, Gress JC (2006) Diversity and phylogenetic relationships of entomopathogenic nematodes (Steinernematidae and Heterorhabditidae) from

the Sky Islands of southern Arizona. *Journal of Invertebrate Pathology* **92**, 66-72.

Stralis-Pavese N, Bodrossy L, Reichenauer TG, Weilharter A, Sessitsch A (2006) 16s rRNA based T-RFLP analysis of methane oxidising bacteria - assessment, critical evaluation of methodology performance and application for landfill site cover soils. *Applied Soil Ecology* **31**, 251-266.

Strong DR (2002) Populations of entomopathogenic nematodes in food-webs. In: *Entomopathogenic Nematology* (ed. Gaugler R), pp. 225-240. CABI International, Wallingford, Oxon.

Strong DR, Whipple AV, Child AL, Dennis B (1999) Model selection for a subterranean trophic cascade: Root-feeding caterpillars and entomopathogenic nematodes. *Ecology* **80**, 2750-2761.

Stuart RJ, Barbercheck ME, Grewal PS, Taylor RAJ, Hoy CW (2006) Population biology of entomopathogenic nematodes: Concepts, issues, and models. *Biological Control* **38**, 80-102.

Suckling DM, Burnip GM, Gibb AR, Daly JM, Armstrong KF (2001) Plant and host effects on the leafroller parasitoid *Dolichogenidia tasmanica*. *Entomologia Experimentalis Et Applicata* **100**, 253-260.

Sulistyanto D, Ehlers RU (1996) Efficacy of the entomopathogenic nematodes *Heterorhabditis megidis* and *Heterorhabditis bacteriophora* for the control of grubs (*Phyllopertha horticola* and *Aphodius contaminatus*) in golf course turf. *Biocontrol Science and Technology* **6**, 247-250.

Sunderland K (1996) Progress in quantifying predation using antibody techniques. In: *The ecology of agricultural pests* (eds. Symondson W, Liddell J), pp. 419-455. Chapman and Hall, London.

Sunderland KD, Crook NE, Stacey DL, Fuller BJ (1987) A study of feeding by polyphagous predators on cereal aphids using Elisa and gut dissection. *Journal of Applied Ecology* **24**, 907-933.

Sutlovic D, Gojanovic MD, Andelinovic S, Gugic D, Primorac D (2005) *Taq* polymerase reverses inhibition of quantitative real time polymerase chain reaction by humic acid. *Croatian Medical Journal* **46**, 556-562.

Symondson WOC (2002) Molecular identification of prey in predator diets. *Molecular Ecology* **11**, 627-641.

Szalanski AL, Taylor DB, Mullin PG (2000) Assessing nuclear and mitochondrial DNA sequence variation within *Steinernema* (Rhabditida : Steinernematidae). *Journal of Nematology* **32**, 229-233.

Tan L, Grewal PS (2001) Infection behavior of the rhabditid nematode *Phasmarhabditis hermaphrodita* to the grey garden slug *Deroceras reticulatum*. *Journal of Parasitology* **87**, 1349-1354.

Thimm T, Hoffmann A, Borkott H, Munch JC, Tebbe CC (1998) The gut of the soil microarthropod *Folsomia candida* (Collembola) is a frequently changeable but selective habitat and a vector for microorganisms. *Applied and Environmental Microbiology* **64**, 2660-2669.

Thompson SN, Redak RA (2005) Feeding behaviour and nutrient selection in an insect *Manduca sexta* l. And alterations induced by parasitism. *Journal of Comparative Physiology a-Neuroethology Sensory Neural and Behavioral Physiology* **191**, 909-923.

Timper P, Kaya HK, Jaffee BA (1991) Survival of entomogenous nematodes in soil infested with the nematode-parasitic fungus *Hirsutella rhossiliensis* (Deuteromycotina: Hyphomycetes). *Biological Control* **1**, 42-50.

Toledo J, Rasgado MA, Ibarra JE, *et al.* (2006) Infection of *Anastrepha ludens* following soil applications of *Heterorhabditis bacteriophora* in a mango orchard. *Entomologia Experimentalis Et Applicata* **119**, 155-162.

Tomalak M (2003) Biocontrol potential of entomopathogenic nematodes against winter moths (*Operophtera brumata* and *O. fagata*) (Lepidoptera : Geometridae) infesting urban trees. *Biocontrol Science and Technology* **13**, 517-527.

Torr P, Spiridonov SE, Heritage S, Wilson MJ (2007) Habitat associations of two entomopathogenic nematodes: A quantitative study using real-time quantitative polymerase chain reactions. *Journal of Animal Ecology* **76**, 238-245.

Triga D, Pamjav H, Vellai T, Fodor A, Buzas Z (1999) Gel electrophoretic restriction fragment length polymorphism analysis of DNA derived from individual nematodes, using the PhastSystem. *Electrophoresis* **20**, 1274-1279.

Tsang WY, Lemire BD (2003) The role of mitochondria in the life of the nematode, *Caenorhabditis elegans*. *Biochimica Et Biophysica Acta-Molecular Basis of Disease* **1638**, 91-105.

Tsiafouli MA, Kallimanis AS, Katana E, Stamou GP, Sgardelis SP (2005) Responses of soil microarthropods to experimental short-term manipulations of soil moisture. *Applied Soil Ecology* **29**, 17-26.

Urzelai A, Hernandez AJ, Pastor J (2000) Biotic indices based on soil nematode communities for assessing soil quality in terrestrial ecosystems. *Science of the Total Environment* **247**, 253-261.

Varga J, Naar Z, Dobolyi C (2002) Selective feeding of collembolan species *Tomocerus longicornis* (Mull.) and *Orchesella cincta* (L.) on moss inhabiting fungi. *Pedobiologia* **46**, 526-538.

Vegter JJ (1983) Food and habitat specialization in coexisting springtails (Collembola, Entomobryidae). *Pedobiologia* **25**, 253-262.

Veremtchuk GV, Issi IV (1970) On the development of the microsporidian of insets in the entomopathogenic nematodes *Neoplectana agriotis* Veremtchuk (Nematodes: Steinernematidae). *Parazitologiya* **4**, 3-7.

Vernava MN, Phillips-Aalten PM, Hughes LA, *et al.* (2004) Influences of preceding cover crops on slug damage and biological control using *Phasmarhabditis hermaphrodita*. *Annals of Applied Biology* **145**, 279-284.

Vincze, T., Posfai, J. and Roberts, R.J. NEBcutter: a program to cleave DNA with restriction enzymes. Nucleic Acids Res. 31: 3688-3691 (2003)

Waite IS, O'Donnell AG, Harrison A, *et al.* (2003) Design and evaluation of nematode 18s rDNA primers for PCR and denaturing gradient gel electrophoresis (DGGE) of soil community DNA. *Soil Biology and Biochemistry* **35**, 1165-1173.

Wajnberg E, Scott J, Quimby P (2001) *Evaluating indirect ecological effects of biological control* CABI, Wallingford, UK.

Walrant A, Loreau M (1995) Comparison of iso-enzyme electrophoresis and gut content examination for determining the natural diet of the ground beetle Species *Abax ater* (Coleoptera, Carabidae). *Entomologia Generalis* **19**, 253-259.

Walsh KT, Webster JM (2003) Interaction of microbial populations in *Steinernema* (Steinernematidae, Nematoda) infected *Galleria mellonella* larvae. *Journal of Invertebrate Pathology* **83**, 118-126.

Walter DE (1987) Trophic behavior of mycophagous microarthropods. *Ecology* **68**, 226-229.

Walter DE, Ikonen EK (1989) Species, guilds, and functional-groups - taxonomy and behavior in nematophagous arthropods. *Journal of Nematology* **21**, 315-327.

Wang Y, Crocker RL, Wilson LT, *et al.* (2001) Effect of nematode and fungal treatments on nontarget turfgrass-inhabiting arthropod and nematode populations. *Environmental Entomology* **30**, 196-203.

Wardle DA (2006) The influence of biotic interactions on soil biodiversity. *Ecology Letters* **9**, 870-886.

Weeden CR, Shelton AM, Hoffman MP (2007). Biological Control: A Guide to Natural Enemies in North America. http://www.nysaes.cornell.edu/ent/biocontrol/ accessed (21/09/2007).

Werner MR, Dindal DL (1987) Nutritional ecology of soil arthropods. In: *Nutritional ecology of insects, mites, spiders and related invertebrates* (eds. Salansky F, Rodriguez JG). John Wiley and Sons.

Wheeler AG (1974) Studies on arthropod fauna of alfalfa .6. Plant bugs (Miridae). *Canadian Entomologist* **106**, 1267-1275.

Wheeler AG (1977) Studies on arthropod fauna of alfalfa .7. Predaceous insects. *Canadian Entomologist* **109**, 423-427.

White GF (1927) A method for obtaining nematode infective larvae from cultures. Science. 66. 302-330.

Williams DA (1976) Improved likelihood ratio tests for complete contingency-tables. *Biometrika* **63**, 33-37.

Willis JC, Bohan DA, Choi YH, Conrad KF, Semenov MA (2006) Use of an individual-based model to forecast the effect of climate change on the dynamics, abundance and geographical range of the pest slug *Deroceras reticulatum* in the UK. *Global Change Biology* **12**, 1643-1657.

Wilson MJ, Glen DM, George SK (1993a) The rhabditid nematode *Phasmarhabditis hermaphrodita* as a potential biological-control agent for slugs. *Biocontrol Science and Technology* **3**, 503-511.

Wilson MJ, Glen DM, George SK, Butler RC (1993b) Mass cultivation and storage of the rhabditid nematode *Phasmarhabditis hermaphrodita*, a biocontrol agent for slugs. *Biocontrol Science and Technology* **3**, 513-521.

Wilson M, Gaugler R (2004) Factors limiting short-term persistence of entomopathogenic nematodes. *Journal of Applied Entomology* **128**, 250-253.

Wilson MJ, Glen DM, Hamacher GM, Smith JU (2004) A model to optimise biological control of slugs using nematode parasites. *Applied Soil Ecology* **26**, 179-191.

Wilson MJ, Glen DM, George SK, Hughes LA (1995a) Biocontrol of slugs in protected lettuce using the rhabditid nematode *Phasmarhabditis hermaphrodita*. *Biocontrol Science and Technology* **5**, 233-242.

Wilson MJ, Glen DM, George SK, Pearce JD (1995b) Selection of a bacterium for the mass-production of *Phasmarhabditis hermaphrodita* (Nematoda, Rhabditidae) as a biocontrol agent for slugs. *Fundamental and Applied Nematology* **18**, 419-425.

Wilson MJ, Glen DM, George SK, Pearce JD, Wiltshire CW (1994a) Biological-control of slugs in winter-wheat using the rhabditid nematode *Phasmarhabditis hermaphrodita*. *Annals of Applied Biology* **125**, 377-390.

Wilson MJ, Glen DM, Wiltshire CW, George SK (1994b) Mini-plot field experiments using the rhabditid nematode *Phasmarhabditis hermaphrodita* for biocontrol of slugs. *Biocontrol Science and Technology* **4**, 103-113.

Wilson MJ, Hughes LA, Hamacher GM, Barahona LD, Glen DM (1996) Effects of soil incorporation on the efficacy of the rhabditid nematode, *Phasmarhabditis hermaphrodita*, as a biological control agent for slugs. *Annals of Applied Biology* **128**, 117-126.

Wilson MJ, Hughes LA, Hamacher GM, Glen DM (2000) Effects of *Phasmarhabditis hermaphrodita* on non-target molluscs. *Pest Management Science* **56**, 711-716.

Wimmer B, Craig BH, Pilkington JG, Pemberton JM (2004) Non-invasive assessment of parasitic nematode species diversity in wild Soay sheep using molecular markers. *International Journal for Parasitology* **34**, 625-631.

Wolesensky W, Joern A, Logan JD (2005) A model of digestion modulation in grasshoppers. *Ecological Modelling* **188**, 358-373.

Wolters V (1985) Resource-Allocation in *Tomocerus flavescens* (Insecta, Collembola) - a Study with C-14-Labeled Food. *Oecologia* **65**, 229-235.

Wolters V (2000) Invertebrate control of soil organic matter stability. *Biology and Fertility of Soils* **31**, 1-19.

Wright RJ, Villani MG, Agudelosilva F (1988) Steinernematid and Heterorhabditid nematodes for control of larval European chafers and Japanese beetles (Coleoptera, Scarabaeidae) in potted yew. *Journal of Economic Entomology* **81**, 152-157.

Yeates GW (1979) Soil nematodes in terrestrial ecosystems. *Journal of Nematology* **11**, 213-229.

Yeates GW (2003) Nematodes as soil indicators: functional and biodiversity aspects. *Biology and Fertility of Soils* **37**, 199-210.

Yeates GW, Bongers T (1999) Nematode diversity in agroecosystems. *Agriculture Ecosystems and Environment* **74**, 113-135.

Yeates GW, Bongers T, Degoede RGM, Freckman DW, Georgieva SS (1993) Feeding-habits in soil nematode families and genera - an outline for soil ecologists. *Journal of Nematology* **25**, 315-331.

Zaidi RH, Jaal Z, Hawkes NJ, Hemingway J, Symondson WOC (1999) Can multiple-copy sequences of prey DNA be detected amongst the gut contents of invertebrate predators? *Molecular Ecology* **8**, 2081-2087.

Zhang G, Lu Z, Wan F, Lovei G (2007a) Real-time PCR quantification of *Bemisia tabaci* (Homoptera: Aleyrodidae) B-biotype remains in predator guts. *Molecular Ecology Notes*.

Zhang GF, Lu ZC, Wan FH (2007b) Detection of *Bemisia tabaci* remains in predator guts using a sequence-characterized amplified region marker. *Entomologia Experimentalis Et Applicata* **123**, 81-90.

Zhou XS, Kaya HK, Heungens K, Goodrich-Blair H (2002) Response of ants to a deterrent factor(s) produced by the symbiotic bacteria of entomopathogenic nematodes. *Applied and Environmental Microbiology* **68**, 6202-6209

Appendices

Appendix 1

Table 1.1 Summary of molecular analyses of predation involving terrestrial and marine invertebrate predators and prey, with details of target gene regions and amplicon sizes

Target groups and species	Target region*	Amplicon size (bp)	Reference
Lepidoptera:			
Helicoverpa armigera	SCAR	1100, 600, 254	Agustí *et al.* (1999)
Ostrinia nubilalis	ITS-1	150, 156, 369, 492	Hoogendoorn & Heimpel (2001)
Scotorythra rara, Eupithecia monticolans, general *Eupithecia* sp. and general geometrid moths	COI	140-170	Sheppard *et al.* (2004)
Plutella xylostella	ITS-1	275	Ma *et al.* (2005)
Hemiptera:			
Trialeurodes vaporariorum	SCAR	2100, 310	Agustí *et al.* (2000)
Schizaphis graminum, Diuraphis noxia, Rhopalosiphum padi, R. Maidis, Sipha flava, Sitobion avenae	COII	77-386	Chen *et al.* (2000)
Cacopsylla pyricola	COI	271, 188	Agustí *et al.* (2003b)
Rhopalosiphum insertum	ND1 and 16S	283	Cuthbertson *et al.* (2003)
Rhopalosiphum maidis	COII	198	Greenstone & Shufran (2003)
Homalodisca coagulate, Homalodisca liturata	SCAR, COI and COII	166-302 166-295	De Leon *et al.* (2005)
Megoura viciae, Sitobion avenae, Metopolophium dirhodum, Rhopalosiphum padi, Myzus persicae, Aphis fabae, general aphids	COI, COII	78-242	Harper *et al.* (2005) (includes two primers developed by Chen *et al.* 2000)
General aphid, *Sitobion avenae*	COI	242, 110	Sheppard *et al* (2005) (using aphid primers developed by Read 2002 and Harper *et al.* 2005)
General aphid	COI	242	Foltan *et al.* (2005) (using primer developed by Harper *et al.* 2005)
General aphids	COI	242	Harper *et al.* (2006)
Bemisia tabaci	SCAR	240	Zhang *et al.* (2007a)
Bemisia tabaci	SCAR	93	Zhang *et al.* (2007b)
Collembola:			
Isotoma anglicana, Lepidocyrtus cyaneus, Entomobrya multifasicata	COI	276 216 211	Agustí *et al.* (2003a)
Coleoptera:			
Sitona sp. weevils	COI	151	Harper *et al.* (2005)
Melolontha melolontha	COI	175, 273, 387, 585	Juen & Traugott (2005)
Amphimallon solstitiale	COI	127, 463	Juen & Traugott (2006)
Leptinotarsa decemilineata, Leptinotarsa juncta	COI	214 219	Greenstone *et al.* (2007)
Phyllopertha horticola	COI	291	Juen & Traugott (2007)

Target groups and species	Target region*	Amplicon size (bp)	Reference
Diptera:			
Culex quinquefasciatus	α esterase	146, 263	Zaidi *et al.* (1999)
Anopheles gambiae	ITS	290	Morales *et al.* (2003)
Copepoda:			
Calanus helgolandicus	COI	172	Vestheim *et al.* (2005)
Crustacea:			
Ceropagis pengoi	16S	154	Gorokhova (2006)
Nematoda:			
Phasmarhabditis hermaphrodita, *Heterorhabditis megidis*, *Steinernema feltiae*	COI	154 150 203	Read *et al.* (2006)
Mollusca:			
Deroceras reticulatum, *Arion hortensis* and *Arion* sp.	12S	109-294	Dodd *et al.* (2003, 2005), Dodd (2004)
Deroceras reticulatum	12S	109	Foltan *et al.* (2005) (using primer developed by Dodd 2004)
Deroceras reticulatum, *Arion hortensis*, *A. intermedius*, *A. distinctus*, general *Arion* sp., *Vallonia pulchella*, *Candidula intersecta*	12S	109-221	Harper *et al.* (2005) (*Deroceras* and *Arion* primers developed by Dodd 2004)
Annelida:			
General earthworms	12S	225-236	Harper *et al.* (2005, 2006)
General earthworm	COI	523	Admassu *et al.* (2005)
General†:			
General invertebrates and vertebrates	16S	500-650	Kasper *et al.* (2004)
General marine invertebrates	COI	~ 700 bp	Blankenship & Yayanos (2005)
General marine vertebrates and invertebrates	16S	183-280	Deagle *et al.* (2005b)
General invertebrates, vertebrates, plants	18S	~ 500	Suzuki *et al.* (2006)
General invertebrates and plants	18S	~240	Martin *et al.* (2006)
General arthropods	Cyt b	358	Pons (2006)
General invertebrates	COI	332	Harper *et al.* (2006)

* Abbreviations are for: COI and COII (cytochrome oxidase I and II genes, mtDNA), 12S and 16S (ribosomal RNA genes, mtDNA), Cyt b (cytochrome b, mtDNA), ND1 (NADH dehydrogenase 1, mtDNA), 18S (ribosomal RNA gene, nuclear DNA), ITS-1 (internal transcribed spacer 1, nuclear DNA), SCAR (sequence characterized amplified region markers, mainly nuclear DNA).

† Several of the other studies used general primers as controls for the presence of amplifiable DNA. These have not been included in the table.

Appendix 2

Table 2.1 Species and groups of invertebrates used for non-target testing with primers *PH-F-(284-304)* and *Ph-R-(479501)*.

No.	Taxon	No.	Taxon
1	*Sitobion avene* (Fabricius, 1794) (Hemiptera: Aphididae)	41	*Deroceras reticulatum* (Muller, 1774) (Pulmonata: Limacoidea)
2	*Rhopalosiphum padi* (Linnaeus, 1758) (Hemiptera: Aphididae)	42	*Arion hortensis* (Ferussaci) (Pulmonata: Arionidae)
3	*Metopolophium dirhodum* (Walker, 1849) (Hemiptera: Aphididae)	43	*Arion distinctus* (Mabille, 1868) (Pulmonata: Arionidae)
4	*Myzus persicae* (Sulzer, 1776) (Hemiptera: Aphididae)	44	*Arion intermedius* (Normand, 1852) (Pulmonata: Arionidae)
5	*Aphis fabae* (Scopoli, 1763) (Hemiptera: Aphididae)	45	*Arion ater* (Linnaeus, 1758) (Pulmonata: Arionidae)
6	*Brevicoryne brassicae* (Linnaeus, 1758) (Hemiptera: Aphididae)	46	*Arion owenii* (Davies, 1979) (Pulmonata: Arionidae)
7	*Nasonovia ribis-nigri* (Mosley, 1841) (Hemiptera: Aphididae)	47	*Limax maximus* (Linnaeus, 1758) (Pulmonata: Limacidae)
8	*Macrosiphum rosae* (Linnaeus, 1758) (Hemiptera: Aphididae)	48	*Limax flavus* (Linnaeus, 1758) (Pulmonata: Limacidae)
9	*Thysanoptera spp* (Thysanoptera)	50	*Heteromurus nitidus* (Templeton, 1835) (Collembola: Entomobryidae)
10	*Oulema melanopus* (Linnaeus) (Coleoptera: Chrysomelidae)	51	*Lepidocyrtus cyaneus* (Tullberg, 1871) (Collembola: Entomobryidae)
11	*Coccinella septempunctata* (Linnaeus) (Coleoptera: Coccinellidae)	52	*Lepidocyrtus curvicolis* (Bourlet, 1839) (Collembola: Entomobryidae)
12	*Adalia bipunctata* (Linnaeus) (Coleoptera: Coccinellidae)	53	*Orchesella villosa* (Linnaeus, 1767) (Collembola: Entomobryidae)
13	*Propylea quatuordecimpunctata* (Linnaeus 1758) (Coleoptera: Coccinellidae)	54	*Orchesella cincta* (Linnaeus, 1758) (Collembola: Entomobryidae)
14	*Thea vigintiduopunctata* (Linnaeus 1758) (Coleoptera: Coccinellidae)	55	*Entomobrya nivalis* (Linnaeus 1758) (Collembola: Entomobryidae)
15	*Forficula auricularia* (Linnaeus, 1758) (Dermaptera: Forficulidae)	56	*Protaphorura armata* (Tullberg, 1869) (Collembola: Onychiuridae)
16	*Tipulidae* sp. (Diptera: Tipulidae)	57	*Isotoma viridis* (Bourlet, 1839) (Collembola: Isotomidae)
17	*Erigone dentipalpis* (Wider, 1834) (Araneae: Linyphiidae)	58	*Isotomurus palustris* (Muller, 1776)(Collembola: Isotomidae)
18	*Erigone atra* (Blackwall, 1833) (Araneae: Linyphiidae)	59	*Folsomia candida* (Willem, 1902) (Collembola: Isotomidae)
19	*Bathyphantes gracilis* (Blackwall, 1841) (Araneae: Linyphiidae)	60	*Isotoma plumosus* (Bagnall, 1940) (Collembola: Isotomidae)
20	*Meioneta rurestris* (Koch, 1836) (Araneae: Linyphiidae)	61	*Stratiolaelaps miles* (Berlese 1892) (Mesostigmata: Laelapidae)
21	*Oedothorax apicatus* (Blackwall, 1841) (Araneae: Linyphiidae)	62	*Hypoaspis aculeifer* (Canestrini, 1884) (Mesostigmata: Laelapidae)
22	*Lepthyphantes tenuis* (Hormiga, 2000) (Araneae: Linyphiidae)	63	*Trombidium* sp. (Prostigmata: Trombidiidae)
23	*Pterostichus melanarius* (Illiger) (Coleoptera: Carabidae)	64	Mesostigmatid mite sp. 1 (Mesostigmata)
24	*Notiophilus biguttatus* (Fabricius, 1779) (Coleoptera: Carabidae)	65	Mesostigmatid mite sp. 2 (Mesostigmata)

No.	Taxon	No.	Taxon
25	*Trechus* sp. (Coleoptera: Carabidae)	66	Mesostigmatid mite sp. 3 (Mesostigmata)
26	*Nebria brevicollis* (Fabricius, 1792) (Coleoptera: Carabidae)	67	Mesostigmatid mite sp. 4 (Mesostigmata)
27	*Poecilus cupreus* (Linnaeus, 1758) (Coleoptera: Carabidae)	68	*Calliphora vomitoria* (Linnaeus, 1758) (Diptera: Calliphoridae)
28	*Tachyporus obtusus* (Linnaeus, 1767) (Coleoptera: Staphylinidae)	69	*Drosophila melanogaster* (Meigen, 1830) (Diptera: Drosophilidae)
29	*Tachyporus chrysomelinus* (Linnaeus, 1758) (Coleoptera: Staphylinidae)	70	*Tenebrio molitor* (Linnaeus, 1758) (Coleoptera: Tenebrionidae)
30	*Rhagonycha fulva* (Scopoli, 1763) (Coleoptera; Cantharidae)	71	*Galleria mellonella* (Linnaeus, 1758) (Lepidoptera: Pyralidae),
31	Scaeva pyrastri (Linnaeus, 1758) (Diptera: Syrphidae	72	*Heterorhabditis megidis* (Poinar, Jackson and Klein) (Rhabditida: Heterorhabditidae)
32	*Metasyrphus luniger* (Diptera: Syrphidae)	73	*Steinernema feltiae* (Filipjev) (Rhabditida: Steinernematidae)
33	*Melanostoma scalare* (Fabricius, 1794) (Diptera: Syrphidae)	74	*Phasmarhabditis hermaphrodita* (Schneider) (Rhabditida: Rhabditidae)
34	*Lumbricus terrestris* (Linnaeus, 1758) (Oligochaeta, lumbricidae)	75	*Phasmarhabditis hermaphrodita* (Schneider) (Rhabditida: Rhabditidae)
35	*Lumbricus rubellus* (Hoff, 1843) (Oligochaeta, lumbricidae)	76	*Phasmarhabditis hermaphrodita* (Schneider) (Rhabditida: Rhabditidae)
36	*Aporrectodea rosea* (Savigny, 1826)	77	
37	Aporrectodea longa (Ude, 1885) (Oligochaeta, lumbricidae)	78	-ve
38	*Aporrectodea caliginosa* (Savigny, 1826) (Oligochaeta, lumbricidae)	79	-ve
39	*Aporrectodea chlorotica 'green'* (Örley 1885) (Oligochaeta, lumbricidae)	80	-ve
40	*Aporrectodea chlorotica 'pink'* (Örley 1885) (Oligochaeta, lumbricidae)	81	-ve

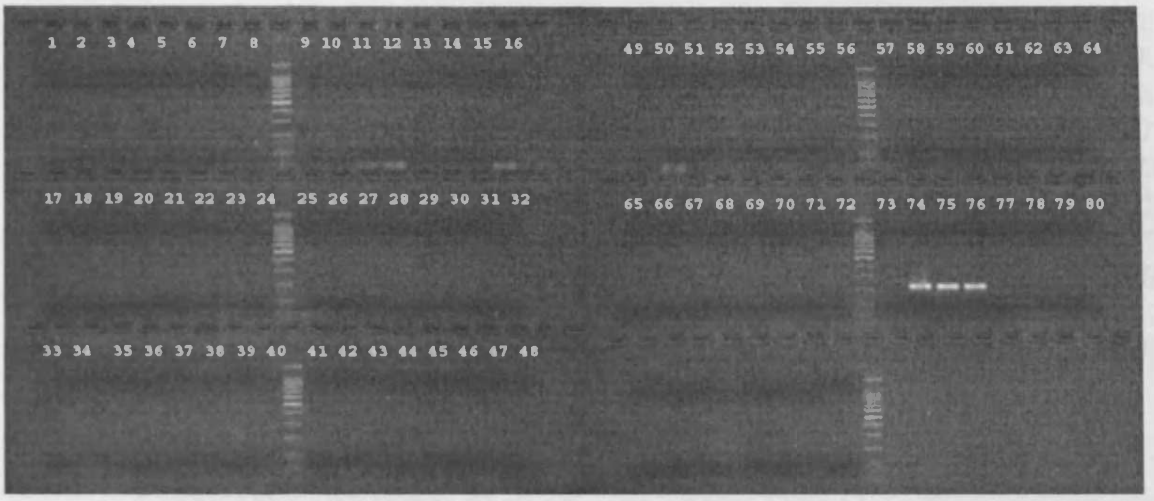


Figure 2.1 Ethidium-bromide stained agarose gel showing non-target testing using primers *PH-F-(284-304)* and *Ph-R-(479501)* against 74 non-target species of invertebrate (see Table 2.1, Appendix 2 for full species listing).

TTGGAGGATTTGGAAATTGATTAGTTCCTCTAATATTGGGGGCACCTGATATGAGTTTT
CCGCGTCTTAATAATTTAAGATTTTGATTGTTGCCTACTTCTATATTTTTAATTTTGGA
TGCTTGTTTTGTTGATATAGGTTGTGGGACGAGATGAACTGTTTATCCGCCTTTGAGAA
CTTTAGGTCATCCGGGAAGAAGGGTTGATTTAGCTATTTTTAGTTTGCATTGTGCTGGT
TTAAGTTCTATTTTAGGTGGAATTAATTTTATAACTACTACTAGTAATTTACGTAGTAG
TTCTATTTCTCTTGAACATATGAGATTGTTTGTTTGAACTGTTTTTGTTACAGTTTTTT
TATTAGTTTTGTCATTACCTGTTTTAGCTGGTGCAATTACTATATTATTGACGGATCGT
AATTTAAATACTTCTTTTTTTGATCCTAGTTCAGGAGGTAATCCTTTAGTATATCAACA
CCTATTTTGATTTTTTGGACACCCTGAAGTTTATATTTTAATTTTACCGGGA

Figure 2.2 Cytochrome Oxidase subunit One (COI) sequence data for *Heterorhabditis megidis* (Rhabditida: Heterorhabditidae).

CTGGAGGATTTGGAAATTGATTAGTTCCTCTTATGTTGGGTGCCCCTGATATAAGATTT
CCACGTTTAAATAATTTAAGTTTTTGATTATTACCTACTTCTATGTTTTTAATCTTGGA
TTCTAGTTTTGTAGATATAGGTTGTGGTACAAGATGAACAGTTTACCCACCATTAAGTA
CCCTTGGTCATCCGGGTAGAAGTGTTGATTTAGCTATTTTTAGTTTACATTGTGCCGGT
TTAAGGTCTATTTTGGGTGGTATTAATTTTATATGTACAACGAAAAATTTACGTAGTAG
TTCTATTTCTTTAGAACATATAAGTTTATTTGTTTGGACTGTTTTTGTTACCGTGTTTT
TATTAGTTTTATCACTTCCTGTTTTAGCAGGTGCTATTACTATATTATTAACTGATCGT
AATTTAAATACTTCTTTTTTTGATCCTAGAACTGGTGGTAATCCTTTAATTTATCAGCA
TTTGTTTTGGTTCTTTGGTCATCCGGAAGTTTATATTTTAATTTTACCGGGAAGG

Figure 2.3 Cytochrome Oxidase subunit One (COI) sequence data for *Phasmarhabditis hermaphrodita* (Rhabditida: Rhabditidae).

TTGGAGGATTTGGAAATTGATTAGTTCCTTTAATACTTGGTGCTCCGGATATAAGATTT
CCTCGTTTAAATAATTTAAGTTTTTGGTTATTACCCACTGCTATATTATTAATTTTAGA
TGCTTGTTTTGTAGATACAGGGTGTGGTACTAGTTGAACTGTTTATCCTCCTTTAAGTA
CTTTAGGCCATCCTGGAAACAGGGTTGATTTAGCTATTTTTAGTCTTCATTGTGCTGGT
GTAAGCTCTATTTTGGGGGCTATTAATTTTATATGTACTACTAAGAATCTTCGTAGAAG
CTCTATTTCTTTAGAGCATATGAGTCTTTTTGTTTGAACTGTTTTTGTTACAGTTTTCC
TTCTTGTTCTTTCTTTACCGGTTTTAGCTGGGGCAATTACTATACTTCTTACAGACCGT
AATTTAAATACTTCTTTTTTTGATCCTAGAGCTGGTGGTAATCCTTTAATTTATCAACA
TCTTTTTTGGTTCTTCGGACATCCTGAAGTTTATATTTTAATTTTACCGGGAA

Figure 2.4 Cytochrome Oxidase subunit One (COI) sequence data for *Steinernema feltiae* (Rhabditida: Steinernematidae).

TTGGAGGATTTGGAAATTGATTAGTTCCTTTAATACTTGGTGCTCCAGATATAAGTTTT
CCTCGTCTTAATAACTTGAGTTTCTGATTACTTCCTACAGCTATGTTACTAATTTTAGA
TGCTTGTTTTGTAGATATAGGTTGTGGTACTAGTTGAACTGTTTATCCTCCTTTAAGAA
CTCTAGGGCACCCTGGTAATAGTGTTGATTTAGCTATTTTCAGTCTTCATTGTGCAGGT
GTTAGCTCTATTCTAGGCGCTATCAACTTTATATGTACCACAAAGAATCTTCGTAGCAG
CTCTATCTCTTTAGAACATATAAGTCTTTTTGTATGGACTGTATTTGTCACTGTTTTTC
TTCTTGTTCTATCACTACCTGTCTTGGCTGGGGCTATCACAATATTGTTAACTGATCGT
AATTTAAACACTTCTTTCTTTGACCCTAGGGCTGGGGGTAATCCTTTAATTTACCAACA
TTTATTTTGGTTTTTTGGTCATCCTGAAGTTTATATTTTAATTTTACCGGGAA

Figure 2.5 Cytochrome Oxidase subunit One (COI) sequence data for *Steinernema kraussei* (Rhabditida: Steinernematidae).

AAAGATATTGGTACTTTATATTTTATTTTTGGAATTTGATCAGGAATAGTTGGAACTTC
CCTAAGATTATTAATTCGAGCTGAACTTGGTAATCCTGGCTCTCTTATTGGAGATGATC
AAATTTATAATACTATTGTTACAGGTCATGCCTTTATTATAATTTTTTTTATAGTAATA
CCTATTATAATTGGTGGATTTGGAAATTGATTAGTTCCCCTAATATTAGGAGCCCCTGA
CATAGCTTTCCCCCGACTTAATAATATAAGTTTTTGATTATTACCTCCTTCTCTTACTT
TATTAATTTTTAGAAGAATTGTTGAAAATGGAGCAGGAACAGGATGAACAGTTTATCCA
CCTTTATCTTCTAATATTGCCCATAGAGGTAGATCTGTAGATTTAGCTATTTTTTCTCT
CCATTTAGCTGGAATTTCTTCTATTTTAGGAGCTGTAAATTTCATTACAACAGTTATTA
ATATAAAATTAAATGGTTTATCATTTGATCAAATACCATTATTTATTTGATCTGTAAGA
ATTACAGCACTATTATTACTTCTTTCATTACCTGTTCTAGCTGGAGCTATTACTATACT
TTTAACTGATCGTAATTTAAACACATCATTTTTTGACCCTGCAGGAGGAGGAGATCCAA
TTCTTTATCAACACTTATTTTGATTTTTTGGTCAC

Figure 2.6 Cytochrome Oxidase subunit One (COI) sequence data for *Galleria mellonella* (Lepidoptera: Pyralidae).

CAAATCATAAAGATATTGGAACATTATACTTCATTTTTGGAGCGTGATCCGGAATAGTC
GGAACCTCCCTAAGAATATTAATCCGTGCAGAATTAGGAAACCCCGGCTCTTTAATTGG
GAACGACCAAATCTACAACGTAATTGTTACAGCACATGCTTTTATCATAATTTTTTTCA
TAGTAATACCAATCATAATTGGAGGATTCGGGAATTGATTAGTACCTCTAATACTAGGT
GCTCCTGACATAGCATTCCCACGAATAAACAACATAAGATTCTGATTGCTACCCCCTTC
ACTAAGACTATTATTAATAAGAAGAATTGTAGAAAACGGGGCGGGAACCGGTTGAACAG
TTTATCCACCCTTATCCTCTAATATCGCCCATGGGGGAGCATCTGTCGATTTAGCAATT
TTCAGGCTACATCTAGCAGGGATTTCATCAATTCTGGGAGCCGTAAATTTTATTACAAC
AGTAATCAACATACGACCACAGGGCATAACGTTCGATCGAATACCGTTATTCGTGTGAG
CAGTAGTAATTACCGCCGTACTATTATTATTATCCTTGCCAGTATTAGCAGGAGCCATC
ACCATATTACTTACAGATCGAAACATTAATACATCCTTCTTTGACCCAGCTGGAGGGGG
AGACCCAATCTTATACCAACACCTATTCTGATTTTTTGGTCACCCT

Figure 2.7 Cytochrome Oxidase subunit One (COI) sequence data for *Tenebrio molitor* (Coleoptera: Tenebrionidae) Haplotype 1.

CATAAAGATATTGGAACATTATACTTCATTTTTGGAGCGTGATCCGGAATAGTCGGAAC
CTCACTAAGACTATTAATCCGTGCAGAATTAGGAAACCCCGGCTCTCTAATTGGAGACG
ACCAAATCTACAACGTAATTGTTACAGCACATGCTTTTATCATAATTTTTTTCATAGTA
ATACCAATCATAATTGGAGGATTCGGAAATTGATTAGTACCTCTAATACTAGGTGCTCC
TGACATAGCATTCCCACGAATAAACAACATAAGATTCTGATTGCTACCCCCTTCACTAA
GACTATTATTAATAAGAAGAATTGTAGAAAACGGGGCGGGAACCGGTTGAACAGTTTAT
CCACCCTTATCCTCTAATATCGCCCATGGAGGAGCATCTGTCGATTTAGCAATTTTCAG
GCTACATCTAGCAGGGATTTCATCAATCCTGGGGGCCGTAAATTTTATTACAACAGTAA
TCAACATACGACCACAGGGCATAACGTTCGATCGAATACCGTTATTCGTGTGAGCAGTA
GTAATTACCGCAGTACTATTATTATTATCCTTGCCAGTATTAGCAGGAGCCATTACCAT
ATTACTTACAGATCGAAACATTAATACATCCTTCTTTGACCCAGCTGGAGGAGGGGACC
CAATCTTATACCAACACCTATTCTGATTTTTTGGTCACCCT

Figure 2.8 Cytochrome Oxidase subunit One (COI) sequence data for *Tenebrio molitor* (Coleoptera: Tenebrionidae) Haplotype 2.

CAAATCATAAAGATATTGGAACATTATACTTCATTTTTGGAGCGTGATCCGGAATAGTC
GGAACCTCACTAAGACTATTAATCCGTGCAGAATTAGGAAACCCCGGCTCTCTAATTGG
AGACGACCAAATCTACAACGTAATTGTTACAGCACATGCTTTTATCATAATTTTTTTCA
TAGTAATACCAATCATAATTGGAGGATTCGGAAATTGATTAGTACCTCTAATACTAGGT
GCTCCTGACATAGCATTCCCACGAATAAACAACATAAGATTCTGATTGCTACCCCCTTC
ACTAAGACTATTATTAATAAGAAGAATTGTAGAAAACGGGGCGGGAACCGGTTGAACAG
TTTATCCACCCTTATCCTCTAATATCGCCCATGGAGGAGCATCTGTCGATTTAGCAATT
TTCAGGCTACATCTAGCAGGGATTTCGTCAATCCTGGGGGCCGTAAATTTTATTACAAC
AGTAATCAACATACGACCACAGGGCATAACGTTCGATCGAATACCGTTATTCGTGTGAG
CAGTAGTAATTACCGCAGTACTATTATTATTATCCTTGCCAGTATTAGCAGGAGCCATT
ACCATATTACTTACAGATCGAAACATTAATACATCCTTCTTTGACCCAGCTGGAGGAGG
GGACCCAATCTTATACCAACACCTATTCTGATTTTTTGGTCACCCT

Figure 2.9 Cytochrome Oxidase subunit One (COI) sequence data for *Tenebrio molitor* (Coleoptera: Tenebrionidae) Haplotype 3.

GGAAATTGATTAGTTCCTTTAATAATCGGGGCCCCTGATATAGCTTTCCCTCGAATAAA
TAATATAAGTTTTTGGTTACTACCTCCTTCTTTAATTCTACTTTTATCCGGAGGATTAG
TAGAAAGAGGAGCTGGAACAGGATGAACCGTTTACCCCCCACTGTCAAGAGGAATCGCT
CATGCAGGGGCTTCCGTAGATTTATCAATTTTTAGACTTCATCTTGCTGGAGCAAGATC
TATTTTAGGGGCTGTAAATTTTATTACAACTATTATTAATATACGAGTCGAAGGAATAA
CATGAGACCGAACACCTTTATTTGTGTGGTCTGTGTTTTTAACCGCCATTCTTCTACTT
TTATCCCTCCCCGTATTAGCAGGGGCTATCACCATACTTCTAACTGACCGTAACCTAAA
TACCTCCTTCTTTGATCCAGCCGGAGGGGGTGACCCAATTTTATATCAACACCTATTCT
GGTTCTTTGGACACCCAGAAGTGTATATTTTAATTTTACCAGGATTTGGTGTAATTTCT
CATATTATTACATTTGAAAGAGGTAAAAAGCAAACATTCGGCCAACTAGGTATAATTTA
TGCTATAATAGCTATCGGCCTACTAGGATTTATCGTATGAGCTCATCATATA

Figure 2.10 Cytochrome Oxidase subunit One (COI) sequence data for *Folsomia candida* (Collembola: Isotomidae).

TTGGAGGATTTGGAAATTGATTAGTTCCTTTAATATTAGGAGCTCCAGATATAGCATTT
CCTCGAATAAATAATATAAGTTTTTGACTTCTTCCCCCATCATTAACTTTATTAATTTC
AAGAAGAATTGTAGAAAATGGAGCAGGAACAGGATGAACAGTTTATCCCCCCCTTTCAT
CCAACATTGCTCATGGAGGTAGATCAGTAGATCTTGCTATTTTTTCCCTTCATTTAGCT
GGTATTTCTTCTATTTTAGGAGCTATTAATTTTATTACCACAATTATTAATATACGATT
AAATAGCTTATCTTTTGATCAAATACCTTTATTTATTTGAGCTGTAGGAATTACTGCAT
TTTTATTATTATTATCTTTACCAGTTTTAGCTGGGGCTATTACTATACTTTTAACAGAT
CGAAATTTAAATACATCTTTTTTTGACCCTGCAGGAGGTGGAGATCCTATTTTATACCA
ACATTTATTTTGATTTTTTGGTCACCCAGAAGTTTATATTTTAATTTTACCGGGA

Figure 2.11 Cytochrome Oxidase subunit One (COI) sequence data for *Agrotis segetum* (Lepidoptera: Noctuidae).

TCCCGGTAAAATTAAAATATAAACTTCAGGATGTCCAAAAAATCAAAATAAATGTTGGT
ATAGAATAGGATCTCCCCCACCAGCCGGATCAAAGAAAGAGGTATTAATATTTCGGTCT
GTGAGTAATATAGTAATAGCCCCAGCTAATACTGGAAGTGAAAGAAGTAATAAGATAGC
AGTAATTTTTACGGCTCAGATAAATAATGATATACGATCTAGGGATATCCCACCTGGGC
GTATATTAATGGCTGTCGAGATAAAATTAATAGCACCTAGGATAGATGAAACCCCTGCT
ATATGTAATCTAAAGATTGCTAAATCTACCGAGGCACCTTCATGTGCAATGTTTGATGA
TAAGGGTGGGTACACAGTTCATCCTGTACCTGCTCCTTTATCAATAATTCTTCTTATTA
ATAAAAGTGATAGAGAAGGTGGGAGTAGCCAAAATCTTATATTATTTAATCGGGGGAAA
GCCATATCTGGGGCCCCAAGTATTAGTGGAACTAATCAATTTCCAAATCCTCCACCA

Figure 2.12 Cytochrome Oxidase subunit One (COI) sequence data for *Otiorhynchus sulcatus* (Coleoptera: Curculionidae).

TTCCCGGTAAAATTAAAATATAAACTTCAGGATGCCCAAAAAATCAAAATAGATGTTGA
TATAAAATTGGATCTCCTCCTCCCGCAGGGTCAAAAAAGGAAGTATTTAAATTTCGATC
TGTTAATAATATAGTAATAGCACCAGCTAAAACTGGTAAAGATAGTAAAAGAAGTAAAG
CTGTAATTACTACAGACCAAACAAATAATGGTATACGGTCAAAGGTAATTCCTGTGGAT
CGTATATTAATTACAGTTGTAATAAAATTTAYAGCTCCTAAAATTGAAGAAATTCCTGC
TAGATGTAAAGAAAAAATTGCTAAATCAACAGAAGCTCCGCCATGAGCGATATTAGAAG
ATAGAGGTGGGTAAACAGTTCATCCTGTCCCAGCTCCATTTTCCACTATACTTCTTACT
AGCAATAAAGTCAATGCAGGAGGTAAGAGTCAAAAACTTATATTATTTATTCGAGGGAA
TGCTATATCTGGAGCACCTAGTATAAGAGGAACTAATCAATTTCCAAATCCTCCA

Figure 2.13 Cytochrome Oxidase subunit One (COI) sequence data for *Delia radicum* (Diptera: Anthomyiidae).

Appendix 3

Tables 3.1 (a) (b) (c) (d) (e) (f) (g) (h) (i) (j) The abundance of microarthropods collected from the soil surface on days -2 to 24 of sampling from both nematode treated and untreated (control) plots. Abbreviations stand for: Ac = Acari *spp.*, Iv = *Isotoma viridis*, Ip = *Isotomurus palustris*, Em = *Entomobrya multifasicata*, En = *Entomobrya nivalis*, Lcu = *Lepidocyrtus curvicolis*, Lcy = *Lepidocyrtus cyaneus*, Ov = Orchesella *villosa*, Oc = *Orchesella cincta*, Sm = *Sminthuridae* spp., Un = *Unidentified collembola.*

Table 3.1 (a) Abundance of surface-dwelling microarthropods from treatment plots on day -2

	Ac	Iv	Ip	Em	En	Lcu	Lcy	Ov	Oc	Sm > 1 mm	Sm < 1 mm	Un > 1 mm	Un < 1 mm
	0	21	2	0	0	0	0	0	0	0	0	0	3
	2	8	8	3	2	2	0	0	0	1	0	0	0
	2	16	12	0	0	2	0	0	0	1	2	0	0
	0	23	25	0	0	1	2	0	0	2	0	0	1
	1	21	2	0	1	0	1	0	1	0	0	0	4
	1	18	6	0	0	1	0	0	0	1	1	0	0
Mean	**1.0**	**17.8**	**9.2**	**0.5**	**0.5**	**1.0**	**0.5**	**0.0**	**0.2**	**0.8**	**0.5**	**0.0**	**1.3**

Table 3.1 (b) Abundance of surface-dwelling microarthropods from control plots on day -2

	Ac	Iv	Ip	Em	En	Lcu	Lcy	Ov	Oc	Sm > 1 mm	Sm < 1 mm	Un > 1 mm	Un < 1 mm
	0	27	6	1	0	1	0	0	0	0	1	1	2
	0	3	7	0	0	0	0	0	0	0	0	0	0
	0	36	2	0	0	2	0	1	0	3	1	0	6
	0	21	15	0	0	0	0	0	1	0	0	0	1
	0	19	3	1	0	5	0	0	0	1	0	0	2
	0	18	19	1	1	0	0	0	0	0	1	1	0
Mean	**0.0**	**20.7**	**8.7**	**0.5**	**0.2**	**1.3**	**0.0**	**0.2**	**0.2**	**0.7**	**0.5**	**0.3**	**1.8**

Table 3.1 (c) Abundance of surface-dwelling microarthropods from treatment plots on day 1

	Ac	Iv	Ip	Em	En	Lcu	Lcy	Ov	Oc	Sm > 1 mm	Sm < 1 mm	Un > 1 mm	Un < 1 mm
	1	39	12	0	0	1	0	0	0	3	0	0	3
	0	30	8	3	0	0	0	0	0	2	2	0	2
	2	31	10	0	0	0	1	0	1	0	5	6	0
	1	17	8	0	2	0	1	0	0	1	1	0	0
	0	39	7	0	0	0	0	0	0	1	2	0	3
	0	17	10	0	0	0	3	0	0	0	1	0	7
Mean	**0.7**	**28.8**	**9.2**	**0.5**	**0.3**	**0.2**	**0.8**	**0.0**	**0.2**	**1.2**	**1.8**	**1.0**	**2.5**

Table 3.1 (d) Abundance of surface-dwelling microarthropods from control plots on day 1

	Ac	Iv	Ip	Em	En	Lcu	Lcy	Ov	Oc	Sm > 1 mm	Sm < 1 mm	Un > 1 mm	Un < 1 mm
	0	14	14	0	0	0	0	0	0	0	0	0	3
	0	8	8	0	0	0	0	0	0	3	0	0	0
	0	13	2	0	2	2	0	0	0	0	2	0	2
	2	38	13	0	0	1	2	0	0	1	0	0	0
	2	21	9	0	0	0	1	0	1	0	0	0	0
	1	18	2	0	1	0	0	0	0	1	1	0	0
Mean	**0.8**	**18.7**	**8.0**	**0.0**	**0.5**	**0.5**	**0.5**	**0.0**	**0.2**	**0.8**	**0.5**	**0.0**	**0.8**

Table 3.1 (e) Abundance of surface-dwelling microarthropods from treatment plots on day 6

	Ac	Iv	Ip	Em	En	Lcu	Lcy	Ov	Oc	Sm > 1 mm	Sm < 1 mm	Un > 1 mm	Un < 1 mm
	0	2	22	5	0	0	1	5	0	0	2	2	0
	1	5	38	17	0	0	0	3	0	0	3	5	0
	0	1	25	12	0	1	1	1	0	0	2	10	0
	0	1	21	1	0	0	1	0	0	0	0	2	0
	0	6	39	27	0	0	0	5	0	0	3	7	0
	4	4	29	13	1	0	0	2	1	1	2	0	2
Mean	**3.2**	**29.0**	**12.5**	**0.2**	**0.2**	**0.5**	**2.7**	**0.2**	**0.2**	**2.0**	**4.3**	**0.3**	**3.0**

Table 3.1 (f) Abundance of surface-dwelling microarthropods from control plots on day 6

	Ac	Iv	Ip	Em	En	Lcu	Lcy	Ov	Oc	Sm > 1 mm	Sm < 1 mm	Un > 1 mm	Un < 1 mm
	0	17	12	0	0	0	0	0	0	0	0	0	0
	1	32	6	0	0	0	1	1	0	0	3	0	0
	0	4	12	0	0	1	0	0	1	2	2	0	2
	0	24	25	0	0	0	3	0	0	0	0	1	7
	0	8	7	0	0	0	0	0	0	1	1	0	0
	4	26	9	3	0	0	0	0	0	0	0	0	2
Mean	**0.8**	**18.5**	**11.8**	**0.5**	**0.0**	**0.2**	**0.7**	**0.2**	**0.2**	**0.5**	**1.0**	**0.2**	**1.8**

Table 3.1 (g) Abundance of surface-dwelling microarthropods from treatment plots on day 12

	Ac	Iv	Ip	Em	En	Lcu	Lcy	Ov	Oc	Sm > 1 mm	Sm < 1 mm	Un > 1 mm	Un < 1 mm
	0	28	8	0	0	0	1	0	1	0	1	0	0
	0	19	8	0	0	2	0	0	0	0	0	1	1
	0	29	5	0	0	0	2	0	0	0	0	0	0
	1	14	12	1	2	0	0	0	0	2	0	0	0
	0	22	3	0	0	0	0	1	0	0	2	0	1
	1	27	13	1	0	0	0	0	0	0	1	0	0
Mean	**0.3**	**23.2**	**8.2**	**0.3**	**0.3**	**0.3**	**0.5**	**0.2**	**0.2**	**0.3**	**0.7**	**0.2**	**0.3**

Table 3.1 (h) Abundance of surface-dwelling microarthropods from control plots on day 12

	Ac	Iv	Ip	Em	En	Lcu	Lcy	Ov	Oc	Sm > 1 mm	Sm < 1 mm	Un > 1 mm	Un < 1 mm
	0	27	11	0	2	0	0	0	0	3	2	0	1
	0	27	10	0	0	0	0	0	0	1	3	0	3
	2	12	18	0	0	0	3	0	0	0	0	0	0
	1	13	11	2	0	0	3	0	0	2	3	0	2
	0	15	4	0	0	0	1	0	0	0	2	1	2
	0	17	0	0	0	0	0	0	0	0	0	0	0
Mean	**0.5**	**18.5**	**9.0**	**0.3**	**0.3**	**0.0**	**1.2**	**0.0**	**0.0**	**1.0**	**1.7**	**0.2**	**1.3**

Table 3.1 (i) Abundance of surface-dwelling microarthropods from treatment plots on day 24

	Ac	Iv	Ip	Em	En	Lcu	Lcy	Ov	Oc	Sm > 1 mm	Sm < 1 mm	Un > 1 mm	Un < 1 mm
	3	9	4	0	0	0	0	0	0	0	4	0	7
	4	4	17	1	0	0	0	0	1	1	1	0	0
	6	14	4	0	0	1	1	0	0	1	1	0	0
	0	19	17	0	0	0	1	0	0	2	8	0	3
	2	18	35	0	1	0	9	0	0	0	0	2	0
	2	17	11	0	0	0	6	0	0	2	7	0	0
Mean	**2.8**	**13.5**	**14.7**	**0.2**	**0.2**	**0.2**	**2.8**	**0.0**	**0.2**	**1.0**	**3.5**	**0.3**	**1.7**

Table 3.1 (j) Abundance of surface-dwelling microarthropods from control plots on day 24

	Ac	Iv	Ip	Em	En	Lcu	Lcy	Ov	Oc	Sm > 1 mm	Sm < 1 mm	Un > 1 mm	Un < 1 mm
	0	12	8	0	0	0	2	1	0	2	7	0	2
	0	7	23	1	0	0	1	0	1	0	0	0	0
	1	14	12	0	0	0	2	0	0	0	1	1	0
	0	12	10	1	0	0	0	0	0	0	3	0	0
	5	8	19	0	0	1	1	0	0	0	4	0	2
	0	12	7	0	1	1	1	0	0	0	0	0	2
Mean	**1.0**	**10.8**	**13.2**	**0.3**	**0.2**	**0.3**	**1.2**	**0.2**	**0.2**	**0.3**	**2.5**	**0.2**	**1.0**

Tables 3.2 (a) (b) (c) (d) (e) (f) (g) (h) (i) (j) Showing the abundance of microarthropods collected from the soil matrix, including the collembola Symphypleona spp., Poduromorpha spp., Entomobryomorpha spp., and the mites (Acari spp.), on days -2 to 24 of sampling from both nematode treated and untreated (control) plots.

Table 3.2 (a) Abundance of soil-dwelling microarthropods from treatment plots on day -2

Plot Number	Symphypleona	Poduromorpha	Entomobryomorpha <1mm	Entomobryomorpha >1mm	Acari	Total
1	2	4	62	1	21	90
2	0	3	21	2	17	43
3	7	8	48	0	11	74
4	1	0	53	2	15	71
5	3	2	39	4	19	67
6	2	7	46	6	10	71
Total	15	24	269	15	93	416
Mean	**2.5**	**4.0**	**44.8**	**2.5**	**15.5**	**69.3**

Table 3.2 (b) Abundance of soil-dwelling microarthropods from control plots on day -2

Plot Number	Symphypleona	Poduromorpha	Entomobryomorpha <1mm	Entomobryomorpha >1mm	Acari	Total
1	2	4	70	2	5	83
2	3	4	66	4	10	87
3	12	5	22	1	20	60
4	0	2	42	3	8	55
5	2	9	72	2	12	97
6	7	1	37	0	23	68
Total	26	25	309	12	78	450
Mean	**4.3**	**4.2**	**51.5**	**2.0**	**13.0**	**75.0**

Table 3.2 (c) Abundance of soil-dwelling microarthropods from treatment plots on day 1

Plot Number	Symphypleona	Poduromorpha	Entomobryomorpha <1mm	Entomobryomorpha >1mm	Acari	Total
1	10	3	188	8	26	235
2	2	0	48	6	10	66
3	1	6	56	1	12	76
4	9	15	82	7	21	134
5	1	3	84	2	6	96
6	2	8	146	4	45	205
Total	25	35	604	28	120	812
Mean	**4.2**	**5.8**	**100.7**	**4.7**	**20.0**	**135.3**

Table 3.2 (d) Abundance of soil-dwelling microarthropods from control plots on day 1

Plot Number	Symphypleona	Poduromorpha	Entomobryomorpha <1mm	Entomobryomorpha >1mm	Acari	Total
1	0	4	36	4	9	53
2	0	7	20	2	11	40
3	8	7	109	2	47	173
4	2	3	51	2	16	74
5	1	0	31	1	19	52
6	2	2	33	1	15	53
Total	13	23	280	12	117	445
Mean	**2.2**	**3.8**	**46.7**	**2.0**	**19.5**	**74.2**

Table 3.2 (e) Abundance of soil-dwelling microarthropods from treatment plots on day 6

Plot Number	Symphypleona	Poduromorpha	Entomobryomorpha <1mm	Entomobryomorpha >1mm	Acari	Total
1	3	5	72	2	29	111
2	4	1	25	0	26	56
3	1	3	56	2	26	88
4	11	10	60	0	36	117
5	1	2	37	0	11	51
6	4	1	71	12	26	114
Total	24	22	321	16	154	537
Mean	**4.0**	**3.7**	**53.5**	**2.7**	**25.7**	**89.5**

Table 3.2 (f) Abundance of soil-dwelling microarthropods from control plots on day 6

Plot Number	Symphypleona	Poduromorpha	Entomobryomorpha <1mm	Entomobryomorpha >1mm	Acari	Total
1	0	0	14	0	8	22
2	5	2	97	4	17	125
3	9	4	74	4	30	121
4	4	1	124	4	42	175
5	5	5	37	1	5	53
6	7	4	44	0	5	60
Total	30	16	390	13	107	556
Mean	**5.0**	**2.7**	**65.0**	**2.2**	**17.8**	**92.7**

Table 3.2 (g) Abundance of soil-dwelling microarthropods from treatment plots on day 12

Plot Number	Symphypleona	Poduromorpha	Entomobryomorpha <1mm	Entomobryomorpha >1mm	Acari	Total
1	11	0	82	1	44	138
2	7	21	53	2	34	117
3	3	9	104	2	32	150
4	5	0	121	1	43	170
5	12	5	70	7	40	134
6	2	2	32	0	22	58
Total	40	37	462	13	215	767
Mean	**6.7**	**6.2**	**77.0**	**2.2**	**35.8**	**127.8**

Table 3.2 (h) Abundance of soil-dwelling microarthropods from control plots on day 12

Plot Number	Symphypleona	Poduromorpha	Entomobryomorpha <1mm	Entomobryomorpha >1mm	Acari	Total
1	2	1	42	2	19	66
2	3	11	46	0	13	73
3	7	9	25	3	14	58
4	2	2	37	1	27	69
5	2	2	47	2	18	71
6	5	13	92	2	31	143
Total	21	38	289	10	122	480
Mean	**3.5**	**6.3**	**48.2**	**1.7**	**20.3**	**80.0**

Table 3.2 (i) Abundance of soil-dwelling microarthropods from treatment plots on day 24

Plot Number	Symphypleona	Poduromorpha	Entomobryomorpha <1mm	Entomobryomorpha >1mm	Acari	Total
1	4	0	23	0	21	48
2	1	0	16	1	12	30
3	0	0	49	2	7	58
4	2	1	62	2	8	75
5	3	0	18	0	19	40
6	1	1	7	1	1	11
Total	11	2	175	6	68	262
Mean	**1.8**	**0.3**	**29.2**	**1.0**	**11.3**	**43.7**

Table 3.2 (j) Abundance of soil-dwelling microarthropods from control plots on day 24

Plot Number	Symphypleona	Poduromorpha	Entomobryomorpha <1mm	Entomobryomorpha >1mm	Acari	Total
1	7	0	25	2	15	49
2	2	1	39	0	12	54
3	3	0	40	0	19	62
4	0	1	12	0	7	20
5	5	1	71	1	11	89
6	1	2	12	2	2	19
Total	18	5	199	5	66	293
Mean	**3.0**	**0.8**	**33.2**	**0.8**	**11.0**	**48.8**

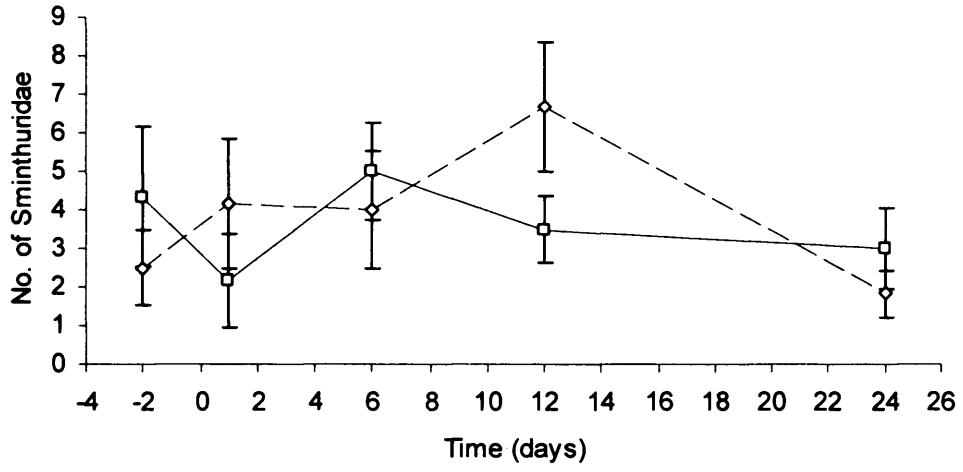


Figure 3.1. Abundance of *Sminthuridae* spp. within the soil matrix against time, in treatment (application of *Phasmarhabditis hermaphrodita* (– –)) and control (water (—)) plots (n = 6). Bars represent standard error (S.E)

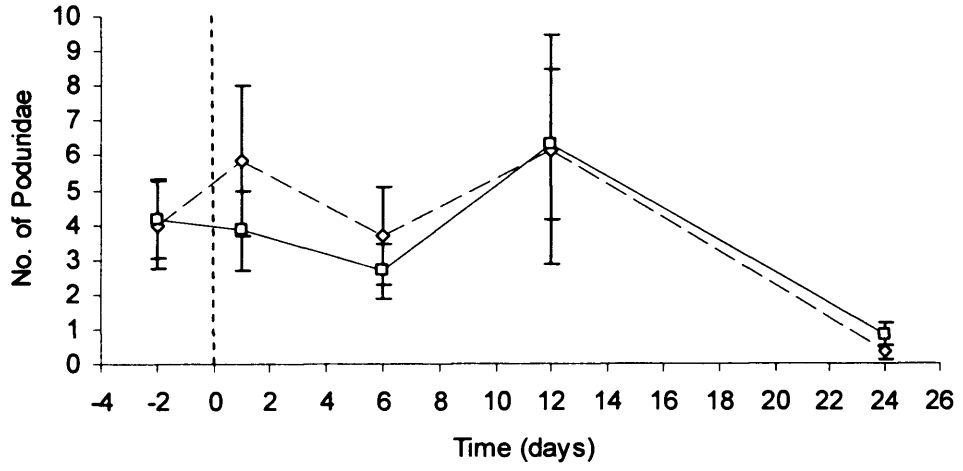


Figure 3.2. Abundance of *Poduroidea* spp. within the soil matrix against time, in treatment (application of *Phasmarhabditis hermaphrodita* (– – –)) and control (water (—)) plots (n = 6). Bars represent standard error (S.E)

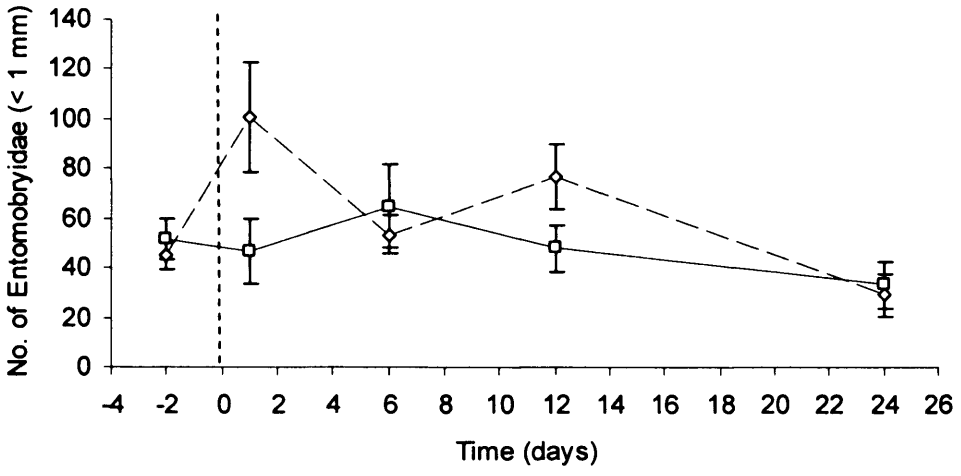


Figure 3.3. Abundance of *Entomobryidae* spp. (< 1 mm) within the soil matrix against time, in treatment (application of *Phasmarhabditis hermaphrodita* (– – –)) and control (water (—)) plots (n = 6). Bars represent standard error (S.E).

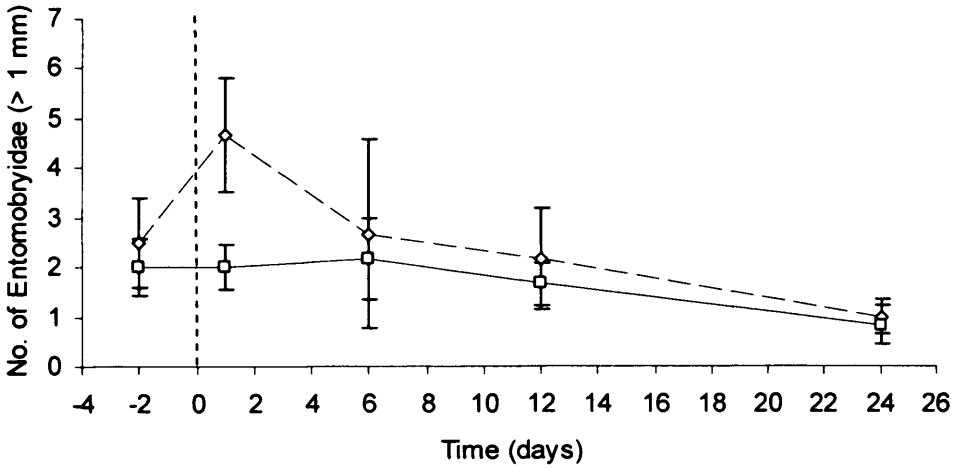


Figure 3.4. Abundance of *Entomobryidae* spp. (> 1 mm) within the soil matrix against time, in treatment (application of *Phasmarhabditis hermaphrodita* (– – –)) and control (water (—)) plots (n = 6). Bars represent standard error (S.E).

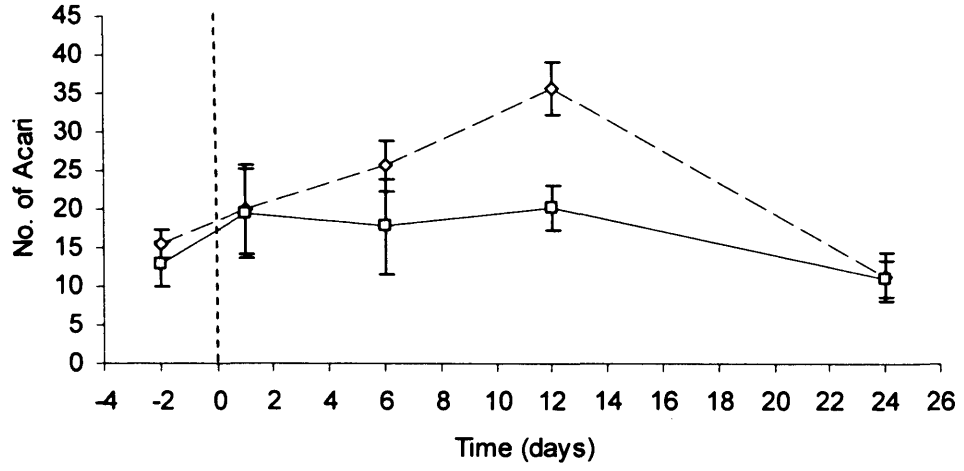


Figure 3.5. Abundance of *Acari* spp. within the soil matrix against time, in treatment (application of *Phasmarhabditis hermaphrodita* (– – –)) and control (water (—)) plots (n = 6). Bars represent standard error (S.E).

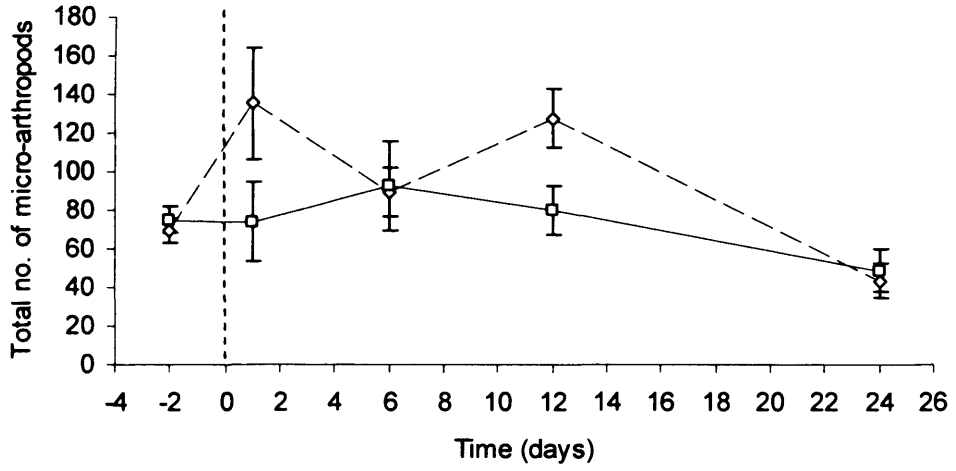


Figure 3.6. Abundance of the total number of microarthropods within the soil matrix against time, in treatment (application of *Phasmarhabditis hermaphrodita* (– – –)) and control (water (—)) plots (n = 6). Bars represent standard error (S.E).

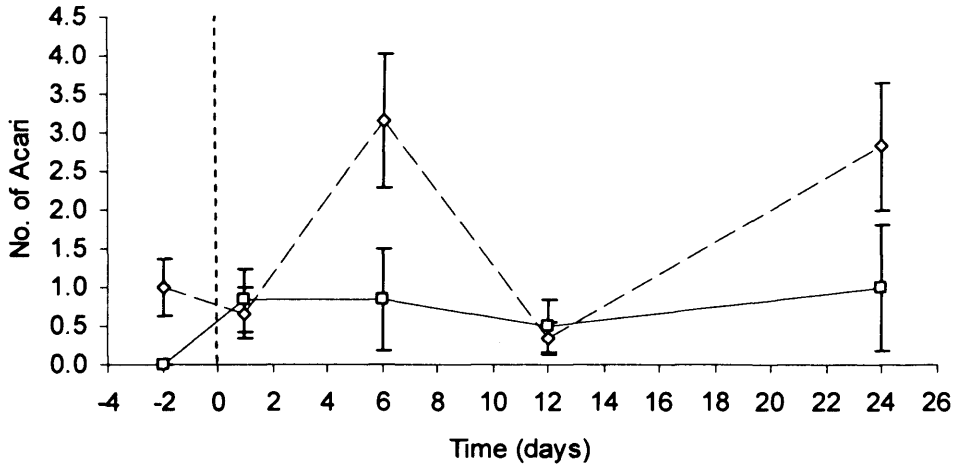


Figure 3.7. Abundance of number of *Acari* spp. on the soil surface against time, in treatment (application of *Phasmarhabditis hermaphrodita* (– – –)) and control (water (—)) plots (n = 6). Bars represent standard error (S.E).

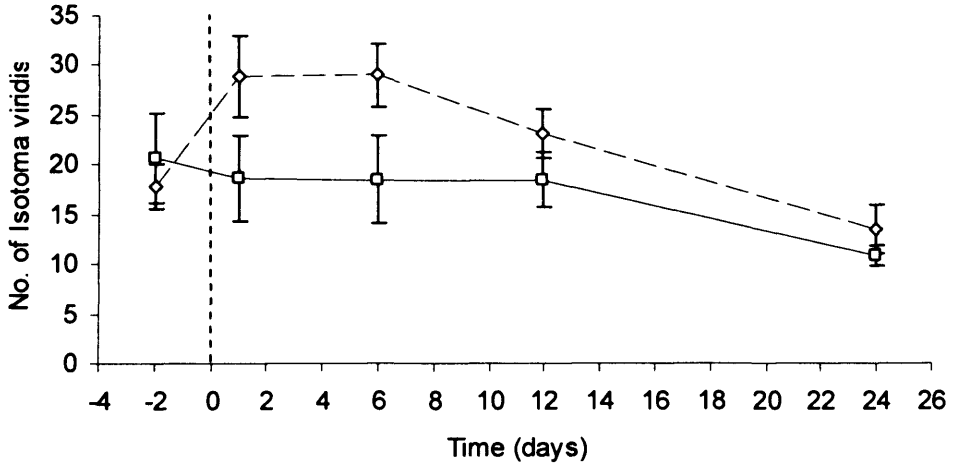


Figure 3.8. Abundance of number of *Isotoma viridis* on the soil surface against time, in treatment (application of *Phasmarhabditis hermaphrodita* (– – –)) and control (water (—)) plots (n = 6). Bars represent standard error (S.E).

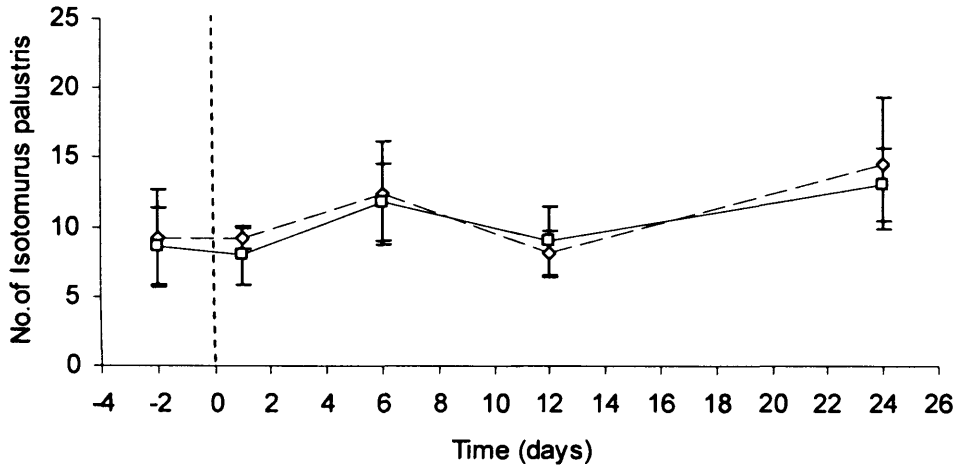


Figure 3.9. Abundance of number of *Isotomurus palustris* on the soil surface against time, in treatment (application of *Phasmarhabditis hermaphrodita* (– – –)) and control (water (—)) plots (n = 6). Bars represent standard error (S.E).

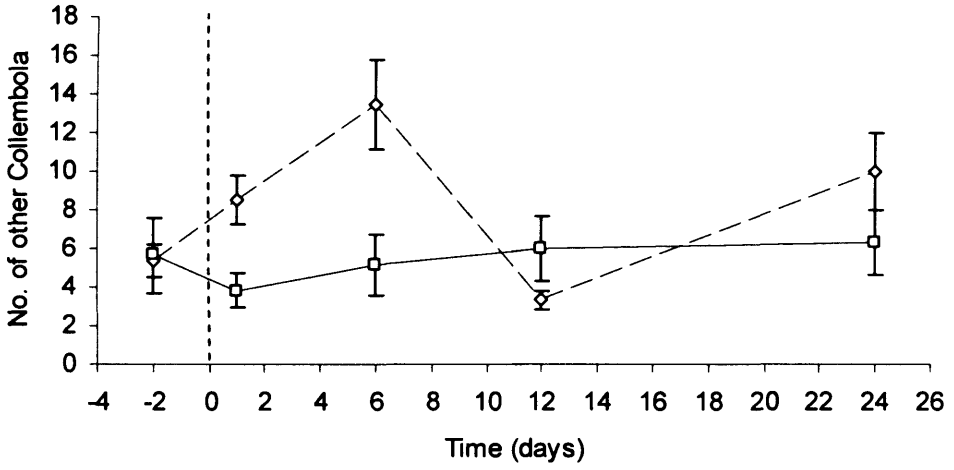


Figure 3.10. Abundance of the number of other collembola on the soil surface against time, in treatment (application of *Phasmarhabditis hermaphrodita* (– – –)) and control (water (—)) plots (n = 6). Bars represent standard error (S.E).

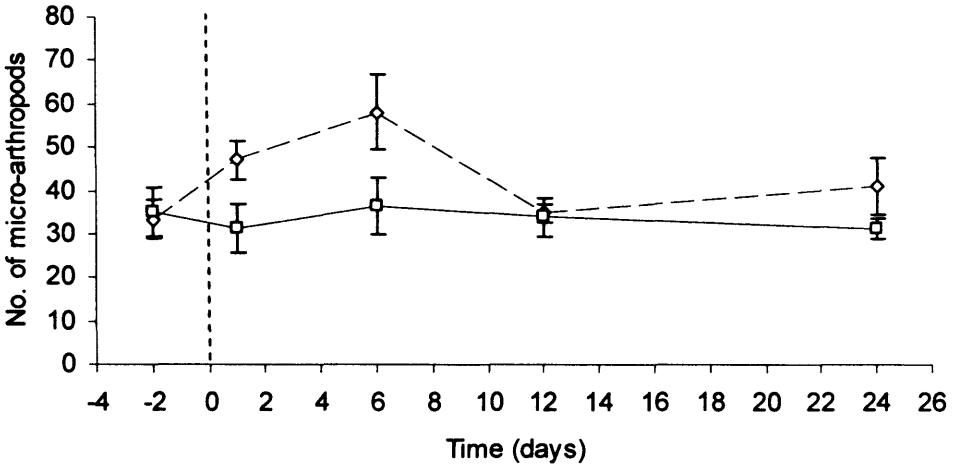


Figure 3.11. Abundance of number of the total number of microarthropods on the soil surface against time, in treatment (application of *Phasmarhabditis hermaphrodita* (– – –)) and control (water (—)) plots (n = 6). Bars represent standard error (S.E).

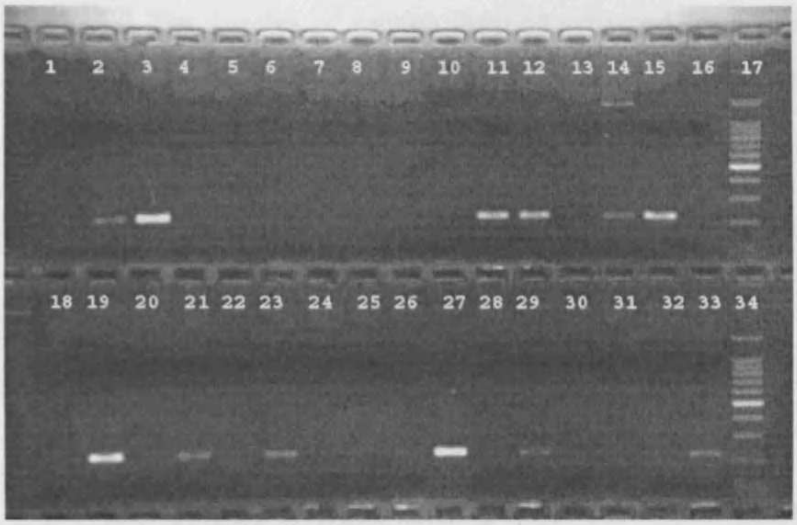


Figure 3.12 Ethidium-bromide stained gel showing the screening of field caught microarthropod predators on day 1 nematode treated plots using primers *Ph-F-(271-291)* and *Ph-R-(417-438)*. Ladder is at 100 bp intervals

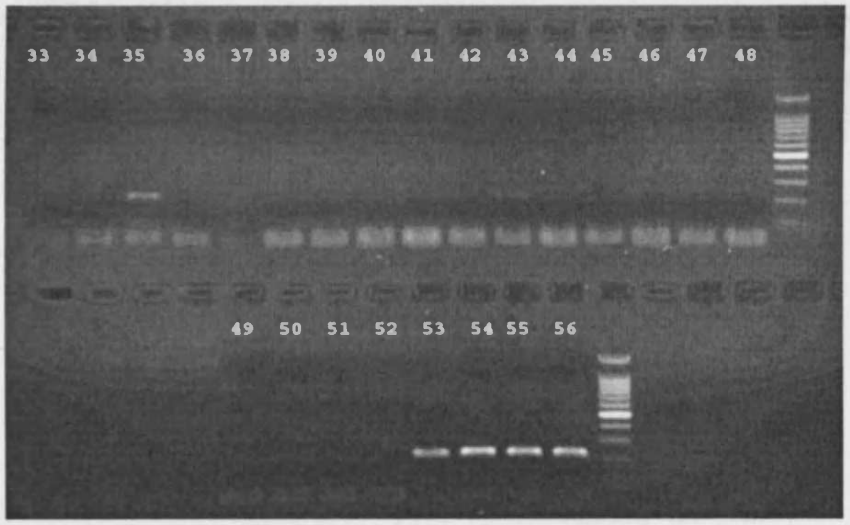


Figure 3.13 Ethidium-bromide stained gel showing the screening of field caught microarthropod predators on day 1 nematode treated plots using primers *Ph-F-(271-291)* and *Ph-R-(417-438)*. Ladder is at 100 bp intervals

Table 3.3 The species of microarthropod screened from treatment plot 1 on day 1 of screening, with gel lane number (Figure 4.5.1) and predation scoring.

No.	Species		No.	Species		No.	Species	
1	*Isotoma viridis*		9	*Sminthuridae sp*		17	*Isotoma viridis*	
2	*Isotoma viridis*	X	10	*Isotoma palustris*		18	*Isotoma viridis*	X
3	*Isotoma viridis*	X	11	*Isotoma viridis*	X	19	*Isotoma palustris*	
4	*Isotoma viridis*		12	*Isotoma palustris*	X	20	*Isotoma palustris*	X
5	*Isotoma viridis*		13	*Isotoma palustris*		21	*Isotoma viridis*	
6	*Isotoma viridis*		14	*Isotoma palustris*	X	22	*Isotoma viridis*	X
7	*Isotoma viridis*		15	*Isotoma palustris*	X	23	*Isotoma viridis*	
8	*Isotoma viridis*		16	*I. viridis*		24	*Isotoma viridis*	

Table 3.4 The species of microarthropod screened from treatment plot 1 on day 1 of screening, with gel lane number (Figures 4.5.1 and 4.5.2) and predation scoring.

No.	Species		No.	Species		No.	Species	
25	*Isotoma palustris*		33	*Isotoma viridis*		41	*Isotoma viridis*	
26	*Isotoma viridis*	X	34	*Sminthuridae sp*		42	*Isotoma viridis*	
27	*Isotoma viridis*		35	*Isotoma viridis*	X	43	*Isotoma palustris*	X
28	*Isotoma viridis*	X	36	*Sminthuridae sp*		44	*Isotoma viridis*	
29	*Isotoma viridis*		37	*Isotoma viridis*		45	*Isotoma viridis*	
30	*Isotoma palustris*		38	*Sminthuridae sp*		46	*E multifasicata*	
31	*Isotoma viridis*		39	*Isotoma palustris*		47	*Isotoma palustris*	
32	*Isotoma viridis*	X	40	*Isotoma viridis*		48	*Sminthuridae sp*	

Appendix 4

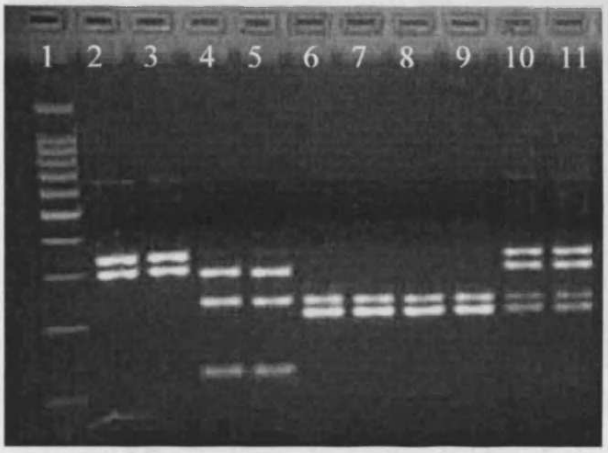


Figure 4.1 Ethidium-bromide stained agarose gel showing restriction digests using enzyme *AseI*. Lane 1: 100bp ladder. Lanes 2-3: *Phasmarhabditis hermaphrodita*. Lanes 4-5: *Heterorhabditis megidis*. Lanes 6-7: *Steinernema feltiae*. Lanes 8-9: *Steinernema kraussei*. Lanes 10-11: Mix of all four nematode species.

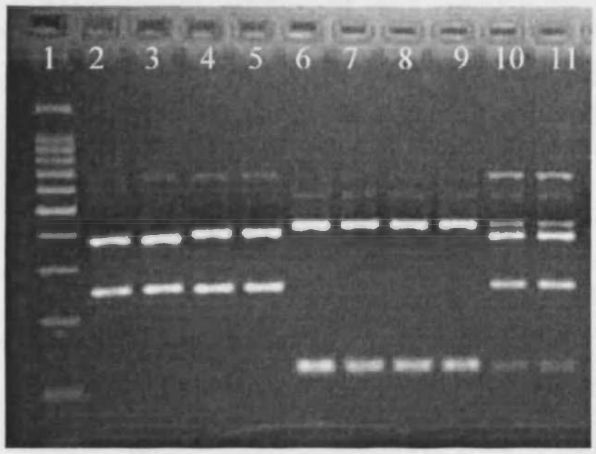


Figure 4.2 Ethidium-bromide stained agarose gel showing restriction digests using enzyme *MboII*. Lane 1: 100bp ladder. Lanes 2-3: *Phasmarhabditis hermaphrodita*. Lanes 4-5: *Heterorhabditis megidis*. Lanes 6-7: *Steinernema feltiae*. Lanes 8-9: *Steinernema kraussei*. Lanes 10-11: Mix of all four nematode species.

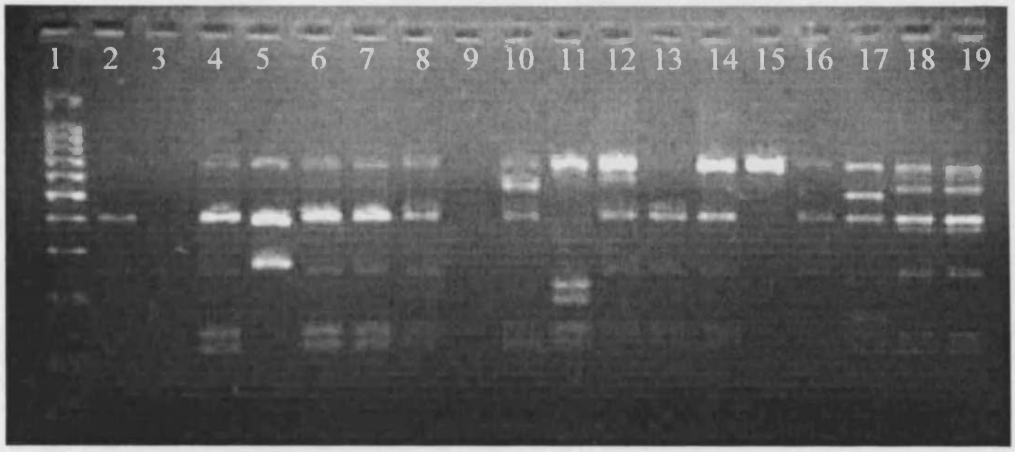


Figure 4.3 Ethidium-bromide stained agarose gel showing restriction digests using enzyme *MboII*. Lane 1: 100bp ladder. Lanes 2-19: digested nematode communities collected from an arable field planted with wheat, at Burdens Farm, Wenvoe, nr. Cardiff.

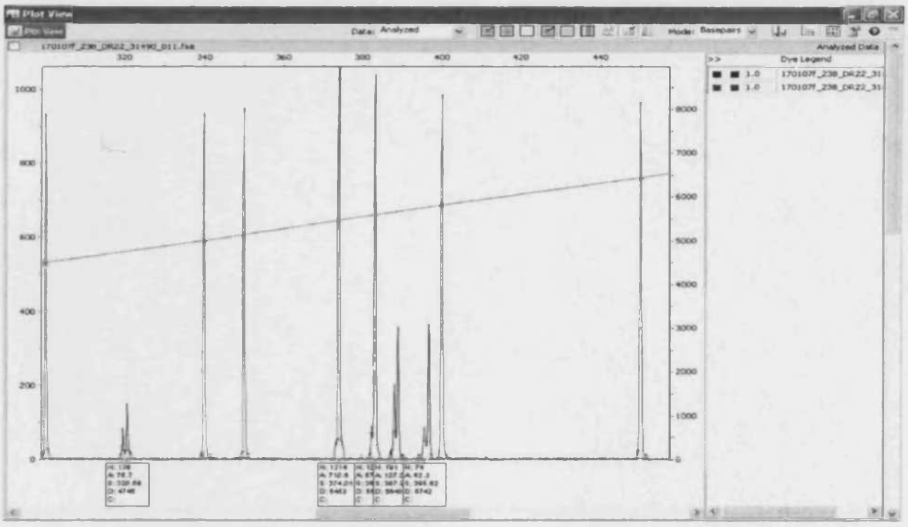


Figure 4.4 Output from Peak Scanner Software V1.0 (Applied Biosystems, Foster City, CA) showing terminal restriction fragments (T-RFs) (blue peaks) and 500 bp size standard (red peaks).