DISCOVERY AND DEVELOPMENT OF NOVEL PARASITIC NEMATODES TO CONTROL SLUGS IN AGRICULTURE

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A thesis submitted in partial fulfilment of the requirements of Liverpool John Moores University for the degree of Doctor of Philosophy

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Declaration

I hereby declare that this thesis has been composed by myself and that it has not been accepted in any previous application for a degree. The work of which it is a record was carried out by myself unless otherwise stated. All sources of information have been acknowledged by means of references and all quotations have been distinguished by quotation marks

James Cutler

September 23, 2021

Abstract

Terrestrial gastropods are highly pestiferous and pose a significant global threat to sustainable agriculture, horticulture and floriculture. Current control methods rely on metaldehyde formulated bait pellets. However, metaldehyde can harm non target organisms and cause environmental pollution. Metaldehyde is also due to be banned for use in the UK by 2022. A viable alternative is the gastropod parasitic nematode *Phasmarhabditis hermaphrodita*, which has been formulated into a biological control agent (Nemaslug®) by BASF Agricultural Specialities. However, the same strain of *P. hermaphrodita* (DMG0001) has been produced in an artificial environment for over 25 years and research suggests this strain is losing beneficial traits (e.g. virulence and host finding) and failing to provide protection against slug damage.

The primary aim of this thesis was to use a collection of wild isolated *Phasmarhabditis* species (*P. hermaphrodita*, *P. neopapillosa* and *P. californica*) to investigate whether they are more virulent than the commercial strain of *P. hermaphrodita* (DMG0001) to the pestiferous slug *Deroceras invadens* and the snail *Cornu aspersum* in laboratory experiments. Host feeding inhibition, numbers of nematode dauers produced on gastropod cadavers and natural variation in chemotaxis behaviour were also investigated. Additionally, the impact of virulent strains on beneficial non- target organisms and the mechanism of host behavioural manipulation were explored. Multiple wild strains were found to perform better than the commercial strain in regards to virulence, feeding inhibition, proliferation on cadavers and locating host cues.

This research has demonstrated that other members of the *Phasmarhabditis* genus can be utilised as safe and effective biological control agents. This thesis presents which *Phasmarhabditis* species show the most potential for pathogenic ability and host seeking. Results obtained from this work have resulted the production of a new slug biocontrol product by BASF, utilising *P. californica*.

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1. USING *Phasmarhabditis hermaphrodita* AS A BIOLOGICAL CONTROL AGENT FOR TERRESTRIAL GASTROPODS AND CURRENT UNDERSTANDING OF OTHER *Phasmarhabditis* SPECIES

1.1. Introduction

Slugs are highly pestiferous and pose a significant global threat to sustainable agriculture, horticulture and floriculture (Barker, 2002). Slugs cause crop damage by eating seeds, stems, growing points and leaves, leading to a reduction in growth (Port & Port, 1986; South, 1992). They can be a major pest throughout the lifecycle of field vegetables and in extreme cases, whole fields have to be re-sown resulting in economic losses (Willis et al., 2006). Contamination of the harvested crop also occurs from slug mucus and faeces, resulting in poor product quality (Port & Ester, 2002). It is estimated that a lack of slug control for crops such as oilseed rape and wheat would lead to £43.5 million a year in loss of product in the UK (Nicholls, 2014). In Europe, wheat and oilseed rape suffer greatly from slug damage (Ester & Wilson, 2005) and in 2010 it was reported that 22% of winter wheat crops suffered damage from slugs, and if left untreated by chemical molluscicides a 5% decrease in yield would be expected (ADAS, 2010). As well as causing damage in agriculture, slug feeding can affect plant community diversity and richness (Wilby & Brown, 2001), with preferential feeding on native species aiding in exotic plant growth (Joe & Daehler, 2008). This also reduces conservation efforts such as forest regeneration (Côté et al., 2005) and threatens endangered species such as lichens (Cameron, 2009). Slugs can also act as invasive pests, causing damage to native plants that may not have anti-herbivore defences due to limited predation pressure (Feeny, 1992) and alter species composition (Schreiner,

1997). Additionally slugs can be responsible for transmitting plant pathogens (Wester et al., 1964; Hasan & Vago, 1966) including *Phytophthora cambivora* (Petri) Buisman, 1927, and *P. plurivora* Jung & Burgess, 2009 (Telfer, 2015). They can also act as vectors of nematode parasites (South, 1992; Grewal et al., 2003a), such as the rat lungworm *Angiostrongylus cantonensis* Chen, 1935, the causal agent of eosinophilic meningitis, which is recognised as an emerging tropic and sub tropic zoonotic disease (Yong et al., 2015). Other veterinary important nematode parasites vectored by slugs include *Angiostrongylus vasorum* Baillet, 1866, *Muellerius capillaris* Mueller, 1889, and *Protostrongylus tauricus* Schulz & Kadenazii, 1949, (Egorov, 1960; Rodonaya, 1977; Simpson & Neal, 1982; Patel et al., 2014; Lange et al., 2018).

Slugs are commonly controlled by chemical bait pellets containing metaldehyde. In the past methiocarb was used, however it is toxic to beneficial invertebrates and other non-target organisms (Purvis & Bannon, 1992; Jones, 2014) and was banned in 2014 (Nicholls, 2014; Jones, 2014). Metaldehyde pellets are used globally (Castle et al., 2017). From 2008 to 2014 an estimated 1640 t of metaldehyde was used in the UK alone (FERA, 2016). Slugs feed on the pellets and exhibit symptoms such as increased levels of mucus secretion and paralysis, and die within several days from water loss (Booze & Oehme, 1986; Bailey, 2002). Though effective, these bait pellets also cause harm to non-target organisms including canines and other vertebrates (Cope et al., 2006). Metaldehyde is also now considered an important emerging pollutant of concern due to leaching into watercourses (Stuart et al., 2012) as it is highly mobile in soil (Kay & Grayson, 2013). Furthermore, in parts of the UK, metaldehyde concentrations in water bodies have exceeded the European Union's regulatory drinking water standard for pesticides (Kay & Grayson, 2013). Metaldehyde is due to be banned for UK use in 2022. An alternative slug pellet (Ferramol®) is composed of iron III phosphate or ferric phosphate and is registered for use in many European countries (Speiser & Kistler, 2001). It has been used to control slugs e.g. *Arion ater* Linnaeus, 1758, however, it has been shown that high doses can lead to mortality and reduced activity in earthworms (Langan & Shaw, 2006).

In agriculture, trapping, drilling at a greater depth, ploughing, crop rotation,

increasing crop diversity and firm seedbed preparation can also help to limit slug damage (Glen et al., 2000). Drilling at depths of 25 – 45 mm has been shown to provide the most effective protection against slug damage (Glen et al., 1990). Ploughing and firm seedbed preparation reduces slug numbers by disrupting their normal surface activity patterns (Glen et al., 2006). Brooks et al. (2006) investigated using alternative food sources to reduce slug damage in winter wheat, by planting red clover before planting winter wheat. Here the slugs continued to feed preferentially on the red clover even when wheat seeds were introduced, although this turned out to be a complex control strategy to implement. There is an increased interest in conservation agricultural practices such as minimal and no till, to improve soil health and reduce environmental damage caused by ploughing (Soane et al., 2012; Kassam et al., 2015). These practices however, provide an ideal environment for slug and snail proliferation (South, 1992; DeFrancesco et al., 1996).

In gardens and greenhouses, damage by gastropods can be limited by cultural control methods such as the use of copper tape, garlic and mulch, although they are inefficient for larger scale agricultural use (Schüder et al., 2003). The use of copper tape or copper-impregnated matting has been shown to reduce the velocity of pest slugs, possibly due to irritation (Schüder et al., 2005). In choice experiments, copper was seen to repel slugs and they nearly always avoided mulch as it dries out quickly (Schüder et al., 2005). The above listed methods however, are time consuming and not always effective.

Due to these issues, the only realistic alternative for slug control is the gastropod parasitic nematode *Phasmarhabditis hermaphrodita* Schneider (Nematoda: Rhabditidae) (Fig. 1.1) (see key diagnostic features in Stock & Hunt, 2005), which has been formulated into a biological control agent (Nemaslug®) produced and sold by BASF Agricultural Specialities (Wilson et al., 1993). *Phasmarhabditis hermaphrodita* (strain DMG0001) is sold in 15 different European countries (Pieterse et al., 2017a) and has been on the market since 1994. It has been successfully used to reduce slug damage in agriculture, floriculture and horticulture, sometimes to comparable levels as metaldehyde treatment (Rae et al., 2007). This literature review will provide information on the *Phasmarhabditis* genus, the susceptibility of slugs and snails,

host interactions, bacterial associations, results of field trials and suggestions for future research to enhance the use of *P. hermaphrodita* in the field.

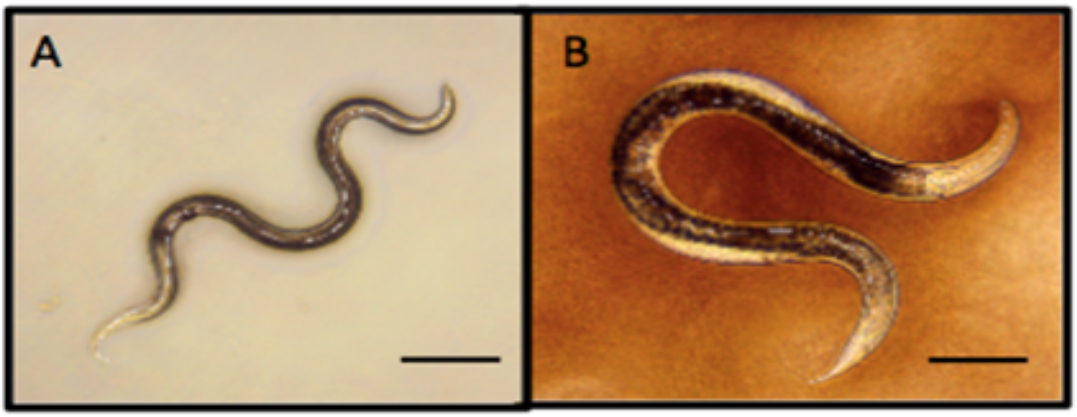

Figure 1.1: Dauer stage *Phasmarhabditis hermaphrodita* (a) seek out slugs in soil and then penetrate inside. Once the slug dies the nematodes exit the dauer stage and grow to young adult nematodes (b) and reproduce on the cadaver. Bars represents 100 µm.

1.2. Slug parasitic nematodes and the genus *Phasmarhabditis*

There are 108 nematodes associated with slugs and snails (Grewal et al., 2003a) used as either definitive, intermediate or necromenic hosts (Pieterse et al., 2017a). Forty-seven species of nematode, belonging to eight families, use molluscs as a definitive host (Grewal et al., 2003a; Pieterse et al., 2017a). However, the only nematodes that can kill slugs and snails are those from the genus *Phasmarhabditis* (Wilson et al., 1993). There are some reports of mortality being caused by another slug parasitic nematode (*Alloionema appendiculatum* Schneider) towards *Arion vulgaris* Moquin- Tandon, 1885, but not at levels considered suitable for a biocontrol agent (Nermut' et al., 2019a).

Phasmarhabditis hermaphrodita is a Rhabditid clade V nematode (Ross et al., 2010a), and along with different *Phasmarhabditis* species, are easy to isolate using protocols described by Wilson *et al.* (1993; 2016), Wilson (2012) and Andrus & Rae (2018), with many different species isolated from around the world. Identification can be accomplished using 18SrRNA primers (Blaxter et al., 1998), species-specific

primers and qPCR methodologies for nematodes isolated from soil and hosts (Macmillan et al., 2006; Jaffuel et al., 2019a). *Phasmarhabditis hermaphrodita* was first described from Germany by Schneider (1859), and it has since been isolated in many different parts of the world. In 1900 Maupas isolated *P. hermaphrodita* in Normandy, France and 50 years later in 1953 it was re-isolated by Mengert in Germany. *Phasmarhabditis hermaphrodita* was isolated in the UK in the early 1990s from diseased grey field slugs (*Deroceras reticulatum* Müller, 1774) at Long Ashton Research Station, University of Bristol (Wilson et al., 1993). For a personal account about its initial isolation in the early 1990s see Glen & Coupland (2017). Further research focused on finding suitable bacteria for mass production (Wilson et al., 1995a,b) and proof the nematode could be used to control slugs under field conditions (e.g. Wilson et al., 1994a; 1995c). Research carried out by Wilson et al. (1993, 1994a, 1995a-c) was used as a blueprint to produce *P. hermaphrodita* commercially, first by MicroBio, then Becker Underwood and now BASF Agricultural Specialities. Subsequently, interest in the species grew with it being found in France (Coupland, 1995), Chile (France & Gerding, 2000), Iran (Karimi et al., 2003), Czech Republic (Nermut' et al., 2010), Egypt (Genena et al., 2011), New Zealand (Wilson et al., 2012; 2016), Norway (Ross et al., 2016) and most recently in Belgium (Singh et al., 2019). One of the biggest markets for slug control is the USA; however, for years *P. hermaphrodita* was never found there despite several surveys (Grewal et al., 2000; Kaya & Mitani, 2000; Ross et al., 2010b). More recently, though, numerous strains of *P. hermaphrodita* and other *Phasmarhabditis* species have been found in North America (see Tandingan De Ley et al., 2017 for an overview; Mc Donnell et al., 2018b; Tandingan De Ley et al., 2014).

Nematodes from the genus *Phasmarhabditis* are problematic to classify as there are some poorly described species, but currently 14 species have been isolated from terrestrial gastropods including *P. apuliae*, *P. bohemica, P. bonaquaense*, *P. californica*, *P. circassica, P. clausiliiae P. hermaphrodita*, *P.kenyaensis, P. meridionalis P. neopapillosa*, *P. papillosa*, *P, safricana*, *P. tawfiki* and *P. zhejiangensis* and one species (*P. huizhouensis*) from rotting leaf litter (Azzam, 2003; Tandingan de Ley et al., 2017; Nermut' et al., 2016a,b, 2017; Ivanov & Spiridonov, 2017, 2020; Ross et al., 2018; Pieterse

et al., 2020; Zhang & Liu, 2020; Huang et al., 2015). There are another two *Phasmarhabditis* species including *P. nidrosienses* (isolated from marine habitat) and *P. valida* (isolated from littoral detritus) (Andrássy, 1983) but after a recent revision they were moved to the genus *Buetschlinema* (Sudhaus, 2011).

It is clear from the numerous surveys carried out over the last 25 years that *Phasmarhabditis* nematodes are commonly found in many countries from diverse terrestrial gastropod hosts. For example, *P. tawfiki* was isolated from the snail *Eobania vermiculata* and the slug *Limax flavus* in Egypt (Azzam, 2003). *Phasmarhabditis bonaquaense* was found in the slug *Malacolimax tenellus* in the Czech Republic (Nermut' et al., 2016a) and *P. apuliae* was isolated from the slugs *Milax sowerbyi* and *M. gagates* from Italy (Nermut' et al., 2016b). Nermut' et al. (2017) described a new species (*P. bohemica*) from the Czech Republic found in *D. reticulatum*. *Phasmarhabditis papillosa* has been isolated from *Deroceras invadens* Reise, Hutchinson, Schunack and Schlitt, 2011, (previously *D. panormitanum*) and *Tandonia sowerbyi* Ferussac, 1823, from the UK, *D. reticulatum* in the USA (Ross et al., 2010b; Tandingan De Ley et al., 2016), South Africa (Pieterse et al., 2017b; Ross et al., 2012) and from *A. vulgaris* in Slovenia (Laznik et al., 2020). *Phasmarhabditis neopapillosa* has been isolated from *D. reticulatum*, *D. invadens* , *Limax flavus Linnaeus, 1758, Arion ater* and *A. distinctus* Mabille, 1868, in Scotland and England (Ross et al., 2010b; Andrus & Rae, 2018). Ross et al. (2018) also found a new species (*P. safricana*) from the slug *D. reticulatum* in South Africa. *Phasmarhabditis californica* has been isolated from the USA from numerous species including *D. reticulatum*, *D. laeve* Müller, 1774, *Arion hortensis* Férussac, 1819, and *Lehmannia valentiana* Férussac, 1822 (Tandingan De Ley et al., 2016), as well as being found in *Geomalacus maculosus* Allman, 1843, in Ireland (Carnaghi et al., 2017) and from the snail *Oxychilus draparnaudi* Beck, 1837 in Wales (Andrus & Rae, 2018). There are other studies that have identified new *Phasmarhabditis* species specifically from snails (see Morand et al., 2004, for further details). Recently, *P. meridionalis* was isolated from snails (*Quantula striata* Grey, 1834) in Vietnam (Ivanova & Spiridonov, 2017) and in 2019, *P. circassica* and *P. clausiliiae* were found in the snails *Oxychilus* sp. and *Clausiliidae* sp., respectively, in Russia (Ivanova et al., 2020).

There are several *Phasmarhabditis* species still awaiting description. For example, Waki (2017) found two *Phasmarhabditis* species in Japan, but could not identify them to species. Also, two species (called "*Phasmarhabditis* sp. SA3" and "*Phasmarhabditis* sp. SA4") were isolated from slugs in nurseries in South Africa (Pieterse et al., 2017c). A possible *Phasmarhabditis* species was also isolated from the earthworm *Lumbricus terrestris* Linnaeus, 1758, by Zaborski et al. (2001) however, it was only identified morphologically and was described as being virulent towards *L. terrestris*, which is highly unusual for a gastropod parasitic nematode. Recently however, two uncharacterised *Phasmarhabditis* isolates (EM434 and DF5056) ,both belonging to the same species, have been recovered from unknown earthworms and found to be virulent towards the earthworms *Eisenia fetida* Savigny, 1826 and *E. hortensis* Michaelsen, 1890 to some degree (Tandingan De Ley et al., 2020)

Out of all the currently described species, *P. hermaphrodita* (Wilson et al., 1993), *P. neopapillosa* (Hooper et al., 1999; Glen et al., 1996), *P. tawfiki* (Azzam & Tawfiki, 2003), *P. papillosa* (Pieterse et al., 2017b), *P. safricana* (Ross et al., 2018) and *P. californica* (Tandingan De Ley et al., 2020) have been shown to kill slugs and snails. There are preliminary reports that *P. bohemica*, *P. bonaquaense* and *P. apuliae* can also kill slugs (Nermut' et al., 2019b). Taken together, these results demonstrate pathogenicity towards terrestrial gastropods is not confined to one species and is an evolutionary conserved trait across the genus *Phasmarhabditis*.

1.3. Life cycle of *P. hermaphrodita*

*Phasmarhabditis hermaphrodita*is a facultative parasite, able to kill several species of terrestrial gastropods and grow and reproduce on a variety of organic matter (Maupas, 1900; Tan & Grewal, 2001a; Macmillan et al., 2009) (Fig. 1.2). It is also able to infect larger host species e.g. *A. ater* where it will remain until the host dies and reproduce on the cadaver, termed "necromeny" by Schulte (1989) (Fig. 1.2b). *Phasmarhabditis hermaphrodita* is a hermaphroditic nematode and the occurrence of males in extremely rare (Andrassy, 1983), with Maupas (1900) finding only 1 male in 14,888 hermaphrodites.

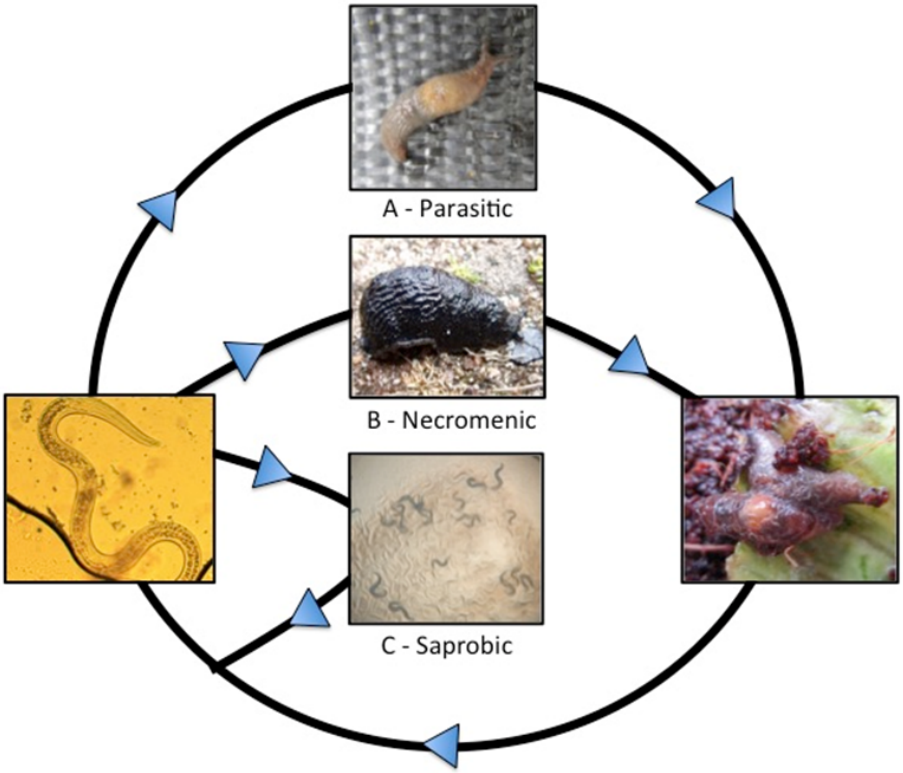

Figure 1.2: *Phasmarhabditis hermaphrodita* can complete its life cycle in three ways. It can parasitise and kill susceptible hosts such as *Deroceras reticulatum* (a), infect resistant slug species such as *A. ater* (b) and wait for it to die (termed "necromeny"- Schulte, 1989) or reproduce on organic matter (c). In each case once the food supply has been depleted it will turn to the infective (dauer) stage and move through soil to find more hosts.

1.4. Chemoattraction of *P. hermaphrodita* to slug and snail host cues

In order to locate hosts *P. hermaphrodita* dauers seek out slugs in the soil by following mucus, faecal and volatile cues (Rae et al., 2006, 2009a; Hapca et al., 2007a-c; 2009; Nermut' et al., 2012a). Nictation (where nematodes stand on their tail hoping to latch on to hosts passing by) and body waving has not been observed in *P. hermaphrodita*, potentially due to their long length (Kruitbos et al., 2009; Brown et al., 2011a). Alternatively, these nematodes employ a "cruiser" based foraging strategy where they actively search for hosts following cues. In one of the first studies to investigate the chemotactic response of *P. hermaphrodita*, Rae et al. (2006) found that *P. hermaphrodita* was attracted to foot and mantle mucus of the pestifer-

ous slug species *D. reticulatum* as well as slug faeces. As many slug species display homing behaviour and return to the same location each night, with species such as *D. reticulatum* displaying high density slug patches with spatial-temporal stability (Duval, 1972, Forbes et al., 2020; 2021), faecal attraction of *P. hermaphrodita* may be beneficial for infecting new hosts. Volatile host cues such as CO2 were found to be the least attractive cues to *P. hermaphrodita* (Rae et al., 2006), potentially due to the vast quantities of CO2 released by microorganisms in soil (Bradley & Wiel, 1999) but also due to *P. hermaphrodita* entering the slug host through the back of the mantle and not the respiratory pore (Wilson et al., 1993). Hapca et al. (2007a,b) observed that there were significant differences in the speed of *P. hermaphrodita* movement, distribution of turning angles and the fractal dimension of nematode foraging trail when exposed to mucus from *D. reticulatum* on agar plates. Furthermore, research by Small & Bradford (2008) showed that *P. hermaphrodita* responded to mucus from an array of slug species. Specifically, they showed that when in contact with slug mucus, nematodes would show increased frequency and duration of head thrusting and head waving but decreased duration and frequency of forward crawling. They found there was little difference between these behaviours when exposed to mucus from six susceptible or non-susceptible slug species. Rae et al. (2009a) found that *P. hermaphrodita* not only responded to mucus from *D. reticulatum* but was positively attracted to a wide range of diverse slug and snail species. Of these species tested, *P. hermaphrodita* showed a preference for slugs such as *Arion subfuscus* Draparnaud, 1805, *D. invadens* and the snail *Cornu aspersum* M"uller, 1774. *Phasmarhabditis hermaphrodita* was found to be more attracted to slugs as opposed to earthworms (*L. terrestris* and *Eisenia hortensis* . Interestingly reproductive success of *P. hermaphrodita* was not greater on attractive species (Rae et al., 2009a).

All these studies have focused on using the commercial strain of *P. hermaphrodita* (strain DMG0001) that has been in culture since 1994. To gain more insight into how wild strains of *P. hermaphrodita* would behave, Andrus & Rae (2019) exposed several wild isolated strains of *Phasmarhabditis* species (*P. hermaphrodita*, *P. neopapillosa* and *P. californica*) to mucus from 7 different slug species. The wild strains

differed in their preference to the slug species tested with *P. neopapillosa* preferring *Arion* sp. In a similar study Andrus et al. (2018) exposed *P. hermaphrodita*, *P. neopapillosa* and *P. californica* to mucus from snails. They found, surprisingly, the commercial strain of *P. hermaphrodita* DMG0001 showed little chemotactic response and remained at the point of application whereas wild isolates of *P. hermaphrodita* and *P. californica* were attracted to mucus of *Cepaea nemoralis*, *C. hortensis* and *Arianta arbustorum* Linnaeus, 1758. There is little information about what the exact compounds in slug and snail mucus *Phasmarhabditis* nematodes are attracted to; however, Andrus et al. (2018) exposed *P. hermaphrodita* to a range of metal ions (e.g. MgCl2, FeSO4) and hyaluronic acid and found that the nematodes showed strong attraction to 1% hyaluronic acid – an abundant component of slug mucus.

The majority of chemotaxis experiments investigating the behaviour of *P. hermaphrodita* have been carried out on agar plates and therefore may not be applicable to their natural soil environment. Hapca et al. (2007b) used a more realistic experimental design and found speed, turning angle distribution, fractal dimension and mean square displacement were reduced when sand grains were placed on agar plates. Nermut' et al (2012a) utilised both agar plates and sand filled olfactometers, and showed that *P. hermaphrodita* was attracted to dead slugs (*D. reticulatum*) but not faeces and mucus, and in soil olfactometers the nematodes were averted from dead slugs, leading the authors to hypothesise that the large variety of decay gases caused *P. hermaphrodita* to suffer from a lack of oxygen and move away (Nermut' et al., 2012a). Macmillan et al.(2009) used columns packed with different substrates and showed *P. hermaphrodita* moved best through organic matter, non-compacted soil and soil containing large aggregates. Dispersal of *P. hermaphrodita* was increased when placed in mineral soils with the earthworm *L. terrestris*. Authors also found that *P. hermaphrodita* (DMG0001) exhibited restricted dispersal through the soil column when compared to a wild isolated strain from Norway.

1.5. How *P. hermaphrodita* kills slugs - the questionable role of bacteria

Once *P. hermaphrodita* locates a slug host, nematodes enter through the back of the mantle and migrate to the shell cavity (Wilson et al., 1993; Tan & Grewal, 2001a). Larvae then develop into self-fertilising hermaphrodites and start to reproduce (Wilson et al., 1993; Tan & Grewal, 2001a). This produces characteristic signs of infection such as a swollen mantle and shell ejection (Fig. 1.3). Host death occurs 4-21 days after initial infection (Wilson et al., 1993), and nematodes feed and reproduce on the cadaver. When the food source is depleted, dauer larvae enter the soil to locate a new host.

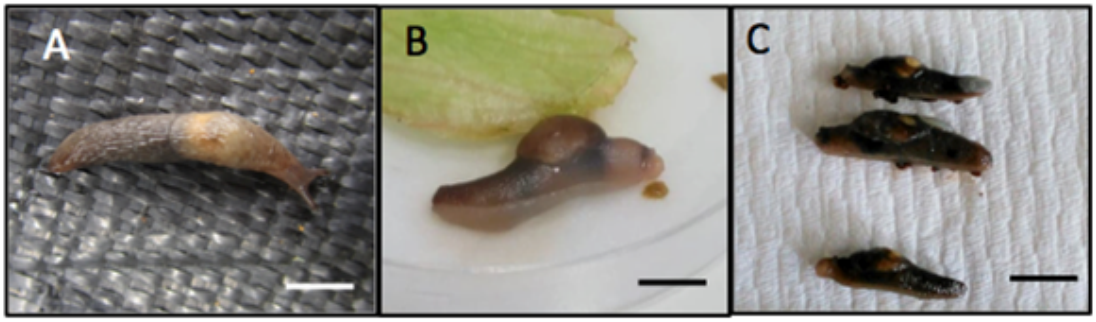

Figure 1.3: *Phasmarhabditis hermaphrodita* produces characteristic signs of infection when parasitising pestiferous hosts such as *Deroceras invadens* (a). Nematodes infect the slug through a pore in the back of the mantle and reproduce, causing a swelling of the mantle area (b), this eventually leads to the internal shell being ejected from the mantle and death (c). Bars represent 0.5 cm in a and b and 1 cm in c.

It is currently unknown how *P. hermaphrodita* kills slugs. Early research focused on a paradigm similar to entomopathogenic nematodes (EPNs) and their symbiotic relationship with bacteria. EPNs of the families Steinernematidae and Heterorhabditidae associate with symbiotic bacteria (Steinernematidae with *Xenorhabdus* spp. and Heterorhabditidae with *Photorhabdus* spp.) that are responsible for killing host insects (Forst et al., 1997). It was previously thought *P. hermaphrodita* functioned similarly to EPNs and acted as a vector for the bacterium *Moraxella osloensis* (Bovre & Henriksen 1967) Bovre, 1979, and the host died due to septicaemia (Tan & Grewal, 2001b). When the first strain of *P. hermaphrodita* (DMG0001) was isolated an attempt was made to identify a bacterium that could be used for industrial

production of these nematodes. Indeed, it is clear that bacterial diet, substrate and inoculation density can have dramatic effects on growth, lipid content and length of nematodes (Wilson et al., 1995a,b; Nermut' et al., 2012b, 2014). Initial studies focused on feeding *P. hermaphrodita* on bacteria from infected slugs and from *P. hermaphrodita* emerging from dead slugs (Wilson et al., 1995a,b). Many different bacterial species were isolated and tested including: *Acinetobacter calcoaceticus* Beijerinck, 1911, *Aeromonas hydrophila* (Chester, 1901) Stanier, 1943, *Aeromonas* sp. Stanier, 1943, *Bacillus cereus* Frankland & Frankland, 1887, *Flavobacterium breve* (Lustig, 1890) Bergey et al., 1923, *FIavobacterium odoratum* Stutzer, 1929, *Moraxella osloensis*, *Providencia rettgeri* Rettger, 1904, *Pseudomonas fluorescens* Migula, 1985, (isolate no. 1a), *P. fluorescens* (isolate no. 140), *P. fluorescens* (isolate no. 141), *P. fluorescens* (pSG), *P. paucimobilis* (Holmes et al., 1977) Yabuuchi et al, 1990, *Serratia proteamaculans* (Paine and Stansfield 1919) Grimont et al., 1978, *Sphingobacterium spiritocorum* Holmes et al., 1982, and *Xenorhabdus bovienii* Akhurst and Boemare, 1993. Successful feeding and growth of *P. hermaphrodita* has also been recorded on *Pseudomonas* sp.1, *Bacillus* sp. 1, *Escherichia coli* Migula, 1895, OP50 and *E. coli* BR (Andrus & Rae, 2018). *M. osloensis* was chosen as it produced consistently high yields of pathogenic nematodes (Wilson et al. 1995a,b). It should be stressed that this bacterium was chosen for commercial production and does not reflect the natural tritrophic interactions that may be occurring between slugs, *P. hermaphrodita* and bacteria in the wild. A study by Rae et al. (2010) showed that *P. hermaphrodita*, when grown on rotting slugs or emerging after parasitising slugs (*D. reticulatum*), had no evidence of *M. osloensis* being present. Therefore, these nematodes do not vertically transmit this bacterium. Similarly, Nermut' et al. (2014) found that *P. hermaphrodita* strain (DMG0001) lost *M. osloensis* after repeated culturing. However, research has shown that injection of 40 and 60 h cultures of *M. osloensis* into the haemocoel of *D. reticulatum* will kill slugs, with the 60 h cultures being more pathogenic than the 40 h cultures (Tan & Grewal, 2001b). This is thought to be due to a lipopolysaccharide (LPS) which acts as an endotoxin (Tan & Grewal 2002, 2003) and *ubiS* and *dsbC* genes that are upregulated by *M. osloensis* when infecting *D. reticulatum* (An et al., 2008). *M. osloensis* is only toxic to

D. reticulatum when injected and showed no contact or oral toxicity to slugs (Tan & Grewal, 2003). The relationship between *M. osloensis* and *P. hermaphrodita* has been categorised as symbiotic yet there are compelling reasons why this may not be the case, which is out of the scope of this review (see Wilson & Rae, 2015 for further details). What is clear is that *P. hermaphrodita* can grow on a multitude of different bacterial species and they can affect the numbers of offspring produced and the nematode's pathogenicity. Whether or not the nematode relies on a strict symbiotic relationship with one bacterium is a matter of debate. It would certainly be worth investigating which bacteria wild isolates of *P. hermaphrodita* associate with in nature. This has not been examined in detail apart from a study by Nermut' et al. (2014) who found *P. hermaphrodita* DMG0001 and a wild *P. hermaphrodita* strain harboured a range of bacterial species, including *Acinetobacter* sp., *Alcaligenes faecalis* Castellani & Chalmers, 1919, *Bacillus cereus* and *Stenotrophomonas* sp. , while *Pseudomonas putida* Trevisan, 1889, was isolated from the wild strain only and *M. osloensis* was isolated from *P. hermaphrodita* DMG0001 only.

1.6. Reproduction

Upon host death, nematodes proliferate on the cadaver, and multiple factors can influence progeny dynamics. Rae et al. (2009a) found *P. hermaphrodita* grown on tissue from different species of slugs and snails yielded different numbers of offspring with *D. invadens* (previously known as *D. panormitanum*) producing the highest number of progeny followed by *Lehmannia marginatus* Müller, 1774, *Milax gagates* Draparnaud, 1801, *C. hortensis* and *D. reticulatum*. Nermut' et al. (2014) found development and quality of *P. hermaphrodita* was severely affected by the growing substrate. *Phasmarhabditis hermaphrodita* was able to grow successfully on multiple substrates including a mixture of homogenised pig kidney with different homogenised slug species (*Arion lusitanicus* Moquin- Tandon, 1855, and *D. reticulatum*) and homogenised moth (*Galleria mellonella* Linnaeus, 1758), and *D. reticulatum* and *A. lusitanicus* faeces and leaf compost. The authors found the yield of *P. hermaphrodita* to be greater on invertebrate-based substrates, but the quality of *P. hermaphrodita* produced remained stable based on body size and lipid size.

Tan & Grewal (2001a) reported similar findings of dauer larvae of *P. hermaphrodita* recovering and multiplying in slug and snail faeces homogenates but not soil samples. These results indicate that reproducing on an invertebrate substrate may have an evolutionary advantage, as it will produce higher numbers with the same quality of progeny as when it kills and reproduces on a host (Nermuť et al., 2014).

Intraspecific competition for resources can also influence *P. hermaphrodita* development and quality (Nermut' et al., 2012b; 2014). Specifically, developmental time, lipid content, yield and body length can be affected by increasing numbers of conspecifics. However, nematodes may leave these areas of dense populations to find other resources (Nermut' et al., 2012b). Temperature can also severely affect the survival and growth of *P. hermaphrodita*. Survival dramatically decreases at 25ºC and 35ºC but there is no difference at 5, 10 and 15ºC (Grewal & Grewal, 2003a) with the optimum growth temperature for *P. hermaphrodita* at 17°C (Wilson et al., 1993).

Figure 1.4: *Phasmarhabditis hermaphrodita* can cause rapid mortality to the susceptible slugs *Deroceras reticulatum* (a), *D. invadens* (b), *Milax gagates* (c) and *Tandonia sowerbyi* (d) but *Arion ater* (e), *A. subfuscus* (f), *Limax maximus* (g) and *L. flavus* (h) are resistant, for reasons unknown. Bars represent 0.5 cm.

1.7. Susceptibility of terrestrial gastropods to *P. hermaphrodita*

There are currently 21 species of slug and 20 species of snail that have been tested for their susceptibility to *P. hermaphrodita* under laboratory conditions (Fig. 1.4,

Supplementary Table 1). To date, 13 slug species and 8 snail species can be killed by *P. hermaphrodita*. There is little research into understanding how *P. hermaphrodita* is able to kill terrestrial gastropods and very little information about why there is this difference in susceptibility of different species. Some studies have shown that younger stages of certain species are susceptible whereas adults are not including *A. lusitanicus* (Speiser et al., 2001; Grimm, 2002), *A. ater* (Wilson et al., 1993, Rae et al., 2009a), *A. vulgaris* (Antzée-Hyliseth et al., 2020) and *C. aspersum* (Glen et al., 1996). Confusingly, studies that have carried out the same experiment have reported different results. For example, the Giant African snail (*Lissachatina fulica* Ferussac, 1821), has been shown to be killed by a wild strain of *P. hermaphrodita* from the USA (Mc Donnell et al., 2018b) but the commercial strain *P. hermaphrodita* DMG0001 has no negative effect on these snails (Williams & Rae 2015). Also, Morley & Morritt (2006) reported the freshwater snail *Lymnaea stagnalis* Linnaeus, 1758, was killed by *P. hermaphrodita* but Whitaker & Rae (2015) observed no mortality when repeating the same experiment. These differences could be due to whether the commercial strain of *P. hermaphrodita* or wild isolates were used in experiments or whether host snails were collected or cultured. For example, Morley & Morritt (2006) used a lab strain of *L. stagnalis* whilst Whitaker & Rae (2015) used wild collected *L. stagnalis*. Similarly, Williams & Rae (2015) used the commercial strain of *P. hermaphrodita* and exposed them to *L. fulica* whilst Mc Donnell et al. (2018a) used a wild strain of *P. hermaphrodita*. It is interesting to speculate why there are such differences, perhaps it could be due to continuous lab culturing, which can have severe effects on the phenotype of laboratory animals (Huey & Rosenzweig, 2009). Nematodes are no different. For example, traits such as heat, UV light and desiccation tolerance and reproductive potential have been shown to be reduced in *Hetorhabditis bacteriophora* Poinar, 1976, through continuous culturing in *G. mellonella* (Wang & Grewal, 2002). The effect of continuous lab culturing in nematodes and hosts could therefore play a role in the differences found in these experiments.

One common symptom of *P. hermaphrodita* infection is host feeding inhibition, which is strongly observed in slugs such as *D. reticulatum* and *D. invadens* but has

also been observed in slug species it cannot kill (Wilson et al., 1993; Rae et al., 2009a). It has been suggested that rapid reduction in slug control in field trials is probably from host feeding inhibition as opposed to slug mortality (Glen et al., 1994, 2000; Wilson et al., 1994a). Feeding inhibition may be a defensive behaviour of slugs to contract and reduce the numbers of nematodes penetrating inside (Grewal et al., 2003b). Some species, however, are not killed by *P. hermaphrodita* and their feeding is not inhibited e.g. *Limax pseudoflavus* Evans, 1978, (Rae et al., 2008).

In contrast to slugs, the effect *P. hermaphrodita* has on snails has not been investigated in detail (although these nematodes have been isolated regularly from snails, see Morand et al., 2004). *Phasmarhabditis hermaphrodita* has been shown to cause high levels of mortality to snails (*Theba pisana* Müller, 1774, *Trochoidea elegans* Gmelin, 1791, and *Monacha cantiana* Montagu, 1803) (Coupland et al., 1995; Tandingan de Ley et al., 2020; Genena & Mostafa, 2010). There are ,however, many snail species resistant to infection by *P. hermaphrodita*. One reason for this may be due to the snail shell. An observation during an infection experiment using *P. hermaphrodita* and *L. fulica* found nematodes permanently fixed in the shell (Williams & Rae, 2015). Evidence of this process has also been shown in live *Cepia nemoralis* Linnaeus, 1758 (Williams & Rae 2016) (Fig. 1.5), *A. arbustorum* (Rae, 2018) and in museum collections of *C. aspersum* and *Helix pomatia* Linnaeus, 1758 (Cowlishaw et al., 2020). This process is remarkably well conserved across the Stylommatophora and has been thought to be present when the two major clades diverged 80-130 MYA (Rae, 2017a) and nematodes have even been observed in the vestigial shell of the slug *L. pseudoflavus* (Rae et al., 2008). Nematodes have been infecting gastropods since they evolved in the late Cambrian (Grewal et al., 2003a) and this evolutionary arms race has resulted in slugs and snails co-opting their shell to encapsulate and encase parasitic nematodes instead of just using the shell for shelter (Rae, 2017a). Interestingly, dark morphs of the snail *Cernuella virgata* Da Costa, 1778, were found to be more resistant to *P. hermaphrodita* than light morphs but this was not due to phenoloxidase levels (Scheil et al., 2014). These authors did not dissect the snails or examine the shells for nematodes but perhaps this

difference in susceptibility was due to the effectiveness of the shell morphs to encase invading nematodes.

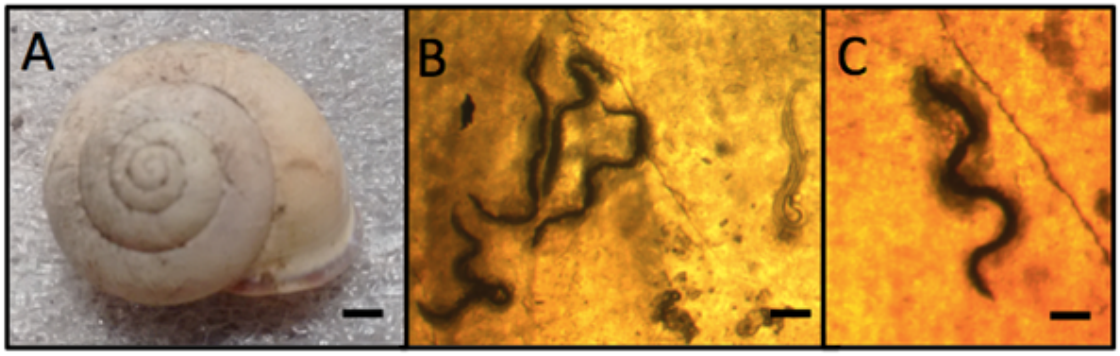

Figure 1.5: Snails such as *Cepaea nemoralis* (a) can be infected with *Phasmarhabditis hermaphrodita* under lab and field conditions and are trapped, encased and killed in the shell (b and c). Scale bars in a represent 2 mm and 100 micrometers in b and c.

As well as use in agriculture, *P. hermaphrodita* could be used to reduce snail populations that vector medically important parasites. Specifically, application of the nematode has been shown to negatively affect freshwater snails *Biomphalaria alexandrina* Ehrenberg, 1831, and *B. pfeifferi* Krauss, 1848 (under lab conditions), which could potentially result in a diminished transmission of schistosomiasis (Abou-Elnour et al., 2015; Okonjo et al., 2015). The potential of these nematodes to control *Biomphalaria* snails warrants significant attention and could be highly promising.

1.8. Host avoidance and behavioural manipulation

In order to reduce parasitism by *P. hermaphrodita* , slugs avoid areas where nematodes are present. Avoidance behaviour is the first strategy an organism can employ to reduce the threat of parasitism (Curtis, 2014). Slugs such as *D. invadens* and *A. ater* are able to detect and avoid areas where *P. hermaphrodita* is present, and subsequently spend less time feeding and resting in such areas (Wilson et al., 1999). Wynne et al. (2016) showed that several diverse slugs from three different families will avoid *P. hermaphrodita* but snails do not avoid the nematodes. They also showed that slugs avoided *P. hermaphrodita* specifically and would not avoid EPNs (*Steinernema kraussei* Steiner, 1923) or the vinegar eelworm (*Turbatrix aceti*

Müller, 1783) - both of which are not parasites of molluscs. Resistant slug species *A. subfuscus*, *A. hortensis* and *L. valentiana* avoid *P. hermaphrodita*, although *L. flavus* is also resistant to *P. hermaphrodita* infection and does not avoid the nematode (Morris et al., 2018; Wynne et al., 2016). Slugs do not avoid areas treated with the supernatant of a liquid suspension of *P. hermaphrodita* suggesting that the slugs are avoiding the mechanical stimulus of the nematodes probing the slug's body, rather than a chemical cue (Wynne et al., 2016). However, when a slug is infected with *P. hermaphrodita* the usual avoidance behaviour is abrogated and slugs are more likely to be found on areas where *P. hermaphrodita* is present (Morris et al., 2018). The exact reason why the nematodes are influencing slug behaviour is unclear, but it could allow for more successful reproduction, survival or dispersal (Morris et al., 2018). It is unclear how *P. hermaphrodita* is able to manipulate slug behaviour, although Morris et al. (2018) and subsequently Cutler et al. (2019) (Chapter 6) found that it could be linked to neurotransmitter signalling as slugs (*D. invadens*) fed fluoxetine or sertraline, which increase serotonin levels, were driven towards nematodes, whereas infected slugs treated with cyproheptadine, which suppresses serotonin levels, were not attracted to the nematodes. Uninfected slugs treated with apomorphine, which stimulates dopamine receptors, failed to avoid *P. hermaphrodita*, and infected slugs treated with a dopamine antagonist (haloperidol) no longer moved towards *P. hermaphrodita* (Cutler et al., 2019 - Chapter 6). This suggests that *P. hermaphrodita* is somehow able to influence levels of biogenic amines to alter slug behaviour (Morris et al., 2018; Cutler et al., 2019 - Chapter 6).

Interestingly, *P. hermaphrodita* has been reported to have extreme effects on host behaviour. For example, infected slugs eat less (Glen et al., 2000), are slower (Bailey et al., 2003), are more likely to be found under refuge traps (Wilson et al., 1994a), move underground to die (Pechova & Foltan, 2008), and infected freshwater snails are more likely to be found outside of the water (Morley & Morritt, 2006). Not only does *P. hermaphrodita* influence host behaviour, it has been suggested they exhibit an anti-feeding effect on scavenging beetles (*Carabus nemoralis* Müller, 1764 and *Pterostichus melanarius* Illiger, 1798) by deterring them from dead, infected slugs where the nematodes are reproducing (Foltan & Puza,

2009). Whether the nematode is actively manipulating the behaviour of the slugs or this is a by-product of infection of sick slugs warrants further investigation.

1.9. The effect of *P. hermaphrodita* on non-target organisms

For its use as a biological control agent, the commercial strain of *P. hermaphrodita* has been tested against non-target beneficial invertebrates. As expected for a parasite of gastropods, *P. hermaphrodita* has been shown not to harm several insect species including *Tenebrio molitor* Linnaeus, 1758 (Wilson et al., 1994b), *G. mellonella* (Wilson, M.J. unpublished) or *Pterostichus melanarius* (Wilson et al., 1993). The earthworms *L. terrestris*, *Eisenia fetida E. hortensis, E. fetida, E. andrei* Bouché and *Dendrodrilus rubidus* Savigny, 1826, are also unaffected by the nematode as well as the platyhelminth *Arthurdendyus triangulatus* Dendy, 1986 (Grewal & Grewal, 2003b; DeNardo et al., 2004; Rae et al., 2005). Zaborski et al. (2001) reported a "*Phasmarhabditis* like" nematode that potentially killed earthworms (e.g. *L. terrestris*) but there has been no subsequent research. This nematode was only identified morphologically and causing earthworm mortality would be highly unusual for a gastropod parasitic nematode. Another *Phasmarhabditis* species (*P. californica*) has also been exposed to earthworms (*L. terrestris* and *E. fetida*) as well as the insect larvae *T. molitor* and *G. mellonella* with no mortality of any species observed (see Chapter 4). However, recent data has found that two *Phasmarhabditis* isolates (EM434 and DF5056) can cause mortality to the earthworms *E. fetida* and *E. hortensis* in laboratory experiments, although between two trials disparity was seen, with EM434 causing no mortality in one trial yet high levels of mortality in a second trial (Tandingan De Ley et al., 2020). Authors did note that earthworm stress may have resulted in the differences observed. 18s rRNA samples placed EM434 and DF5056 at the base of a well supported *Phasmarhabditis* clade, however D2-D3 sequences did not show a supported clade for these isolates (Tandingan De Ley et al., 2020). Also, mtDNA COI sequences did not match any *Phasmarhabditis* COI sequences when checked in GenBank, although this data base contains few

mtDNA sequences for Phasmarhabditis (Tandingan De Ley et al., 2020). Interestingly, more recent phylogenetic analysis utilising mtDNA by Howe et al. (2020) did place isolates EM434 and DF5056 in a strongly supported internal clade of *Phasmarhabditis*.

The effect of *P. hermaphrodita* on non-target molluscs has also been investigated under lab conditions and in the field. From seven snail species commonly found in hedgerows Wilson et al. (2000) found that high doses of *P. hermaphrodita* caused mortality to just two (*Moncha cantiana* Montagu, 1803, and *C. hortensis*). Also, over a two-year field trial Iglesias et al. (2003) found no effect of *P. hermaphrodita* on the snail species *Ponentina ponentina* Morelet, 1845, and *Oxychilus helveticus* Blum, 1881 or on acarids, collembolans or earthworm populations. Therefore, the effect of *P. hermaphrodita* on non-target organisms seems limited.

1.10. Production and field application of *P. hermaphrodita*

Consistent and efficacious control as well as cost, storage, delivery, handling and marketing are required for any biocontrol product to become commercial (Georgis et al., 2006), including nematode products (Askary, 2010). *P. hermaphrodita* has successfully been in production since 1994 by MicroBio, then was taken over by Becker Underwood and now BASF Agricultural Specialities. *Phasmarhabditis hermaphrodita* is grown in *in vitro* liquid culture with the bacterium *M. osloensis* (Wilson et al., 1995a,b) with upwards of 100,000 dauers per ml being produced (Glen et al., 1994). Monoxenic liquid culture of nematodes for mass production allows for more predictable and high virulent yields (Wilson et al., 1995a,b, Ehlers & Shapiro-Ilan, 2005). After monoxenic fermentation, dauers are harvested. Young et al. (2002) investigated the most effective dauer recovery methodology and concluded that using a combination of continuous phase density and flotation by adjustment was best. The same authors also found that the introduction of an air supply to break apart and clear insoluble spent media was recommended. To separate dauers and other life stages, the product can be sieved at an aperture size

of 75-106 µl (Wilson et al., 2001) or by using vibrating membrane filtration (Wilson et al., 2003). Centrifugation and repeated washing can also be used (Rae et al., 2007). After extraction, dauers are mixed with an inert gel polymer and packaged ready for the consumer to apply to garden or farm soil (Pieterse et al., 2017a).

1.11. Field use and application

The water dispersible formulation containing *P. hermaphrodita* is suspended in tap water and applied to soil at a rate of 3 x 10^9 third stage infective juveniles (dauer larvae) per hectare (Rae et al., 2007), via spraying equipment (Glen et al., 1994) such as backpack sprayers and irrigation lines (Wilson & Gaugler, 2000). As well as being applied to the soil surface *P. hermaphrodita* can be incorporated into soil through cultivation to kill subterranean slugs though this has mixed results in terms of efficacy at reducing slug damage and slug numbers (Wilson et al., 1996). *Phasmarhabditis hermaphrodita* has been used successfully to control slug damage in an array of plants including lettuce (Wilson et al., 1995c; Grubišić et al., 2018), winter wheat (Wilson et al., 1994a; 1996), oilseed rape (Speiser & Andermatt, 1996; Wilson et al., 2000; Kozlowski et al., 2012), cabbage (Grubišić et al., 2003, 2018; Kozlowski et al., 2012), asparagus (Ester et al., 2003a), Brussels sprouts (Ester et al., 2003b), glasshouse orchids (Ester et al., 2003c) and sugar beet (Ester & Wilson, 2005).

There have been few field trials using *P. hermaphrodita* since 2009 but many before, for a complete list of field trials and results see Rae et al. (2007). Failure to reduce slug damage has also been observed. For example, Wilson et al. (1995c) reported no reduction in slug damage in lettuce. Also, Iglesias et al. (2003) found *P. hermaphrodita* treated areas did not affect slug numbers contaminating the harvested crop (but did reduce slug damage). This is similar to Rae et al. (2009b) who also reported a lack of reduction in slug numbers in lettuce.

Other factors may influence the efficacy of *P. hermaphrodita* in the field such as watering regime and earthworm activity, which was investigated in comparison to chemical controls by Dörler et al. (2019). No effect on slug feeding or mortality was seen, however this could be due to the use of the slug *A. vulgaris*, which

is known to be resistant to *P. hermaphrodita* (Speiser et al., 2001; Grimm, 2002). It has ,however, been suggested that failures could be avoided by following recommended protocols (Wilson et al., 2000).

The effect of treatment of crops before nematode application has also been investigated. For example, Iglesias et al. (2001a) found when manure was applied prior to *P. hermaphrodita*, dauer larvae were rendered ineffective, possibly due to poor dauer survival, manure interfering with chemoreception or the manure attracting more slugs. In contrast, Vernavá et al. (2004) and France et al. (2002) found no effect of cover crops or lupin on the ability of nematodes to control slugs in the next crop planted.

Novel application strategies that improve efficiency and economic use of nematode biological control products will improve their attractiveness (Grewal et al., 2005) and have been investigated with *P. hermaphrodita*. Ester & Geelen (1996) found that the best control of slugs in sugar beet utilised nematode application and methiocarb pellets in furrow treatment; however, Wilson et al. (2000) found methiocarb reduced nematode survival, but not infectivity. However, there is limited scope for this combination as methiocarb has since been banned (Jones, 2014; Nicholls, 2014). Multiple lower rate applications of *P. hermaphrodita* can sometimes offer better control (Ester & Wilson, 2005), or the same level of control as standard recommended broadcast rate (Ester et al., 2003a,b; Grewal et al., 2001; Rae et al., 2009b) but they require more time to achieve a reduction in slug damage (Hass et al., 1999a,b; Iglesias et al., 2001a). Lower application rates and concentration could be beneficial for larger areas of crops, with *P. hermaphrodita* being applied via irrigation lines (Brown et al., 2011b), instead of broadcast application. However, *P. hermaphrodita* has been applied in bands but offered no economic advantage over recommended broadcast application at the standard rate, possibly due to too few nematodes being applied (Hass et al., 1999a,b). Other application strategies such as dipping root plugs in a nematode/carboxymethyl cellulose solution have also been found to be successful therefore providing protection against slugs using a lower number of nematodes and reducing the cost (Rae et al., 2009b; Kozlowski et al., 2014). More targeted application methods have been proposed by Brown

et al. (2011b) who described nematode application machinery (Wroot water Nemaslug xtra applicator) that injects nematodes into irrigation water and aerates and agitates the nematode suspension allowing nematodes to be applied over a longer time scale. Also, in plots of hostas, targeted application of *P. hermaphrodita* to slug shelters at a reduced application rate provided similar protection to that of uniform broadcast application (Grewal et al., 2001). Similarly, damage to oilseed rape by *A. lusitanicus* was reduced for 25 days by spraying *P. hermaphrodita* on the plants at a rate of 2 x 10 nematodes/cm² (Jaskulska & Kozlowski, 2012). In order to examine and optimise the numbers of *P. hermaphrodita* used for slug control several models have been developed (Glen et al., 2000; Hass et al., 1999b; Wilson et al., 2004; Schley & Bees, 2006).

1.12. Persistence and environmental factors affecting the success of *P. hermaphrodita* in the field

In order for *P. hermaphrodita* to be successfully used as a biological control agent, it must persist in soil after application. There is little research on this. However, soil type can affect the movement and persistence of *P. hermaphrodita* (MacMillan et al., 2006, 2009). The persistence of *P. hermaphrodita* in soil has been monitored using real time qPCR techniques (Macmillan et al., 2006) showing that the *P. hermaphrodita* population declines sharply after two weeks (Hatteland et al., 2013). However, in other studies survival of *P. hermaphrodita* has been recorded for much longer. Nermut' (2012) found *P. hermaphrodita* could survive up to 5 months in wet sand, and even 8 months in garden soil and organic horticultural substrate. In field trials *P. hermaphrodita* can survive up to 6 weeks in soil (Kozlowska et al., 2014) and even up to 99 days (Vernavá et al., 2004). Under lab conditions, Grewal & Grewal (2003a) showed survival of *P. hermaphrodita* was best at 5, 10 and 15ºC and osmotic desiccation in 10% glycerol could increase survival of the nematodes at temperature extremes.

Unfavourable abiotic and biotic conditions including UV light, temperature and desiccation affect nematode survival and persistence (Wilson & Gaugler, 2004).

This can be reduced by cultivating the land immediately after application (Wilson et al., 1996), with the best cultivation equipment to limit plant damage after nematode application being tines or roterra (Hass et al., 1999a). Upon application, nematodes are predated by mites, collembolans and fungi (Strong, 2002; Wilson & Gaugler, 2004). DNA analysis has shown mites and Collembola, e.g. *Heteromurus nitidus* Templeton, 1835, eat *P. hermaphrodita* under lab conditions and in the field (Read et al., 2006; Heidemann et al., 2011; Fiera, 2014) and fungi have been speculated to affect the survival of these nematodes (Nermut', 2012).

With temperature increasing due to climate change the efficacy of *P. hermaphrodita* to control slugs may be affected. Wilson et al. (2015a) found evidence of this as slug feeding was not reduced in infected slugs as temperatures increased from 14°C to 24°C. It is thought *P. hermaphrodita* is well adjusted to the cooler climate of northern Europe (Glen & Wilson, 1997); however, Iglesias et al. (2001b) found *P. hermaphrodita* could be used to reduce slug damage in warmer conditions in Spain, where the mean air temperature was 19.8 ± 2.6°C (mean ± SD). The impact of temperature on the efficacy of *P. hermaphrodita* was also investigated through field trials using predicted winter warming conditions by El-Danasoury & Iglesias-Piñeiro (2017). They found damage to plants and slug survival was much lower in the predicted wintering conditions than under normal wintering conditions. Therefore, *P. hermaphrodita* may perform better at controlling slug damage under winter warming conditions.

1.13. Combining chemical and biological control methods with *P. hermaphrodita*

There is evidence to show *P. hermaphrodita* combined with other methods could enhance control. Karamaz et al. (2007) exposed the snail *C. aspersum* (previously known as *H. aspersa*) to combined treatments of *P. hermaphrodita* with cadmium and *Bacillus thuringiensis* Berliner, 1915 (BT). The growth rate of *C. aspersum* was reduced by both BT and cadmium and increasing doses of *P. hermaphrodita*. Also, snails exposed to the highest doses of *P. hermaphrodita* accumulated the highest

level of cadmium (Kramarz et al., 2007).

The repellent effect of Birch tar oil (BTO) has been investigated and suggested for possible complementary use with *P. hermaphrodita* to control the slugs *A. arbustorum* and *A. lusitanicus* (Lindqvist et al., 2010). Authors found that BTO repels *A. arbustorum* and *A. lusitanicus* in confined heavily nematode infested areas and repeated application of BTO over several weeks was required to deter *A. lusitanicus*, with weekly treatments offering the best slug control.

Other more novel strategies have also been investigated, such as combining *P. hermaphrodita* with venom from the wasp *Pimpla hypochondriaca* Miller, 1759, to kill and inhibit feeding of *D. reticulatum* (Richards et al., 2008). Authors found that separately, *Pimpla hypochondriaca* venom and *P. hermaphrodita* increased mortality and feeding inhibition of *D. reticulatum*, yet when combined no synergistic or additive effect was seen. A suggested strategy for future research is to genetically engineer *P. hermaphrodita* to express individual venom factors, releasing venom into the host haemocoel (Richards et al., 2008)

More recently the behaviour and feeding of *Tetanocera elata* Fabricius, 1781, fly larvae, (a parasitoid and predator of slugs) has been explored for potential combined use with *P. hermaphrodita* (Ahmed et al., 2019). It was found that *T. elata* larvae development and pupation suffers if larvae are feeding from an infected slug, with only 20% pupating. Interestingly, larvae did show a preference for slugs previously infected with *P. hermaphrodita*. Ultimately further work is needed to examine if they can provide a consistently efficient synergistic level of slug control.

1.14. Future directions and conclusions

Over the 25 years since *P. hermaphrodita* was first implemented as a biological control agent, interest in this nematode has slowly increased as chemical usage is being reduced. However, compared to other nematodes used in biological control such as EPNs, the number of researchers investigating *P. hermaphrodita* is low (Wilson & Rae, 2015) and there are still many unanswered questions about the use and basic biology of *P. hermaphrodita.* Here I outline several research avenues to improve the use of *P. hermaphrodita*, principally:

- An appreciation of co-evolution between host and parasite
- Genetic improvement and genomic understanding of *P. hermaphrodita* and other *Phasmarhabditis* species
- Investigating new application strategies of *P. hermaphrodita* in the field.

1.15. The importance of understanding the co-evolution between host and parasite

Nematodes and slugs have been co-evolving in an arms race for 540 MY (Grewal et al., 2003a). The geographic mosaic theory of co-evolution predicts there will be genetic variation in the ability of hosts to combat parasites as well as pathogenicity of parasites (Thompson, 2005). There is little information on natural variation in pathogenicity of *P. hermaphrodita* strains or whether local and global populations of slugs differ in their susceptibility to the nematode. It seems highly likely there would be genetic variation in both host defence and pathogenic potential of the parasite, which has been observed in other animals. For example, there is considerable variation in the resistance of the crustacean *Daphnia magma* Straus, 1820, to the obligate parasite *Pasteuria ramosa* Metchnikoff, 1888, (as well as variation in parasite infectivity) (Carius et al., 2001). Similarly, wild isolates of *Caenorhabditis elegans* Maupas, 1900, exposed to different strains of the pathogen *Serratia marcescens* Bizio, 1823, show different levels of resistance as well as variation in bacterial virulence (Schulenburg & Ewbank, 2004). For *Phasmarhabditis* nematodes this has only been investigated at the interspecies level (see the "Susceptibility of terrestrial gastropods to *P. hermaphrodita*" section), where species such as *A. ater* are resistant and *D. invadens* and *D. reticulatum* are highly susceptible (Wilson et al., 1993; Tan & Grewal, 2001a; Rae et al., 2009). There is limited data on host susceptibility to *P. hermaphrodita* at the intraspecies level. The only evidence is focused on the snail *C. hortensis*. Wilson et al. (2000) found the population used in their *P. hermaphrodita* infection experiments (from England) were resistant, yet Rae et al. (2009a) collected *C. hortensis* from Aberdeen, Scotland and found them

to be susceptible to the nematode. This has important ramifications for gastropod control. If different populations have evolved resistance to *P. hermaphrodita* then application of the current strain (DMG0001) will be futile. Therefore, it is important to understand the mechanism of how different populations of slugs overcome parasitism and infection by *P. hermaphrodita*. Furthermore, examining the pathogenic potential of wild *P. hermaphrodita* strains should also be carried out, with the aim of them being formulated and developed to overcome any potential resistance (see Chapter 2 and Chapter 3). This approach is commonly used in EPN research. For example, wild strains of *Steinernema* and *Heterorhabditis* have been isolated and screened for superior virulence (Shapiro & McCoy, 2000), host finding and stress tolerance e.g. UV light (Gaugler et al., 1989), heat tolerance (Mukuka et al., 2010) and longevity (Grewal et al., 2002) (to name but a few). This approach has never been utilised for *P. hermaphrodita* as researchers tend not to keep their wild isolated strains in culture. Therefore, natural variation of different traits has not been investigated in great detail for *P. hermaphrodita* apart from tolerance to extreme pHs and temperature (Andrus & Rae, 2018), as well as chemotactic response (Andrus & Rae, 2019; Cutler & Rae, 2021- Chapter 5).

1.16. Genetic tools and genomic sequencing of parasitic nematodes

Coupled with the isolation of wild strains, the development of genetic techniques could enhance the efficacy of *P. hermaphrodita* in the field. This is also inspired by approaches used within EPN research. There have been numerous successful examples of the selection of different EPN strains for advantageous traits. For example, Hiltpold et al. (2010) selected strains of *H. bacteriophora* for high responsiveness to foraging cues and Grewal et al. (1996) selected for cold tolerance in *S. feltiae* Filipjev, 1934, which potentially increased their viability as biological control agents. Other techniques such as inbreeding, hybridization and mutagenesis have been employed to improve oxidative stress tolerance and longevity in *H. bacteriophora* (Sumaya et al., 2018; Okolo et al., 2019). More sophisticated genetic

techniques have been shown to work in EPNs, such as RNAi in *S. carpcocapsae* Weiser, 1955, (Morris et al., 2017) and *H. bacteriophora* (Ratnappan et al., 2016) and even transgenic techniques in *H. bacteriophora* (Hashmi et al., 1995). Engineering of the nematode does not have to be the principal focus. Recent research by Machado et al. (2020) found selection experiments using the symbiotic bacteria (*Photorhabdus*) of *Heterorhabditis* could be used to control pests by selecting for benzoxazinoid resistance (which is sequestered by western corn rootworm when eating maize and used to increase resistance to the parasites). Although *P. hermaphrodita* has been proposed as a model nematode to understand the genetic mechanisms of parasitism (Wilson et al. 2015b, Rae, 2017b, 2019; Luong & Mathot, 2019) development of techniques for genetic manipulation are in their infancy (Andrus & Rae 2019). With the subsequent sequencing of the genome on going (Sheehy, Rae, unpublished), the unravelling of the genetic blueprint of *P. hermaphrodita* may aid in the development of molecular tools. As seen with *C. elegans* and parasitic helminths, genomic investigations can lead to valuable insights regarding the evolution of these organisms (Hunt et al., 2016; International Helminth Genomes, 2019; Stevens et al., 2019) as well as the development of beneficial online resources such as WormBase and WormBase ParaSite. The availability of genomic data would enable the identification of key genes such as those for pathogenicity, dauer formation and chemoattraction. Knowledge of how these genes influence the above mechanisms could lead to improvements in the use of *P. hermaphrodita* as a biological control agent. In terms of genomics, research on EPNs is well ahead of *P. hermaphrodita* with the genomes and transcriptomes of several *Steinernema* species including *S. carpocapsae*, *S. scapterisci* Nguyen & Smart, 1990, *S. monticolum* Stock, Choo & Kaya, 1997, *S. feltiae* and *S. glaseri* Steiner, 1929, already sequenced (Dillman et al., 2015) as well as *Heterorhabditis bacteriophora* (Bai et al., 2013) and their bacterial symbionts *Xenorhabdus* and *Photorhabdus* (Chaston et al., 2011).

1.17. Novel application strategies of *P. hermaphrodita*

Novel application strategies can reduce the cost of the use of nematodes and attractiveness to the consumer (Grewal et al., 2005). Instead of broadcast spraying

these techniques include dipping roots of plants into adhesive mixtures containing nematodes, using lower, more frequent applications of nematodes as well applying infected cadavers or applying nematodes to slow releasing bags. Some of the techniques have been shown to work well in field trials e.g. mixing *P. hermaphrodita* with carboxymethylcellulose to adhere to root plugs and smaller more frequent doses of nematodes to control slug damage in Chinese cabbage (Rae et al., 2009b). However, methods such as using already infected hosts or slow release bags have not received attention using *P. hermaphrodita*. Another promising method is encapsulating nematodes in alginate beads providing a more targeted approach, which has been shown to work with EPNs to control *Diabrotica balteata* LeConte, 1865, larvae (Jaffuel et al., 2019b). These methods could also be combined with other methods to allow synergistic slug control e.g. using essential oils such as clove bud oil that kill snail eggs (Mc Donnell et al., 2016) coupled with nematodes to kill juvenile snails (Mc Donnell et al., 2018a) or combining with other biocontrol agents such as the fly *T. elata* (Ahmed et al., 2019).

With the discovery of *Phasmarhabditis* nematodes from across the world (Pieterse et al., 2017a), including the USA (Tandingan de Ley et al., 2014) there is ample opportunity for expansion of the Nemaslug® product across the globe. Ultimately, by focussing on the approaches suggested, *P. hermaphrodita* (and other *Phasmarhabditis* species) could be developed and used as successful biological control agents of slugs for the next 25 years.

1.18. Aims of thesis

There are reports of *P. hermaphrodita* being used in field studies and failing to provide protection against slug damage (e.g. Wilson et al. 1995c) and reducing slug numbers (e.g. Rae et al., 2009). As this nematode has been grown for 25 years under artificial conditions being fed on one food source (*M. osloensis*) and going through hundreds of thousands of generations, suffering from low genetic diversity due to being a hermaphroditic nematode, traits such as virulence and host finding could be severely affected. Indeed, it has been shown by Andrus & Rae (2019) the current strain of *P. hermaphrodita* (DMG0001) is poor at responding

to slug host cues. Hence, the main aim of this thesis was to use a collection of wild isolated *Phasmarhabditis* species (*P. hermaphrodita*, *P. neopapillosa* and *P. californica*) (collected by Andrus & Rae, 2018 and housed at LJMU) to investigate whether they are more virulent than the commercial strain of *P. hermaphrodita* (DMG0001) to the pestiferous slug *D. invadens*, and for *P. neopapillosa* and *P. californica: D. invadens* and the snail *Cornu aspersum.* As well as looking at the pathogenic potential of these strains, feeding inhibition and the numbers of dauers growing on the cadaver of dead gastropods was also investigated (see Chapter 2 and Chapter 3). The main outcome of this research could be that the most virulent strains may potentially be developed as alternative biological control agents superior to *P. hermaphrodita* DMG0001. The results of these studies using wild strains of *P. hermaphrodita* in Chapter 2 were published in the Journal of Invertebrate Pathology (Cutler & Rae, 2020). At the time of writing, results regarding the virulence of *P. californcia* towards *C. aspersum* (Chapter 3) are under review for publish in the Journal of Biocontrol Sciences and Technology.

There have been some unfounded reports that *Phasmarhabditis* nematodes may negatively affect earthworms. If true, this would be detrimental for the use of these nematodes. It has been shown in many studies (e.g. Rae et al., 2005) *P. hermaphrodita* does not affect earthworms but this is unknown for other *Phasmarhabditis* species. I therefore exposed *P. californica* to earthworms and insects to examine whether they had the potential to affect non-target organisms in Chapter 4.

In Chapter 5, natural variation in chemotaxis behaviour to slug mucus and hyaluronic acid (a component of slug mucus these nematodes are highly attracted to) was investigated. Several wild strains of *P. hermaphrodita*, *P. neopapillosa* and *P. californica* were assessed and I could show there are some strains that are better at responding to these cues compared to *P. hermaphrodita* DMG0001. The results of this chapter were published in the Journal of Helminthology (Cuter & Rae, 2021).

Finally, in Chapter 6, I investigated how *P. hermaphrodita* can affect slug behaviour. It had been shown previously (Morris et al., 2018) these nematodes can manipulate the behaviour of slugs by making them move to areas where other *P.*

hermaphrodita nematodes are present by altering levels of biogenic amines (Morris et al., 2018). I repeated the experiment of Morris et al. (2018) and used a selection of agonists and antagonists of serotonin and dopamine receptors and showed their application severely affected slug behaviour. This was published in Behavioural Processes (Cutler et al., 2019).

2. Pathogenicity of wild and commercial *P. hermaphrodita* exposed to the pestiferous slug *D. invadens*

2.1. Introduction

On average, 32% of crop production is lost on a global scale due to pests (Dhaliwal et al., 2010). Terrestrial gastropods (slugs and snails) inhabit all continents and are important agricultural pests in Europe, America and Australia (South, 1992; Barker, 2002). The main method to control slugs is the use of metaldehyde based slug pellets (Castle et al., 2017),but metaldehyde will be banned for use in the UK in 2022. A viable alternative is *P. hermaphrodita*, which is capable of killing several pestiferous slug species (Wilson et al., 1993) and has been used to control slug damage in many crops, including winter wheat (Wilson et al., 1994), cabbage (Grubišič et al., 2003, 2018; Kozlowski et al., 2012) and sugar beet (Ester & Wilson, 2005). The DMG0001 strain *of P. hermaphrodita* has been used in the production of Nemaslug® for 25 years and there is little information on the pathogenicity of wild strains of *P. hermaphrodita*. Therefore, we investigated the pathogenicity and host feeding inhibition of nine recently isolated *P. hermaphrodita* strains (Andrus & Rae, 2018) to the common pest slug *D. invadens* and compared results to those of *P. hermaphrodita* DMG0001. *D. invadens* was chosen as the host in this study as it has been reported invading new areas over the last century and is an important pest of UK agricultural crops (Williams et al., 2010) with a worldwide distribution (Hutchinson et al., 2014). Studies of natural isolates of *P. hermaphrodita* that vary in pathogencity could determine the mechanism of infection causing host mortality.

2.2. Materials and Methods

2.2.1. Source and maintenance of invertebrates

Phasmarhabditis hermaphrodita commercial strain DMG0001 (Nemaslug®) was supplied by BASF Agricultural Specialities and stored at 10°C before use. Wild *P. hermaphrodita* strains (DMG0002, DMG0003, DMG0005, DMG0006, DMG0007, DMG0008, DMG0009, DMG0010 and DMG0011) were isolated from slugs collected from locations around Liverpool, UK (Andrus & Rae 2018). They have been in culture at Liverpool John Moores University (LJMU) since 2014 on modified White traps (White, 1927). Each strain began as an isogenic line from a single hermaphrodite mother and was identified to species via amplification and sequencing of the ITS1, 18SrRNA and the D2-D3 domain of large subunit (LSU) rDNA genes (Andrus & Rae, 2018). For experimentation, the nematodes were grown on decaying slug (*Limax flavus*) on modified White traps until they had reached the dauer stage and were then stored in cell culture flasks at 10 °C (see Andrus & Rae, 2018 for more details). *L. flavus* were frozen at 80 °C before use to kill any existing nematodes. For each experiment, fresh dauers were grown.

Deroceras invadens (mean weight ± SE 0.70 g ± 0.55, n = 660) were collected from parks around Liverpool. Slugs were stored in non-airtight plastic containers in the dark at 10°C. Damp paper towel was used to retain moisture and slugs were fed lettuce *ad libitum*. Slugs were stored like this for 1 week before use to screen for any prior infection.

2.2.2. Survival and feeding inhibition of *D. invadens* exposed to *P. hermaphrodita*

A method adapted from Wilson et al. (1993) was used to test the pathogenicity and feeding inhibition of *D. invadens* exposed to *P. hermaphrodita*. Two *D. invadens* were placed in a 20 ml plastic tube with a cotton wool bung pushed to the bottom and 2 ml water was added to the tube. Slugs were exposed to 2 ml of 500 or 1000 nematodes per ml, or 2 ml of water without nematodes as a control. A cotton wool bung was used to prevent the slugs from escaping and the lid was loosely screwed on to allow airflow. Slugs were then incubated at 10 °C in the dark for

5 days. After exposure, each slug was placed on a 5 cm Petri dish containing a 3 cm diameter disc of lettuce (area 700 mm^2). Petri dishes were then incubated at 10 °C for 9 days. Mortality was recorded every 2–3 days and the volume of lettuce disc eaten was recorded 8 and 14 days after initial infection by tracing the remaining lettuce disc on 1 mm^2 graph paper (Rae et al., 2009a). Ten *D. invadens* were used per experiment, repeated three times for each wild *P. hermaphrodita* strain. A no nematode control and *P. hermaphrodita* DMG0001 (concentration 500, 1000 nematodes) were run with each group of wild *P. hermaphrodita* tested.

2.2.3. Data analysis

A Log Rank test was used in OASIS (Yang et al., 2011) to analyse *D. invadens* survival after exposure to *P. hermaphrodita* at 0, 500 and 1000 nematodes per ml. The amount of lettuce consumed by *D. invadens* on days 8 and 14 was analysed using a One Way ANOVA with Tukey's post hoc test in R (R Core Team, 2020).

2.3. Results

2.3.1. Survival of *D. invadens* exposed to wild *P. hermaphrodita* strains and commercial *P. hermaphrodita* DMG0001

At a concentration of 500 nematodes per ml, the commercial strain of *P. hermaphrodita* DMG0001 and all wild strains except *P. hermaphrodita* DMG0010, caused significant mortality to *D. invadens* when compared to an uninfected control after 14 days ($P < 0.05$) (Fig. 2.1a). The wild *P. hermaphrodita* strains DMG0002, DMG0005, DMG0007, and DMG0008 were significantly more pathogenic than commercial *P. hermaphrodita* strain DMG0001. Wild *P. hermaphrodita* DMG0002 and DMG0008 caused rapid and consistent mortality, killing 12.2% and 10.6% slugs per day, respectively ($P < 0.05$) (Fig. 2.1a).

At the higher concentration of 1000 nematodes per ml, *P. hermaphrodita* DMG0001 and all wild strains caused significant mortality to *D. invadens* compared to an uninfected control after 14 days ($P < 0.05$) (Fig. 2.1b). Wild *P. hermaphrodita* strains DMG0007, DMG0008, DMG0009 and DMG0010 caused significantly more

D. invadens mortality than the commercial *Phasmarhabditis hermaphrodita* strain (DMG0001) at 1000 nematodes per ml after 14 days. The fastest mortality rate was seen in *P. hermaphrodita* strains DMG0009 (15% per day) and DMG0010 (13.9% per day) at 1000 nematodes per ml ($P < 0.05$) (Fig. 2.1b).

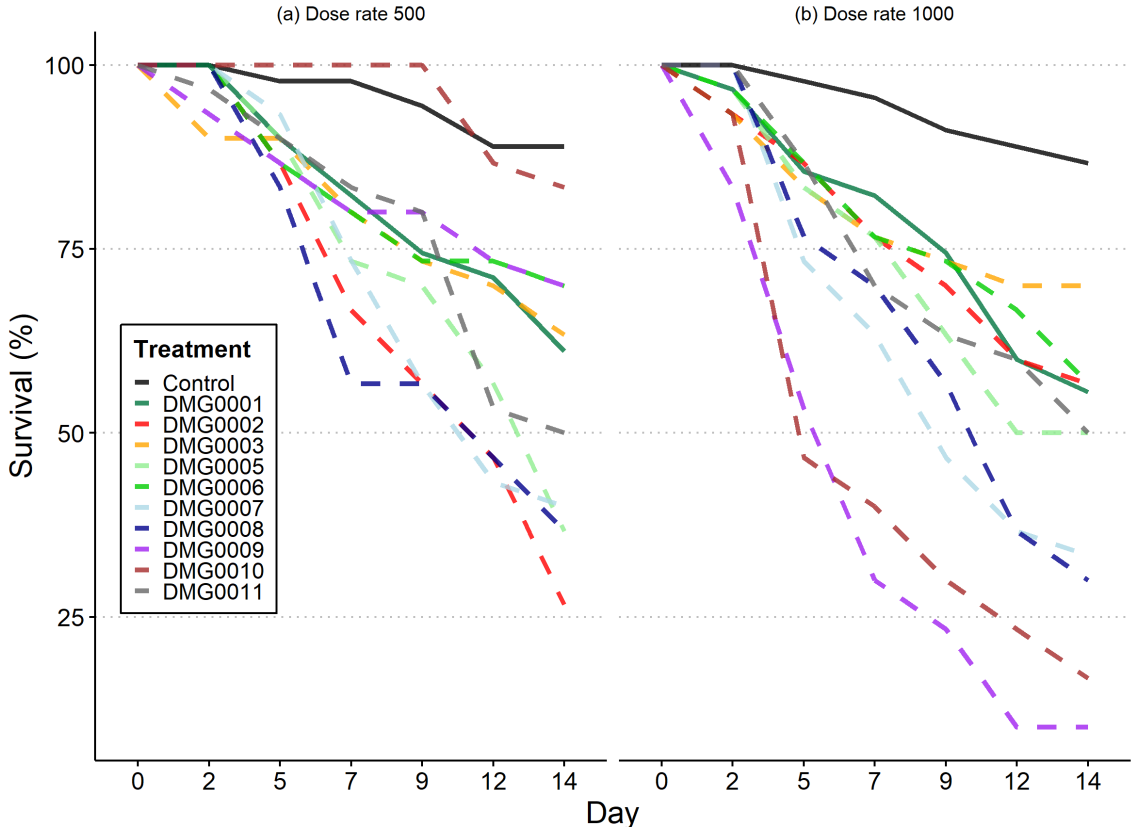

Figure 2.1: Percentage survival of *Deroceras invadens* exposed to 0 (black line), 500 (a), and 1000 (b) nematodes per ml of *Phasmarhabditis hermaphrodita* commercial and wild strains over 14 days.

2.3.2. Feeding inhibition of *D. invadens* caused by *P. hermaphrodita* infection

There were significant differences in the amount of lettuce consumed by *D. invadens* exposed to all treatments after 8 days ($F(10, 272) = 3.716$, $P < 0.0001$) and 14 days ($F(10, 227) = 5.922$, $P < 0.0001$) at a concentration of 500 nematodes/ml (Fig. 2.2a). Specifically, *P. hermaphrodita* DMG0008 (43.3 ± 18.3 mm^2, $P < 0.01$) and DMG0009 (114.9 ± 32.4 mm^2, $P < 0.05$) caused significant feeding inhibition in *D. invadens* compared to the uninfected control (317.6 ± 38.6 mm^2) at a concentration of 500 nematodes/ml (Fig. 2.2a).

After 14 days *P. hermaphrodita* DMG0003 (297.4 ± 58.5 mm^2, $P < 0.01$), DMG0005 (215.4 ± 47.5 mm^2, $P = 0.001$), DMG0006 (296.8 ± 60.9 mm^2, $P < 0.05$), DMG0007 (255.4 ± 64.8 mm^2, $P < 0.01$), DMG0008 (81.6 ± 25.1 mm^2, $P < 0.001$), DMG0009 (208.9 ± 60.2 mm^2, $P < 0.001$), DMG0010 (244.8 ± 49.6 mm^2, $P < 0.001$) and DMG0011 (301.7 ± 42.4 mm^2, $P < 0.05$) caused significant feeding inhibition to *D. invadens* compared to the uninfected control (534.5 ± 33.6 mm^2) at a concentration of 500 nematodes per ml (Fig. 2.2a). Only *P. hermaphrodita* DMG0008 (81.6 ± 25.1 mm^2, $P < 0.05$) inhibited *D. invadens* feeding significantly more than commercial strain DMG0001 (354.9 ± 45.9 mm^2) after 14 days at 500 nematodes per ml (Fig. 2.2a).

After exposure to a concentration of 1000 nematodes per ml, there were significant differences among treatments in the amount of lettuce consumed by *D. invadens* after 8 days ($F(10, 246) = 11.890$, $P < 0.0001$) and after 14 d ($F(10, 169) = 9.156$, $P < 0.0001$). Feeding was inhibited significantly more than the uninfected control (348.9 ± 36.4 mm^2) after 8 days by *P. hermaphrodita* DMG0001 (180.4 ± 30.5 mm^2, $P < 0.01$) and the wild strains DMG0002 (76.8 ± 22.6 mm^2, $P = 0.001$), DMG0003 (57.0 ± 22.3 mm^2, $P < 0.01$), DMG0005 (28.6 ± 9.6 mm^2, $P < 0.05$), DMG0006 (139.8 ± 34.2 mm^2, $P < 0.001$), DMG0007 (111.5 ± 25.5 mm^2, $P < 0.001$) and DMG0009 (30.3 ± 14.5 mm^2, $P < 0.001$) (Fig. 2.2b).

After 14 days *P. hermaphrodita* DMG0001 (214.5 ± 38.7 mm^2, $P < 0.001$) and the wild strains DMG0002 (164.1 ± 46 mm^2, $P < 0.001$), DMG0003 (148.1 ± 41.3 mm^2, $P < 0.001$), DMG0005 (138.4 ± 52.8 mm^2, $P < 0.001$), DMG0006 (283.4 ± 68.5 mm^2, $P < 0.001$), DMG0007 (185.4 ± 43.8 mm^2, $P < 0.001$), DMG0009 (85.0 ± 59.7 mm^2, $P < 0.01$) and DMG0011 (285.7 ± 48.2 mm^2, $P < 0.01$) caused more feeding inhibition than the uninfected control (534.6 ± 33.5 mm^2) at 1000 nematodes per ml ($P < 0.05$) (Fig. 2.2b). None of the wild strains inhibited feeding significantly more than *P. hermaphrodita* DMG0001 after 8 or 14 days at a concentration of 1000 nematodes per ml.

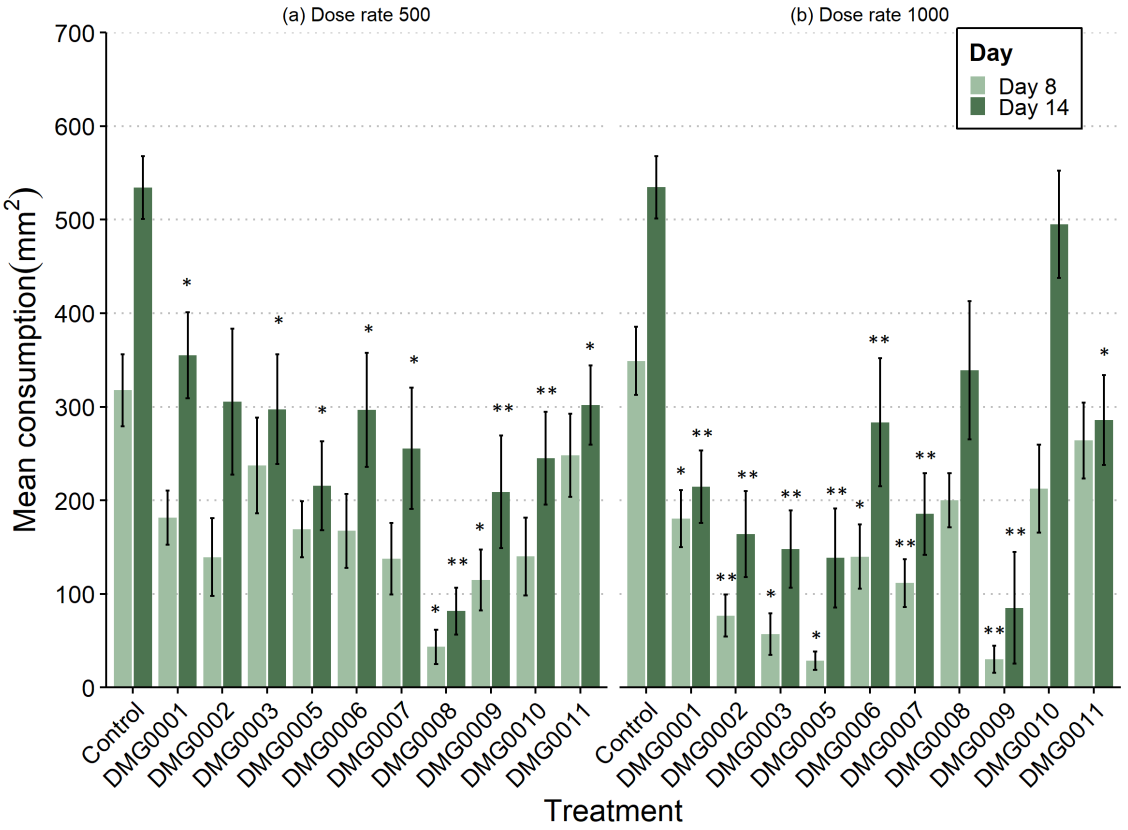

Figure 2.2: Feeding inhibition of *Deroceras invadens* exposed to 0, 500 (a) and 1000 (b) nematodes per ml of *Phasmarhabditis hermaphrodita* (DMG0001, DMG0002, DMG0003, DMG0005, DMG0006, DMG0007, DMG0008, DMG0009, DMG0010 and DMG0011) after 8 and 14 days. Significant differences between the amount consumed by the control and treatments at $p < 0.05$ are denoted by * and at $p < 0.001$ denoted by **. Bars represent ± 1 standard error.

2.4. Discussion

The results of this study showed that wild isolated *P. hermaphrodita* from the UK are highly pathogenic to *D. invadens*. Although *P. hermaphrodita* has been isolated globally (Pieterse et al., 2017a), there have been few reports on the pathogenic potential of wild *P. hermaphrodita* strains apart from Wilson et al. (2012) showing a New Zealand strain of *P. hermaphrodita* is pathogenic to the slug *D. reticulatum* (Wilson et al., 2012). Also, Tandingan De Ley et al. (2020) found a USA strain of *P. hermaphrodita* kills the snail *T. pisana* and neonate life stages of *L. fulica* (McDonnell et al., 2018a). In contrast, Williams & Rae (2015) showed commercial *P. hermaphrodita* DMG0001 could not kill 12-week-old *L. fulica*, but a wild USA *P. hermaphrodita* strain ITD290, was highly virulent (Mc Donnell et al., 2018a). The reasons for this difference could be due to the larger snails used by Williams & Rae

(2015) as larger and older snails and slugs tend to be more resistant to nematode infection. This has been observed in infection studies exposing *C. aspersum*, *A. ater* (Glen et al., 1996) and *A. lusitanicus* (Speiser et al., 2001; Grimm, 2002) to *P. hermaphrodita*. Only *P. hermaphrodita* DMG0007 and DMG0008 caused significantly more mortality than *P. hermaphrodita* DMG0001 at both nematode concentrations. *P. hermaphrodita* DMG0002 and DMG0005 only caused significantly more pathogenicity at the lower concentration of 500 nematodes per ml. Higher pathogenicity at a lower concentration indicates that these wild isolates are highly virulent, and a lower worm burden is required for infection, proliferation, and death of the host. Interestingly, DMG0010 caused no mortality at 500 nematodes per ml, yet was highly virulent at 1000 nematodes per ml. Presumably a higher worm burden is required for DMG0010 to establish a lethal infection and when such infection is established this strain kills more rapidly than others. Natural variation in pathogenicity between nematode strains is well documented in entomopathogenic nematodes, such as wild isolated strains of *Heterorhabditis bacteriophora* and *Steinernema carpocapsae* that were found to be more virulent to their targeted hosts (*Cephalcia tannourinensis* Chevin and *Agrotis ipsilon* Hufnagel, 1766, respectively) than the commercial formulations (Noujeim et al., 2015; Bélair et al., 2013). Further host range testing of the more virulent wild isolated *P. hermaphrodita* strains is needed. We found wild *P. hermaphrodita* strains DMG0002, DMG0005, DMG0007, DMG0008, DMG0009 and DMG0010 were more pathogenic than the commercial strain DMG0001 depending on nematode concentration. The reasons for this difference could be due to their associated bacteria. Infectivity and pathogenicity of *P. hermaphrodita* are strongly influenced by bacterial diet (Wilson et al., 1995a,b). *M. osloensis* was chosen for commercial production from a selection of 16 isolates as it consistently produced high yields of pathogenic nematodes (Wilson et al., 1995a,b). *Phasmarhabditis hermaphrodita* is still grown on this bacterium. It is thought that *M. osloensis* is released into the slug's haemocoel by dauer stage nematodes and causes septicaemia by production of an endotoxin (Tan & Grewal, 2001b; 2002). However, there is evidence suggesting that *M. osloensis* is not vertically transmitted to the next generation of pathogenic nematodes (Rae et al., 2010; Nermut' et al., 2014).

Therefore, the relationship between *P. hermaphrodita*, and especially wild strains, with bacteria warrants further investigation. One such approach could be to use 16S metagenomics of bacteria present in the nematode gut, which has worked well in profiling the associated microflora in other nematodes like *C. elegans* (Dirksen et al., 2016). As well as killing slugs, the wild strains of *P. hermaphrodita* inhibited feeding of *D. invadens*. This is a common symptom of *P. hermaphrodita* infection and enhances the use of these nematodes as a biological control agent. Host feeding inhibition is also caused by species that resist infection (Glen et al., 2000), such as juvenile *A. lusitanicus* (Grimm, 2002). Variation across exposure concentration, day and nematode strain was observed. Wild *P. hermaphrodita* strain DMG0009 had the largest effect, inhibiting slug feeding more than the control at both doses and more than the commercial strain at 1000 nematodes per ml.

Other factors may influence the pathogenic potential of these nematodes including the ability of each strain to locate a host. For example, wild strains may display better chemoattraction, a trait that has diminished in the commercial strain (Andrus et al., 2018; Andrus & Rae, 2019; Cutler & Rae 2021 – Chapter 5). In these experiments, using the same wild strains as above, the commercial strain (DMG0001) responded poorly to multiple attractants, with the majority of the nematodes remaining at the point of application. Both authors also observed high levels of natural variation in the chemoattraction behaviour of *P. hermaphrodita* wild strains exposed to multiple slug mucus samples as well as 1% and 5% hyaluronic acid, a key component of mucus (Andrus et al., 2018; Cutler & Rae 2021 – Chapter 5).

We suggest that understanding natural variation between strains used for biological control purposes could result in a more effective product. Genetic variation in virulence could also help with understanding which genes are associated with pathogenicity, aid in understanding the evolution of parasitism and even enhance *P. hermaphrodita* as a biological control agent (Rae, 2017b). Additional research on the better performing strains is needed, including understanding the effects associated bacteria have on yield and virulence, and optimising *in vitro* culture techniques.

3. Pathogenic potential of wild isolated *P. neopapillosa* and *P. californica* exposed to the slug *D. invadens* and the snail *C. aspersum*

3.1. Introduction

To date, there are 15 known species of *Phasmarhabditis*: *P. hermaphrodita* (Schneider, 1859), *P. papillosa* (Schneider, 1866), *P. neopapillosa* (Mengert, 1953), *P. tawfiki* (Azzam, 2003), *P. huizhouensis* (Huang et al., 2015), *P. bonaquaense* (Nermut' et al., 2016a), *P. apuliae* (Nermut' et al., 2016b), *P. californica* (Tandingan De Ley et al., 2016), *P. bohemica* (Nermut' et al., 2017), *P. meridionalis* (Ivanova & Spiridonov, 2017), *P. safricana* (Ross et al., 2018), *P. circassica* , *P. clausiliiae* (Ivanova et al., 2020), *P. zhejiangensis* (Zhang & Liu, 2020) and *P. kenyaensis* (Pieterse et al., 2020). Currently, *P. hermaphrodita, P. papillosa, P. neopapillosa, P. tawfiki*, *P. safricana P. bohemica, P. bonaquaense, P. apuliae,* and *P. californica* have been reported to kill slugs (Wilson et al.,1993; Hooper et al., 1999; Azzam & Twafiki, 2003; Pieterse et al., 2017b; Ross et al., 2018; Nermut et al., 2019; Mc Donnell et al., 2020). One such species (*P. neopapillosa*, first described by Mengert, 1953) has received little attention in terms of pathogenic ability. It is morphologically indistinguishable from closely related *P. hermaphrodita* (Hooper et al., 1999) apart from male incidence which can be a distinguishing factor, being far greater in *P. neopapillosa* (25% - 50% male production) and is very rare in *P. hermaphrodita* (Andrassy, 1983; Hooper et al., 1999; Andrus & Rae, 2018). Although *P. neopapillosa* has been described as being a lethal parasite of slugs (Glen et al., 1996), there are no quantitative data investigating its pathogenic potential.

As well as *P. neopapillosa* there is little information about the pathogenicity of the recently described species *P. californica*, which since discovery in the USA (Tandingan De Ley et al., 2016), has been isolated in New Zealand (Wilson et al.,

2012, 2016), Canada (Brophy et al., 2020), Ireland (Carnaghi et al., 2017) and Wales (Andrus & Rae, 2018). In recent studies by Tandingan De Ley et al. (2020) and Mc Donnell et al. (2020), *P. californica, P. hermaphrodita, P. papillosa* and an undescribed *Phasmarhabditis* species isolated from the USA were shown to be lethal to the snail *T. pisana* and the slug *D. reticulatum*, under laboratory conditions. There is currently no information on the pathogenicity of UK strains of *P. californica*.

To investigate the pathogenic potential of these wild species we exposed five recently isolated strains of *P. neopapillosa* and three strains of *P. californica* to the slug *D. invadens* and the snail *C. aspersum*, a notorious pest species found across Europe and America (Guiller et al., 2012), and compared nematode pathogenicity to the commercial strain of *P. hermaphrodita* DMG0001. The nematodes were applied at two different doses and the survival and feeding inhibition was recorded over 14 days. We monitored host feeding as *P. hermaphrodita* infection causes slug feeding inhibition (Glen et al., 2000), (it is unknown for *P. neopapillosa* and *P. californica*), a useful trait for pest control. We also recorded the number of dauer larvae produced on the cadavers of *D. invadens* post infection to understand the reproductive capacity these nematodes have on this host. If *P. neopapillosa* and *P. californica* killed *D. invadens* and *C. aspersum* they could to be developed as new biological control agents potentially superior to the current strain of *P. hermaphrodita* which is not as virulent as wild strains (Cutler & Rae, 2020; Chapter 2).

3.2. Materials and Methods

3.2.1. Source and maintenance of invertebrates

Five wild strains of *P. neopapillosa* (DMG0012, DMG0013, DMG0014, DMG0015 and DMG0016) and three wild strains of *P. californica* (DMG0017, DMG0018 and DMG0019) housed at LJMU were grown to the dauer stage using the same methodology as Chapter 2. *D. invadens* (mean weight ± SE 0.70g ± 0.55, n= 720) were collected from Sefton park, Liverpool, UK and were treated as per Chapter 2. Neonate *C. aspersum* (mean weight ± SE 0.06g ± 0.05g, n= 360) were supplied by Escargotsni online retailers (Armagh, Northern Ireland) and housed under the

same conditions as *D. invadens*.

3.2.2. Survival and feeding inhibition of *D. invadens* and *C. aspersum* exposed to *P. neopapillosa* and *P. californica*

Deroceras invadens were exposed to *P. neopapillosa, P. californica* and *P. hermaphrodita* using methodology from Chapter 2. To assess the susceptibility of neonate snails a protocol by Cutler and Rae (2020)(Chapter 2) was adapted. Briefly, a piece of cotton wool was added to the bottom of fifteen 20 ml universal tubes. One thousand dauer stage *P. hermaphrodita* (DMG0001) were added to five tubes and this process was repeated for *P. neopapillosa* (DMG0012, DMG0013, DMG0014, DMG0015 and DMG0016) and *P. californica* (DMG0017, DMG0018 and DMG0019). Five tubes had the equivalent volume of water and no nematodes added and acted as the control. Two *C. aspersum* were added to each tube and the tubes were loosely sealed and stored at 15°C. After 5 days infection the snails were removed and allowed to feed on a 3.5 cm diameter disc of lettuce. Feeding was quantified on days 8 and 14 using methods by Cutler and Rae (2020)(Chapter 2) and the survival of the snails was monitored every 2-3 days. The experiment was repeated three times in total with 30 snails being exposed to each treatment.

3.2.3. Number of *P. neopapillosa* and *P. californica* produced on *D. invadens* cadavers

The number of dauer larvae produced on the cadaver of *D. invadens* infected by each strain of *P. neopapillosa* (DMG0012, DMG0013, DMG0014, DMG0015 and DMG0016), *P. californica* (DMG0017, DMG0018 and DMG0019) and *P. hermaphrodita* (DMG0001) was recorded. Dead *D. invadens* were gently washed with distilled water and placed on modified White traps (White, 1927) and incubated at 20°C for 21 days. After 21 days, the number of dauers in the water was quantified. This was repeated for a minimum of ten *D. invadens* cadavers for each nematode strain.

3.2.4. Data analysis

A Log Rank test was used in OASIS (Yang et al., 2011) to analyse *D. invadens* and *C. aspersum* survival after exposure to *P. neopapillosa* (DMG0012, DMG0013, DMG0014, DMG0015 and DMG0016), *P. californica* (DMG0017, DMG0018 and DMG0019) and *P. hermaphrodita* (DMG0001) at 0, 500 and 1000 nematodes per ml. The amount of lettuce consumed by *D. invadens* and *C. aspersum* on days 8 and 14 was analysed using a One-Way ANOVA with Tukey's post hoc test in R. The number of dauers emerging from *D. invadens* cadavers was analysed using a One-Way ANOVA with Tukey's post hoc test in R (R Core Team, 2020).

3.3. Results

3.3.1. Survival of *D. invadens* exposed to *P. neopapillosa*

All *P. neopapillosa* strains and *P. hermaphrodita* (DMG0001) caused significantly higher mortality to *D. invadens* compared to the uninfected control at a dose rate of 500 nematodes per ml ($P < 0.05$) (Fig. 3.1a) and 1000 nematodes per ml ($P < 0.001$) (Fig. 3.1b). All *P. neopapillosa* strains applied at both 500 and 1000 nematodes per ml were significantly more pathogenic than *P. hermaphrodita* DMG0001, apart from *P. neopapillosa* DMG0016 ($P > 0.05$) (Fig. 3.1).

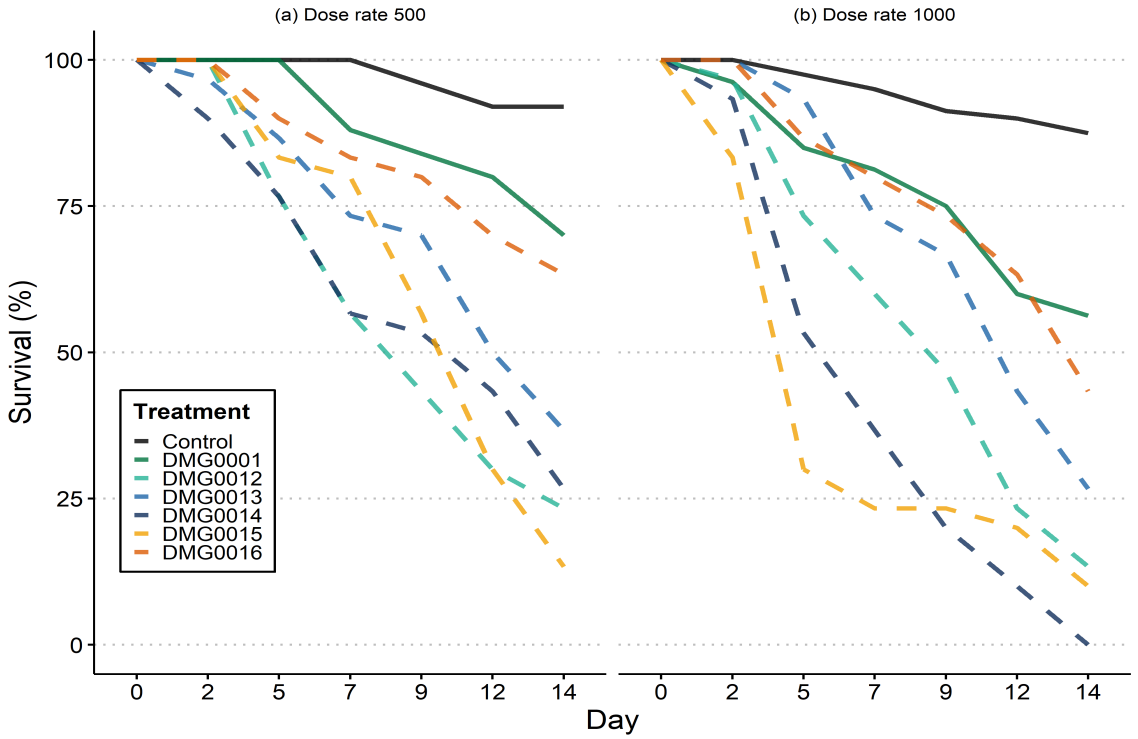

Figure 3.1: Percentage survival of *Deroceras invadens* exposed to *Phasmarhabditis hermaphrodita* DMG0001, *P. neopapillosa* (DMG0012, DMG0013, DMG0014, DMG0015, and DMG0016) and an uninfected control at dose rates of 500 (a) and 1000 (b) nematodes per ml.

3.3.2. Survival of *D. invadens* exposed to *P. californica*

At a dose of 500 nematodes per ml, *P. californica* (DMG0017 and DMG0019) and *P. hermaphrodita* (DMG0001) caused significant mortality to *D. invadens* compared to the uninfected control after 14 days ($P<0.05$) (Fig. 3.2a), whereas *P. californica* (DMG0018) did not ($P>0.05$) (Fig. 3.2a). At a dose of 500 nematodes per ml, no *P. californica* strains (DMG0017, DMG0018 and DMG0019) caused significantly more mortality than *P. hermaphrodita* (DMG0001) (Fig. 3.2a) ($P>0.05$)

Phasmarhabditis californica (DMG0017, DMG0018 and DMG0019) and *P. hermaphrodita* (DMG0001) caused significant mortality to *D. invadens* when applied at 1000 nematodes per ml compared to the untreated control after 14 days (Fig. 3.2b) ($P<0.001$). Surprisingly, *P. californica* (DMG018) killed *D. invadens* significantly faster than *P. hermaphrodita* (DMG0001) (Fig. 3.2b) ($P<0.05$).

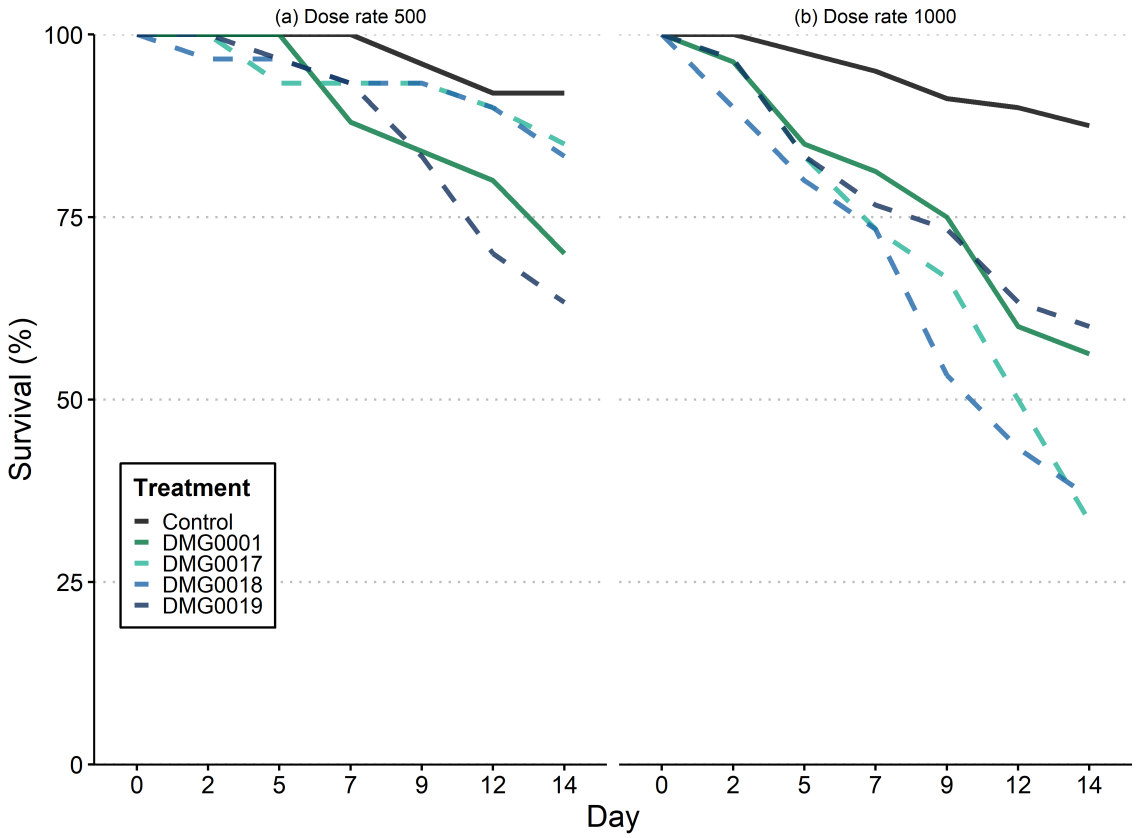

Figure 3.2: Percentage survival of *Deroceras invadens* exposed to *Phasmarhabditis hermaphrodita* DMG0001, *P. californica* (DMG0017, DMG0018, DMG0019) and an uninfected control at dose rates of 500 (a) and 1000 (b) nematodes per ml.

3.3.3. Feeding inhibition of *D. invadens* exposed to *P. neopapillosa*

All *P. neopapillosa* strains ($P < 0.01$) and *P. hermaphrodita* DMG0001 ($P < 0.01$) caused significant feeding inhibtion to *D. invadens* compared to an uninfected control 8 and 14 days after initial exposure to 500 nematodes per ml (Fig. 3.3a). After 8 and 14 days all *P. neopapillosa* strains inhibited feeding significantly more than *P. hermaphrodita* DMG0001 (Fig. 3.3a)

Eight and fourteen days after being exposed to a dose of 1000 nematodes per ml, all *P. neopapillosa* strains caused significant feeding inhibition to *D. invadens* when compared to an uninfected control ($P > 0.05$) (Fig. 3.3b). There was no significant difference between the feeding of *D. invadens* exposed to *P. hermaphrodita* DMG0001 and the uninfected control after 8 days ($P > 0.05$) (Fig. 3.3b). Only *P. neopapillosa* DMG0016 did not cause significant feeding inhibtion when compared to *P. hermaphrodita* DMG0001 after 8 and 14 days.

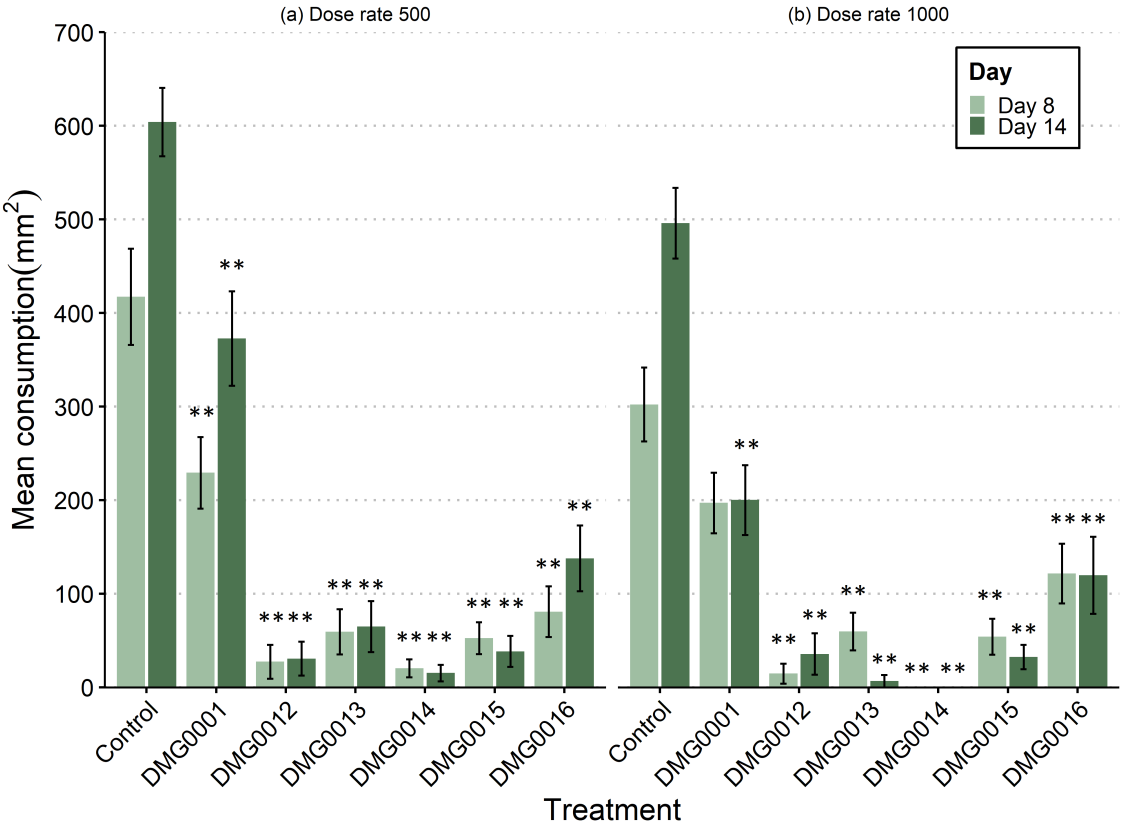

Figure 3.3: Feeding inhibition of *Deroceras invadens* exposed to 0, 500 (a) and 1000 (b) nematodes per ml of *Phasmarhabditis neopapillosa*(DMG0012, DMG0013, DMG0014, DMG0015 and DMG0016) and *P.hermaphrodita*(DMG0001). Significant differences between the amount consumed by the control and treatments at $p < 0.05$ are denoted by * and at $p < 0.001$ denoted by **. Bars represent ± 1 standard error.

3.3.4. Feeding inhibition of *D. invadens* exposed to *P. californica*

There was a significant difference between the amount *D. invadens* consumed when exposed to 500 *P. californica* (DMG0017 and DMG0019) per ml and the control after 8 days (P<0.05) (Fig. 3.4a). After 14 days, *P. californica* (DMG0017) (P<0.001) and (DMG0018) (<0.05) and *P. hermaphrodita* (DMG0001) (P<0.05) applied at 500 nematodes per ml caused significant feeding inhibition compared to untreated *D. invadens* (Fig. 3.4a). *Phasmarhabditis californica* (DMG0017) caused significantly more feeding inhibition than DMG0019 after 14 days at 500 nematodes per ml (P<0.05) (Fig. 3.4a).

When exposed to 1000 nematodes per ml, feeding of *D. invadens* was significantly reduced (compared to the untreated *D. invadens*) by *P. californica* (DMG0019) (P<0.001) and *P. hermaphrodita* (DMG0001) (P<0.001) on day 8 (P<0.001) (Fig. 3.4b).

However, after 14 days *D. invadens* infected with 1000 *P. californica* DMG0017 (P<0.05), DMG0018 (P<0.001), DMG0019 (P<0.001) and *P. hermaphrodita* (DMG0001) (P<0.001) per ml was significantly reduced compared to untreated *D. invadens* (Fig. 3.4b).

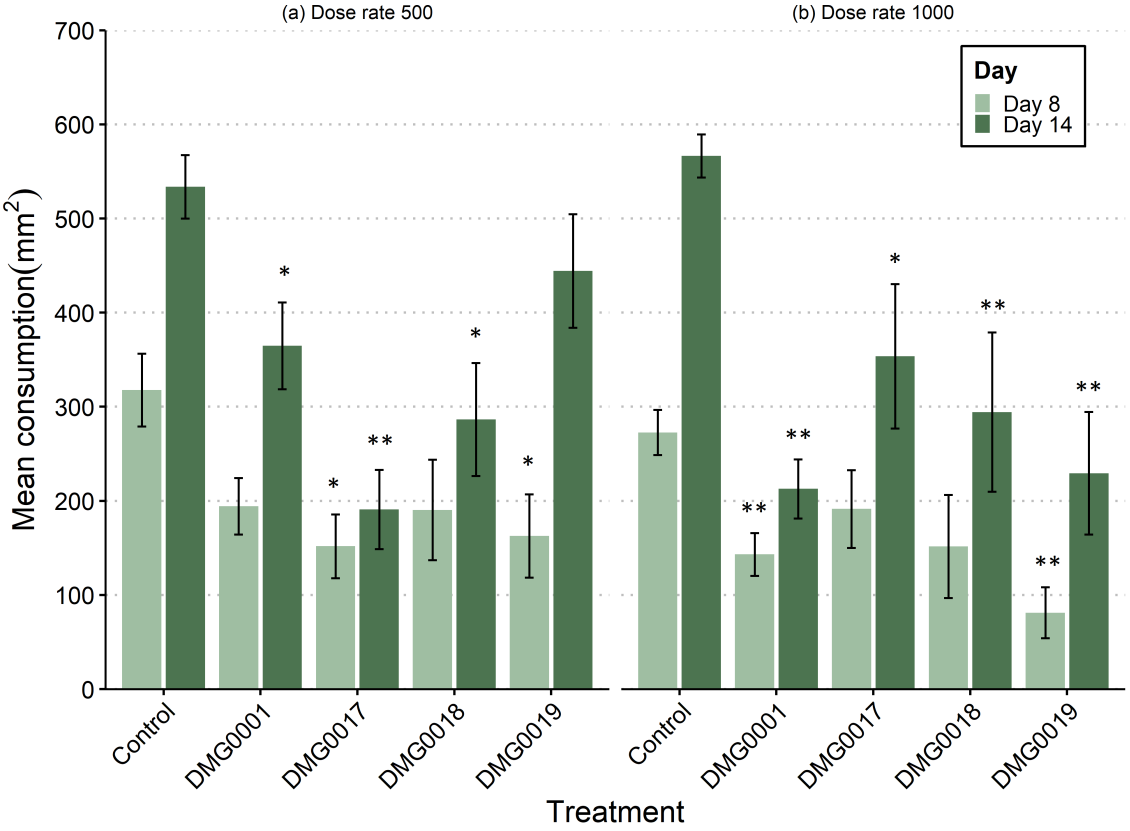

Figure 3.4: Feeding inhibition of *Deroceras invadens* exposed to 0, 500 (a) and 1000 (b) nematodes per ml of *Phasmarhabditis californica*(DMG0017, DMG0018 and DMG0019) and *P.hermaphrodita*(DMG0001). Significant differences between the amount consumed by the control and treatments at $p < 0.05$ are denoted by * and at $p < 0.001$ denoted by **. Bars represent ± 1 standard error.

3.3.5. Survival of *C. aspersum* exposed to *P. neopapillosa*

When *C. aspersum* were exposed to the *P. neopapillosa* strains and *P. hermaphrodita* DMG0001 there was significantly more mortality compared to uninfected *C. aspersum* (P< 0.05) (Fig. 3.5a). Only *P. neopapillosa* DMG0012 caused significantly more mortality to *C. aspersum* than *P. hermaphrodita* DMG0001 (P<0.05) (Fig. 3.5a).

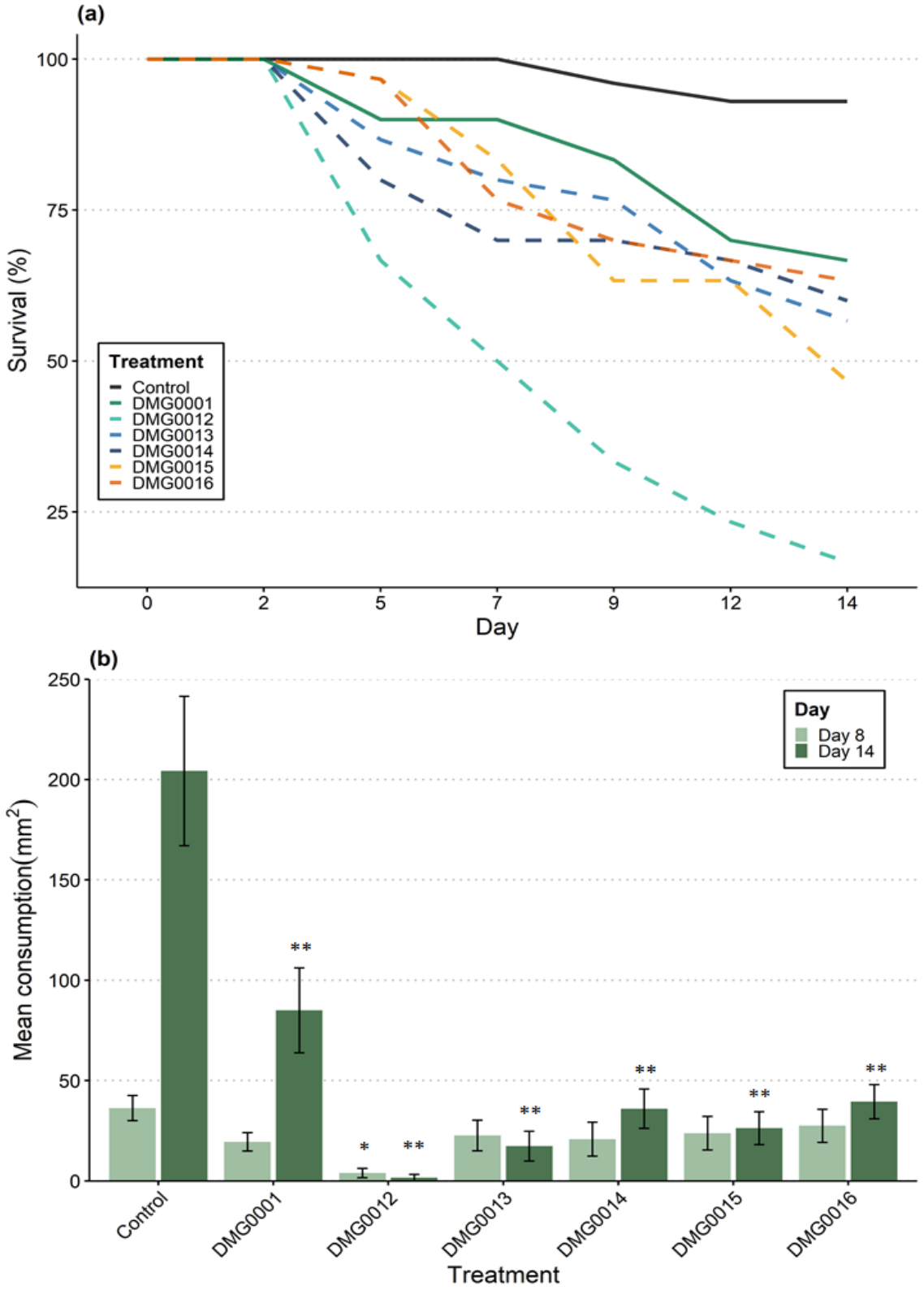

Figure 3.5: (a) Percentage survival of *Cornu aspersum* exposed to no nematodes, *Phasmarhabditis hermaphrodita* DMG0001 and *P. neopapillosa* (DMG0012, DMG0013, DMG0014, DMG0015 and DMG0016). (b) Mean consumption of lettuce discs by *C. aspersum* exposed to *P. hermaphrodita* (DMG0001), *P. neopapillosa* (DMG0012, DMG0013, DMG0014, DMG0015 and DMG0016) after 8 and 14 days. Significant differences between the amount consumed by the control and treatments at $p < 0.05$ are denoted by * and at $p < 0.001$ denoted by **. Bars represent ± 1 standard error.

3.3.6. Feeding inhibition of *C. aspersum* exposed to *P. neopapillosa*

No significant difference in feeding of *C. aspersum* was recorded between any treatments after 8 days (P>0.05) (Fig. 3.5b). After 14 days all *P. neopapillosa* strains significantly inhibited *C. aspersum* feeding more than an uninfected control (P<0.05) (Fig. 3.5b). *Phasmarhabditis hermaphrodita* DMG0001 did not significantly inhibit feeding more than the uninfected control (P>0.05) (Fig. 3.5b). No significant difference was found in lettuce consumption between *C. aspersum* exposed to *P. neopapillosa* and *P. hermaphrodita* (P>0.05) (Fig. 3.5b).

3.3.7. Survival of *C. aspersum* exposed to *P. californica*

There was a significant difference between the survival of untreated neonate *C. aspersum* compared to snails exposed to *P. hermaphrodita* (DMG0001) (P <0.05), *P. californica* (DMG0018) (P <0.05) and *P. californica* (DMG0019) (P <0.05) over 14 days (Fig. 3.6a). However, *P. californica* (DMG0017) (P >0.05) had no significant effect on the survival of neonate *C. aspersum*.

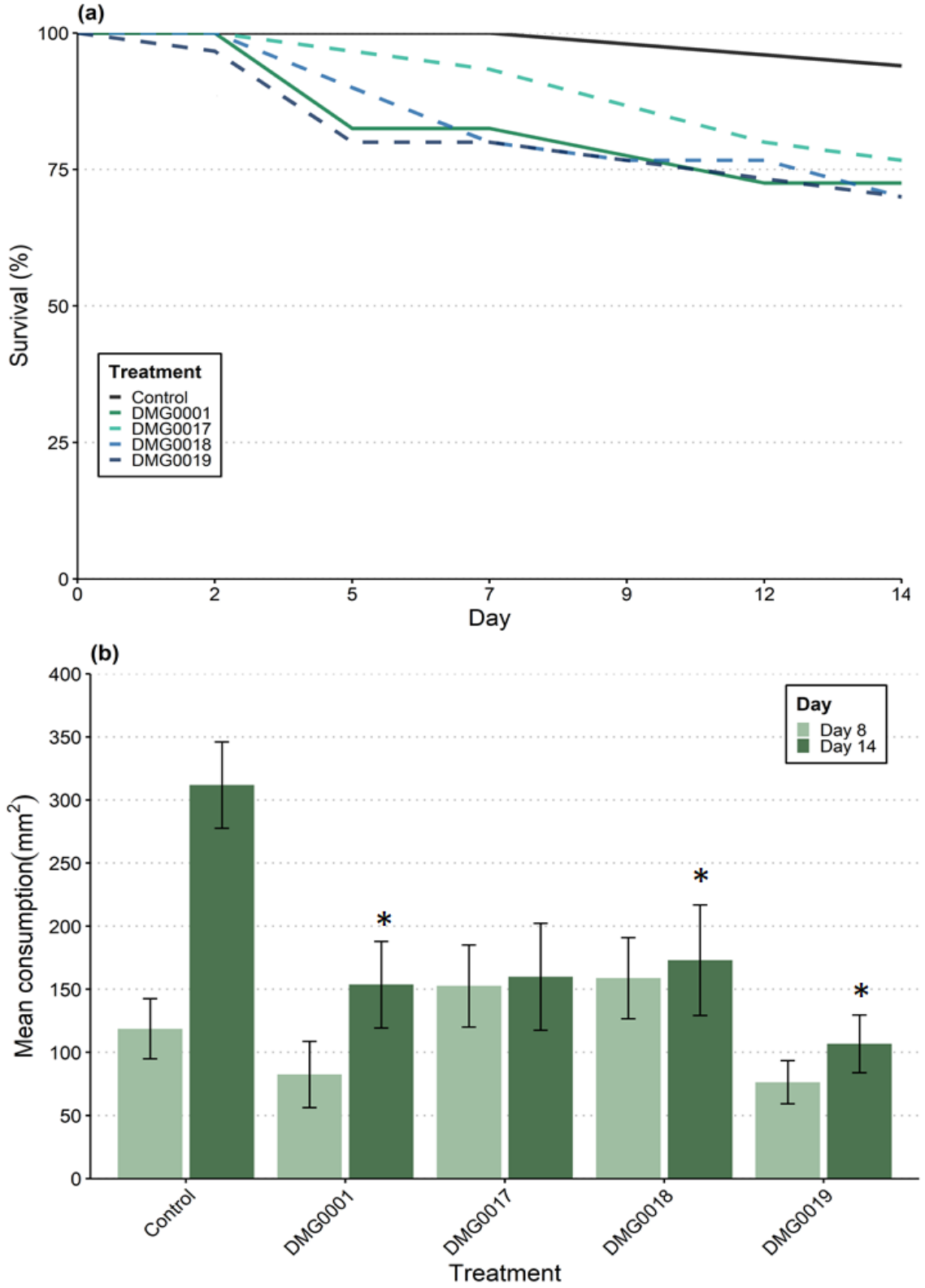

Figure 3.6: (a) Percentage survival of *Cornu aspersum* exposed to no nematodes, *Phasmarhabditis hermaphrodita* DMG0001 and *P. californica*(DMG0017, DMG0018, DMG0019). (b) Mean consumption of lettuce discs by *C. aspersum* exposed to *P. hermaphrodita* (DMG0001) and *P. californica*(DMG0017, DMG0018 and DMG0019) after 8 and 14 days. Significant differences between the amount consumed by the control and treatments at $p < 0.05$ are denoted by * and at $p < 0.001$ denoted by **. Bars represent ± 1 standard error.

3.3.8. Feeding inhibition of *C. aspersum* exposed to *P. californica*

The amount of lettuce (mm^2) neonate *C. aspersum* consumed when exposed to no nematodes and *P. californica* (DMG0019) ($P < 0.05$) 8 days after exposure to the nematodes differed significantly (Fig. 3.6b). However, there was no difference in snails treated with *P. hermaphrodita* (DMG0001), *P. californica* (DMG0017 or DMG0018) ($P > 0.05$) (Fig. 3.6b).

After 14 days all nematodes had a significant effect on the amount of lettuce neonate *C. aspersum* ate compared to the untreated control ($P < 0.01$) (Fig. 3.6b). There was no difference between the amount of lettuce eaten by *C. aspersum* exposed to *P. hermaphrodita* (DMG0001), or *P. californica* (DMG0017, DMG0018 and DMG0019) ($P > 0.05$).

3.3.9. Proliferation of *P. neopapillosa* on *D. invadens* cadavars

All *P. neopapillosa* strains, other than DMG0013, produced significantly more dauers on *D. invadens* cadavers than *P. hermaphrodita* DMG0001 ($P < 0.001$) (Fig. 3.7a) after 21 days at a dose rate of 500 nematodes per ml. Similarly, all *P. neopapillosa* strains produced significantly more dauers on *D. invadens* cadavers after 21 days than *P. hermaphrodita* DMG0001 ($P < 0.001$) at a dose rate of 1000 nematodes per ml (Fig. 3.7b). No nematodes were found on any of the uninfected control cadavers.

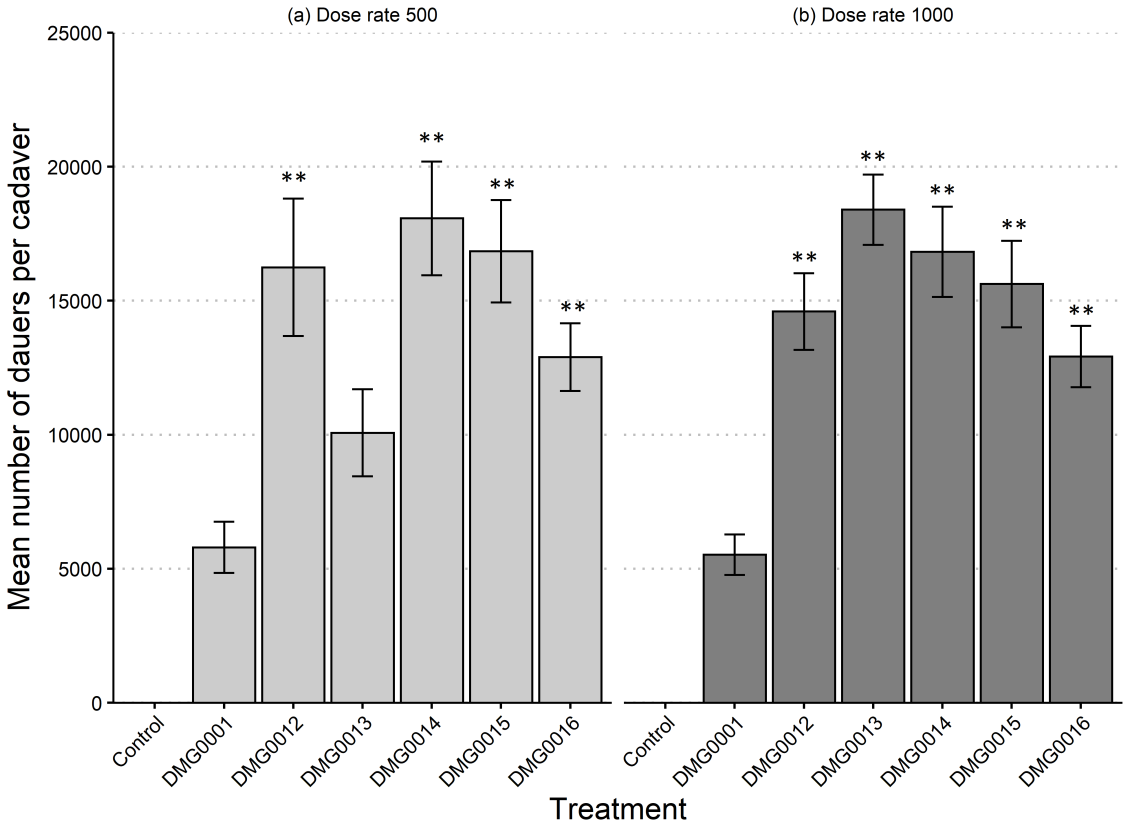

Figure 3.7: Mean number of dauers produced on *Deroceras invadens* cadavers by *Phasmarhabditis hermaphrodita* (DMG0001), *P. neopapillosa* (DMG0012, DMG0013, DMG0014, DMG0015, DMG0016) and an uninfected control at 500 (a) and 1000 (b) nematodes per ml. Significant differences between the amount of dauers produced by commercial *P. hermaphrodita* DMG0001 and wild *P. neopapillosa* strains at $p < 0.05$ are denoted by * and $p < 0.001$ are denoted by **.Bars represent ± 1 standard error.

3.3.10. Proliferation of *P. californica* on *D. invadens* cadavars

The numbers of *P. californica* (DMG0019) dauers produced after 21 days on *D. invadens* was significantly greater than *P. hermaphrodita* (DMG0001) when applied at a rate of 500 nematodes per ml ($P<0.001$) (Fig. 3.8a). After 21 days, only *P. californica* DMG0017 and DMG0018 produced significantly more dauers than *P. hermaphrodita* (DMG0001) on *D. invadens* exposed to 1000 nematodes per ml ($P<0.05$) (Fig. 3.8b).

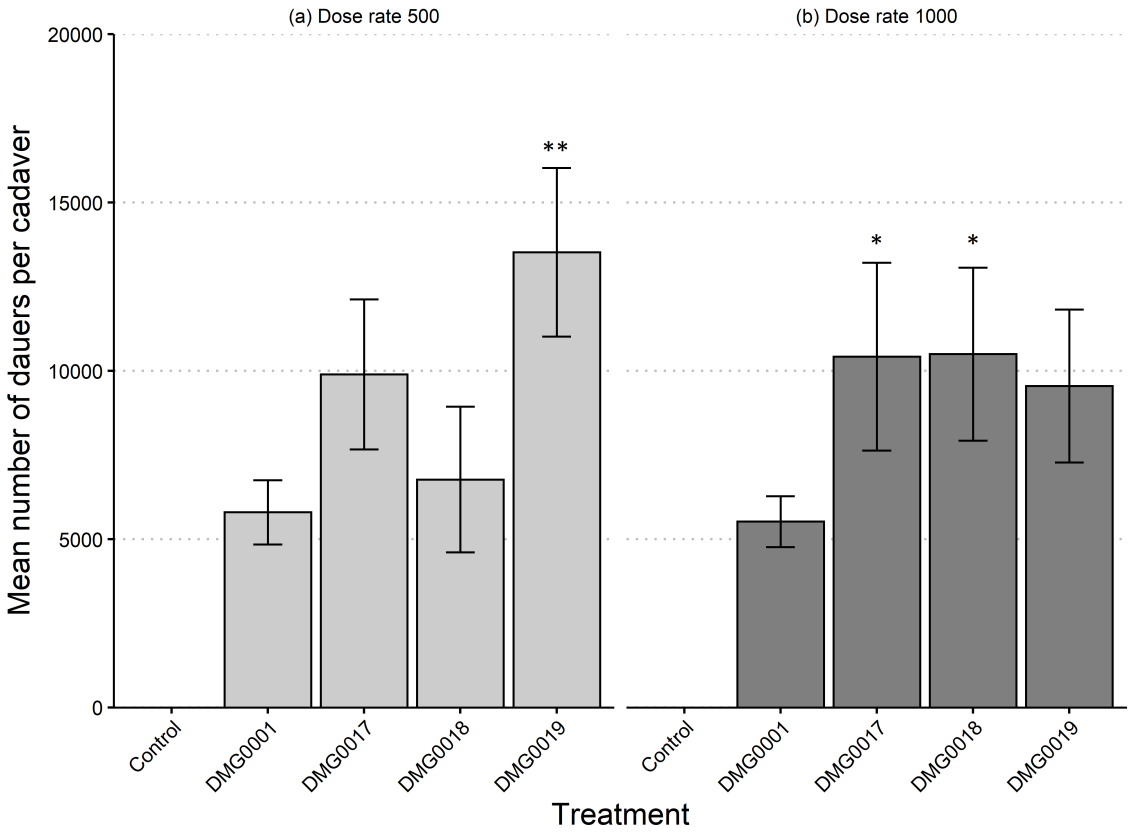

Figure 3.8: Mean number of dauers produced on *Deroceras invadens* cadavers by *Phasmarhabditis hermaphrodita* (DMG0001), and *P. californica* (DMG0017, DMG0018 and DMG0019) infected with 0, 500 (a) and 1000 (b) nematodes per ml. Significant differences between the amount of dauers produced by commercial *P. hermaphrodita* DMG0001 and wild *P. californica* strains at $p < 0.05$ are denoted by * and $p < 0.001$ are denoted by **. Bars represent ± 1 standard error.

3.4. Discussion

Previous research has stated *P. neopapillosa* parasitises slugs in a similar way to *P. hermaphrodita* (Glen et al., 1996), but there is no quantitative data on this. We found that all wild *P. neopapillosa* strains caused significantly more host mortality, feeding inhibition and proliferation on cadavers than an uninfected control. When compared to *P. hermaphrodita* DMG0001, four out of five wild isolated *P. neopapillosa* strains (DMG0012, DMG0013, DMG0014 and DMG0015) were more lethal to *D. invadens*. *Phasmarhabditis neopapillosa* DMG0012 could be considered the best performing wild *P. neopapillosa* strain, consistently outperforming the commercial *P. hermaphrodita* DMG0001 regarding *D. invadens* mortality, and was the only strain to cause higher *C. aspersum* mortality than *P. hermaphrodita* DMG0001. This

strain also inhibited *D.invadens* feeding more than *P. hermaphrodita* (DMG0001). *Phasmarhabditis neopapillosa* DMG0012 was also among the strains that displayed higher levels of proliferation than *P. hermaphrodita* DMG0001 on dead *D. invadens*. In contrast, *P. neopapillosa* DMG0016 was the only strain to not kill hosts or inhibit *D. invadens* feeding significantly more than *P. hermaphrodita* DMG0001.

There are only two recent studies (Tandingan De Ley et al., 2020; Mc Donnell et al., 2020) that have investigated the pathogenic ability of *P. californica* against terrestrial gastropods. Both of these studies used only a single USA isolated strain of *P. californica*, which was compared to wild isolated *P. hermaphrodita*. Here we show multiple *P. californica* strains are lethal to *D. invadens*, compared to commercial *P. hermaphrodita* (DMG0001) and can inhibit feeding and reproduce prolifically on this host. Specifically, *P. californica* (DMG0018) was significantly more pathogenic than the commercial strain *P. hermaphrodita* (DMG0001) when applied at 1000 nematodes per ml. However, at 500 nematodes per ml *P. californica* (DMG0018) did not cause mortality compared to the uninfected control, yet *P. californica* (DMG0017 and DMG0019) did. In general, the pathogenic potential of *P. californica* is poorly researched, especially for the UK strains. Tandingan De Ley et al. (2020) showed *P. californica* from the USA could kill the snail *T. pisana* (albeit with high doses of 150 nematodes per cm^2) and was equally as pathogenic as *P. hermaphrodita* and *P. papillosa*. Mc Donnell et al. (2020) tested the infectivity of *P. hermaphrodita*, *P. californica* and *P. papillosa* to *D. reticulatum* and found *P. papillosa* was the most pathogenic. From this research it is clear that *P. californica* is pathogenic to neonate stages of *C. aspersum* but this is strain dependant. Specifically, both *P. californica* DMG0018 and DMG0019 were pathogenic to neonate *C. aspersum* but *P. californica* DMG0017 was not.

Natural variation in pathogenicity of *Phasmarhabditis* nematodes towards other terrestrial gastropods has been reported. For example, Cutler and Rae (2020) (Chapter 2) reported that several wild strains of *P. hermaphrodita* were more virulent to slugs (*D. invadens*) than the commercial strain and other wild strains. Interestingly, the three strains of *P. californica* used in this study were isolated from one single snail (*O. draparnaudi*) (Andrus & Rae, 2018). This highlights how strains

isolated in close proximity to each other and phylogenetically similar (Howe et al., 2020) differ in their pathogenic abilities (Cutler & Rae, 2020) as well as host seeking abilities (Andrus et al., 2018). Similarly, *P. neopapillosa* DMG0016 displayed lower pathogenic potential than *P. neopapillosa* DMG0014 and DMG0015, yet these strains were isolated from *L. flavus* slugs located in Sefton Park, Liverpool (Andrus & Rae, 2018). Therefore, in order to produce and develop a suitable biological control agent for slugs and snails, intraspecific variation of strains should be investigated.

This is the first time that *P. neopapillosa* and *P. californica* have been reported as being lethal to snails such as juvenile *C. aspersum*. Although members of the *Phasmarhabditis* genus are parasitic towards both slugs and snails, most research has focussed on slugs. However, *P. hermaphrodita* is capable of infecting and killing numerous snails from the families Achatinidae, Helicidae, Cochlidellidae, Hygromiidae, and Lymnaeidae (Rae et al., 2007; Mc Donnell et al., 2018b). Understanding the host range of potential biological control agents is important for control of multiple pest species but also to protect against non-target mortality, as there are few mono-specific biological control agents (Sands & Van Driesche, 1999).

The ability of *Phasmarhabditis* species to induce host feeding inhibition is a major benefit in the use of this nematode as a biological control agent and is one reason rapid slug control is recorded (Glen et al., 2000). Here we found that all *P. neopapillosa* wild strains significantly inhibited feeding more than commercial *P. hermaphrodita* DMG0001 at the lower dose rate of 500 nematodes and only *P. neopapillosa* DMG0016 was unable to inhibit feeding more than the commercial strain at 1000 nematodes per ml. Interestingly, commercial *P. hermaphrodita* DMG0001 was unable to cause significant feeding inhibition after 8 days at the higher does of 1000 nematodes per ml when compared to an uninfected control. The ability of all wild *P. neopapillosa* strains to inhibit feeding more than commercial *P. hermaphrodita* at the lower dose rate of 500 nematodes per ml suggests these strains are highly virulent, able to establish an infection quicker and display greater feeding inhibition.

Our results corroborate that *P. californica* and *P. neopapillosa* are two other *Phas-*

marhabditis species that have evolved a terrestrial gastropod parasitic lifecycle. This suggests pathogenicity is an evolutionary conserved ability in the *Phasmarhabditis* genus. This is intriguing. There are 108 species of nematodes associated with gastropods (Grewal et al., 2003a), with members spanning four out of five clades of the Nematoda (Blaxter et al., 1998). The majority of species have co-evolved with gastropods for 500 MY (Grewal et al., 2003a) using gastropods as definitive, intermediate, necromenic and phoretic hosts (Pieterse et al. 2017a), yet the only species that kill gastropods are members from the *Phasmarhabditis* genus. Interestingly, *Phasmarhabditis* nematodes such as *P. hermaphrodita* do not have to rely on killing gastropods to complete their lifecycle. As facultative parasites they have been shown to infect larger slug species where they wait until the slug dies to reproduce (termed 'necromeny') (Wilson et al., 1993; Rae et al., 2009a) and they can reproduce on decaying matter such as rotting leaves and even slug faeces (Tan & Grewal, 2001a). It remains to be seen whether the other species also kill gastropods and whether any of these species have evolved stricter relationships with slugs and snails, such as obligate parasitism. There is little information on the parasitic lifestyle of *P. californica* and *P. neopapillosa*. We have shown, like *P. hermaphrodita*, they are facultative parasites that can be grown on rotting slugs and parasitise and kill slugs and snails. When the numbers of dauers growing on cadavers were quantified, only *P. californica* DMG0019 and DMG0017 produced more dauers than *P. hermaphrodita* DMG0001 at 500 and 1000 nematodes per ml respectively. All *P. neopapillosa* wild strains produced more dauers than *P. hermaphrodita* DMG0001 on *D. invadens* cadavers when applied at both doses. High proliferation levels on host cadavers is beneficial to biological control, as more dauer larvae will be entering the soil to locate other hosts. Successful proliferation on host cadavers also indicates a suitable host (Gaugler et al., 1997). Having wild strains that display higher proliferation, inhibit feeding more and cause higher levels of host mortality than commercial *P. hermaphrodita* at the lower dose rate shows potential for a more economic control agent due to lower application dose requirements. One major issue for implementing nematode biological control agents in large scale agriculture is cost. *Phasmarhabditis hermaphrodita* costs associated with production

efficiency, shelf life, need for refrigeration and high application dose disadvantage the use of this nematode (Rae et al., 2007). However, utilising wild strains that are far superior in terms of pathogenic ability and potentially producing higher yields could reduce costs.

In summary, both *P. californica* and *P. neopapillosa* can parasitise and kill *D. invadens* and *C. aspersum* as well as inducing feeding inhibition and reproducing prolifically on cadavers. As both host species are dominant pests in British nurseries, wild strains of these nematodes could be developed as superior biological control agents than *P. hermaphrodita* DMG0001.

4. *P. californica* DOES NOT KILL BENEFICIAL EARTHWORMS (*L. terrestris* AND *E. fetida*) OR INSECTS (*G. mellonella* AND *T. molitor*)

4.1. Introduction

Phasmarhabditis hermaphrodita is a parasite of gastropods and does not affect earthworms under lab (Rae et al., 2005) or field conditions (Iglesias et al., 2003). This is contrary to findings by Zaborski et al. (2001) who reported finding a '*Phasmarhabditis* like' nematode parasitising earthworms. Even recently it has been found that two *Phasmarhabditis* isolates (EM434 and DF5056) cause mortality to the earthworms *Eisenia hortensis* and *E. fetida* (Tandingan De Ley et al., 2020) This is alarming. Earthworms are integral to the functioning of the soil ecosystem by improving soil fertility through accelerating decomposition of organic matter and releasing nutrients in forms that are available for uptake by plants (Edwards & Lofty, 1977). Therefore, we decided to investigate whether a strain of the closely related species *P. californica* from the UK would affect the survival of earthworms (*Lumbricus terrestris* and *E. hortensis*) and insects (*Galleria mellonella* and *Tenebrio molitor*).

4.2. Materials and Methods

4.2.1. Testing the susceptibility of earthworms, slugs and insects

Nine non-airtight plastic boxes with lids (170 x 110 x 50 mm) were filled with 50 g of soil. *Phasmarhabditis californica* DMG0018 was formulated and supplied by BASF Agricultural Specialities and stored at 10°C. Nematodes were applied at the field application rate of *P. hermaphrodita* (30 nematodes per cm^2) (Wilson et al., 1993) to three boxes and five times the recommended rate (150 nematodes per cm^2) to another three boxes. Three boxes received water with no nematodes and acted

as the control. Nematodes were suspended in 50 ml of water and applied evenly to the soil surface. After nematode application, five individuals of each earthworm species (*L. terrestris* and *E. fetida*) were added to each box (mean weight ± SE: *L. terrestris* 1.52 ± 0.65 g; *E. fetida* 1.25 ± 0.81 g). Earthworms were purchased from online suppliers Yorkshireworms.co.uk and Wigglywigglers.co.uk. Boxes were incubated at 10°C in the dark for 14 days. At the end of the experiment, numbers of surviving earthworms were counted. The experiment was replicated twice (30 individuals in total) using fresh batches of earthworms and *P. californica* DMG0018. The pathogencity of *P. californica* DMG0018 was monitored using a standard assay with slugs (*D. invadens*) (see Chapter 2 and Chapter 3). Ten slugs were used per treatment and the experiment was repeated twice.

To assess whether *P. californica* DMG0018 could potentially affect insects nine 10 cm Petri dishes were inverted and a single 90 mm Whatman number 1 filter paper discs were added to each lid. *Phasmarhabditis californica* DMG0018 were applied in 1 ml of water at 30 and 150 nematodes per cm² to three separate Petri dishes. Three plates received 1 ml of water and no nematodes and acted as the control. Ten individuals of *G. mellonella* or *T. molitor* (mean weight ± SE *G. mellonella* 0.23 ± 0.11 g; *T. molitor* 0.19 ± 0.06 g) (ordered from Livefoods Direct) were added to each plate with plates stored in an unsealed plastic bag, to limit moisture loss, for 5 days at 20°C in the dark. At the end of the experiment the numbers of surviving insects were recorded. Experiments were repeated twice (60 individuals in total) using fresh *G. mellonella* and *T. molitor*. As a positive control we used the same experimental set up but exposed *G. mellonella* and *T. molitor* to 1 ml of the entomopathogenic nematode *Heterorhabditis bacteriophora* (approx. 1000 nematodes per ml), which can kill insects in 24-48 hours

4.2.2. Data analysis

A one-way ANOVA was used to determine if there was a significant difference between the numbers of alive insects exposed to 0, 30 and 150 *P. californica* DMG0018 per cm^2. Statistical analysis was conducted in R (R Core Team, 2020).

4.3. Results

Phasmarhabditis californica (DMG0018) killed 16 out of 20 *D. invadens* (compared to an uninfected control where 1 out of 20 *D. invadens* died) after 14 days. *Phasmarhabditis californica* (DMG0018) had no effect on the survival of *L. terrestris* or *E. fetida* when exposed to 0, 30 or 150 nematodes per cm^2 after 14 days. All earthworms (n = 30) were alive at the end of the experiment when exposed to both doses of *P. californica* (DMG0018) or the untreated control. *H. bacteriophora* killed both *G. mellonella* (60 were dead compared to 3 in the control) and *T. molitor* (57 were dead compared to 0 in the control) within 4 days. There was no significant difference in the survival of both *G. mellonella* and *T. molitor* when exposed to 0, 30 and 150 *P. californica* (DMG0018) per cm^2 ($P>0.05$) (Fig. 4.1).

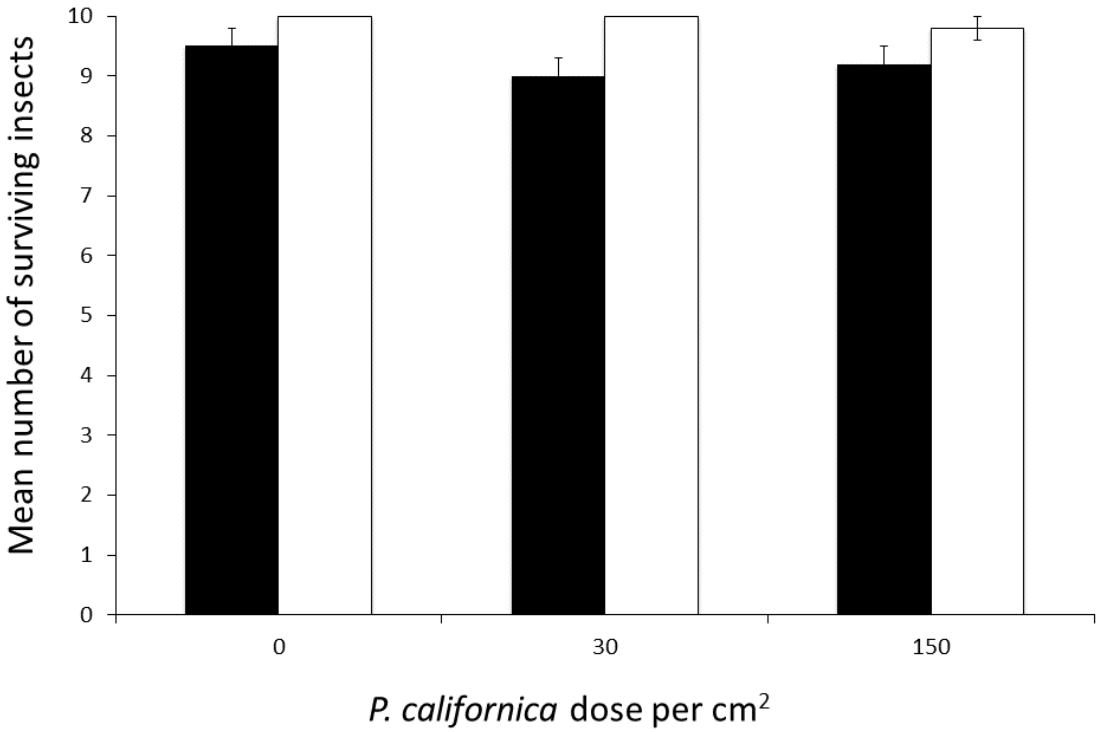

Figure 4.1: The survival of *Galleria mellonella* (black bars) and *Tenebrio molitor* (white bars) exposed to *Phasmarhabditis californica* DMG0018 at 0, 30 and 150 nematodes per cm^2 after 5 days. Bars represent ± one standard error.

4.4. Discussion

Here we have shown *P. californica* does not kill beneficial earthworms or insects. There was no effect on either group of organisms. There are two reports of *Phasmarhabditis* nematodes causing detrimental effects to earthworms. Zaborski et al. (2001) described a"*Phasmarhabditis* like" nematode from earthworms causing mortality, yet to date, there were no further studies. As there was no molecular verification of this nematode it could be entirely possible this nematode was not even a *Phasmarhabditis* species. Recently, however, two *Phasmarhabditis* isolates (EM434 and DF5056), identified to the *Phasmarhabditis* genus through 18s and mtDNA sequences, have been shown to kill the earthworms *E. hortensis* and *E. fetida* (Tandingan De Ley et al., 2020; Howe et al., 2020). Studies (e.g. Rae et al., 2005) have demonstrated that the UK strain of *P. hermaphrodita* cannot kill five species of earthworms (*L. terrestris*, *E. hortensis*, *E. andrei*, *E. fetida*, *Dendrodrilus rubidus*) or the New Zealand flatworm (*Arthurdendyus triangulatus*). Also, in field studies, application of *P. hermaphrodita* had no effect on earthworm populations, acarids or collembolans (Iglesias et al., 2003). Furthermore, it has been shown that *P. hermaphrodita* does not affect insects including *Pterostichus melanarius*, *T. molitor*, *Zophobas morio* Fabricius, 1776, and *G. mellonella* (see Rae et al., 2007). This stands to reason, as both groups of organisms are anatomically different to gastropods. Our results show similar results to *P. hermaphrodita* (e.g. Rae et al., 2005), in that *P. californica* does not kill earthworms or insects, even at high doses. Therefore, the use of *P. hermaphrodita* and *P. californica* on farms and in gardens poses little risk to non-target organisms.

5. Natural variation in host finding behaviour of gastropod parasitic nematodes (*Phasmarhabditis* spp.) exposed to associated host cues

5.1. Introduction

Parasitic nematodes use many diverse cues exuded from hosts to ensure successful infection (Lee, 2002). For example, *Howardula aoronymphium* Welch, 1959, parasitises the mushroom fly (*Drosophila falleni* Wheeler, 1960) and is attracted to mushroom-derived odorants such as 3-octanone and 1-octen-3-one (Cevallos et al. 2017). The mammalian parasite *Heligmosomoides polygyrus* Dujardin, 1845, is attracted to mouse faeces and compounds such as geranyl acetone and 2-butanone and averted from carbon dioxide (Ruiz et al. 2017). Entomopathogenic nematodes (*Steinernema carpocapsae* and *Heterorhabditis bacteriophora*), which are lethal pathogens of insects, are attracted to different host derived compounds. For example, Hallem et al. (2011) found *S. carpocapsae* was attracted to propanoic acid and hexanal, 2,3-butanedione (as well as others) yet *H. bacteriophora* was only attracted to 1-propanol. The relationships between olfaction of parasitic nematodes and hosts are poorly understood (Cevallos et al. 2017), hence we are attempting to develop a model nematode (Rae, 2017b) to elucidate the molecular nature of host location by parasites. The model proposed is *P. hermaphrodita*. The host cues *P. hermaphrodita* uses to find gastropods have been studied in detail. In agar based chemotaxis assays the nematodes positively chemotax towards slug and snail mucus and faeces (Rae et al., 2006, 2009a; Hapca et al., 2007a,b; Andrus et al. 2018; Andrus & Rae, 2019). They have also been shown to be attracted to alive and dead slugs in soil-based experiments (MacMillan et al., 2009; Nermut' et al., 2012a). Mucus from some slug species is more attractive to *P. hermaphrodita* than others. More specifically, Rae et al. (2009a) showed that *P. hermaphrodita* was

strongly attracted to mucus from slugs such as *Arion subfuscus* but less attracted to *Limax marginatus* and the snail *C. hortensis* (for reasons unknown). Small & Bradford (2008) recorded a selection of behaviours when *P. hermaphrodita* came in contact with mucus from six gastropod species including forward crawling, head thrusting and head waving but observed few differences between species. In order to investigate the specific components of slug mucus the nematodes are attracted to, Andrus et al. (2018) showed that *Phasmarhabditis* nematodes were strongly attracted to hyaluronic acid – a common component of slug mucus, in a dose dependant manner.

The majority of chemotaxis experiments using *P. hermaphrodita* have concentrated on studying DMG0001, which has been used in commercial production since 1994. There is little information about whether there is natural variation in chemoattraction of not just wild strains of *P. hermaphrodita* but any of the 14 other species in the *Phasmarhabditis* genus (*P. bohemica, P. circassica, P. clausilliae*, *P. meridionalis*, *P. apuliae*, *P. bonaquaense*, *P. californica*, *P. safricana, P. huizhouensis*, *P. neopapillosa*, *P. papillosa, P. kenyaensis*, *P. zhejiangensis* and *P. tawfiki*) (Tandingan De Ley et al. 2017; Ivanova et al. 2020; Zhang & Liu, 2020; Pieterse et al., 2020). One study (Andrus & Rae, 2019) reported natural variation in chemoattraction of *P. hermaphrodita*, *P. californica* and *P. neopapillosa* to certain slug species. For example, *P. hermaphrodita* isolates differed in their preference of slug species but *P. neopapillosa* preferred *Arion* sp. However, there is little information about whether these species differ in their response to mucus from a single slug species or other attractants like hyaluronic acid.

To understand whether there is natural variation in the ability of these nematodes to chemotax to slug mucus and associated cues we exposed 9 isolates of *P. hermaphrodita* (including the commercial strain DMG0001), 5 isolates of *P. neopapillosa* and 3 isolates of *P. californica* to mucus from *D. invadens*, as well as two concentrations of hyaluronic acid (1% and 5%). Ultimately, this will unravel whether behavioural mechanisms responsible for host chemoattraction in the *Phasmarhabditis* genus are under selective pressure.

5.2. Materials and Methods

5.2.1. Source of invertebrates

Phasmarhabditis hermaphrodita (DMG0002, DMG0003, DMG0005, DMG0007, DMG0008, DMG0009, DMG0010 and DMG0011), *P. neopapillosa* (DMG0012, DMG0013, DMG0014, DMG0015 and DMG0016) and *P. californica* (DMG0017, DMG0018 and DMG0019) were grown on White traps for 21 days to the dauer stage and stored in tissue culture flasks until use. *Phasmarhabditis hermaphrodita* (DMG0001) was purchased from BASF Agricultural Specialities and stored at 10°C until use. *D. invadens* were collected from Sefton Park in Liverpool and stored in non-airtight plastic boxes lined with moist paper at 15°C until use.

5.2.2. Chemotaxis assay

A chemotaxis assay was used to assess the ability of *Phasmarhabditis* nematodes to locate slug mucus and hyaluronic acid (Rae et al. 2006, 2009a; Andrus et al. 2018). Briefly, 10 cm Petri dishes were half filled with 1.2% technical agar and left to solidify. A 1 cm^2 piece of Whatman number 1 filter paper was placed 0.5 cm from edge of the Petri dish and 10 µl of distilled water was added and acted as the control. Another piece of filter paper (same size) was placed equidistant from the control after being used to swab 0.01 g of mucus from adult *D. invadens* (or 10 µl of 1% or 5% hyaluronic acid was added). Approximately 50 dauer larvae of each *Phasmarhabditis* isolate were added to the middle of each Petri dish. They were then sealed with Parafilm® and stored at 20°C overnight. The following morning the numbers of nematodes that had moved to the control or treated piece of filter paper were quantified (as well as those that remained in the middle). For each isolate of *Phasmarhabditis* three Petri dishes were used to test the response to *D. invadens* mucus or hyaluronic acid (1 or 5%) and each experiment was repeated three times (n = 9 dishes per isolate).

5.2.3. Data analysis

The numbers of *Phasmarhabditis* nematodes found in the mucus or hyaluronic acid (1% or 5%) vs. water was analysed using a Student's *t* test. To understand which *Phasmarhabditis* strain responded strongest to mucus or hyaluronic acid (1 or 5%) the numbers of nematodes found in each treatment from each species was compared using a One-Way ANOVA with Tukey's post hoc test.

5.3. Results

5.3.1. Natural variation in the ability of *Phasmarhabditis* nematodes to chemotax to *D. invadens* mucus

There were significantly more *P. hermaphrodita* (DMG0001, DMG0003, DMG0005, DMG0007, DMG0008, DMG0009, DMG0010 and DMG0011), *P. neopapillosa* (DMG0012, DMG0013, DMG0014, DMG0015 and DMG0016) and *P. californica* (DMG0017, DMG0018 and DMG0019) found in the *D. invadens* mucus compared to the water control (Fig. 5.1) ($P<0.05$). However, this was not the case for *P. hermaphrodita* (DMG0002) as there was no significant difference in the number of nematodes found on each side (Fig. 5.1) ($P>0.05$).

Of all the *P. hermaphrodita* isolates tested, isolate DMG0010 chemotaxed significantly more to *D. invadens* mucus compared to *P. hermaphrodita* (DMG0001, DMG0002, DMG0003, DMG0005, DMG0008, DMG0009 and DMG0011) ($P<0.05$) (Fig. 5.1).

There was no difference in the numbers of *P. neopapillosa* (DMG0012, DMG0013, DMG0014, DMG0015 and DMG0016) that were found in the *D. invadens* mucus ($P>0.05$) (Fig. 5.1).

Significantly more *P. californica* (DMG0018) were found in the *D. invadens* mucus than *P. californica* (DMG0019) ($P>0.05$) (Fig. 5.1), but there was no difference between *P. californica* (DMG0018) and *P. californica* (DMG0017) ($P<0.05$).

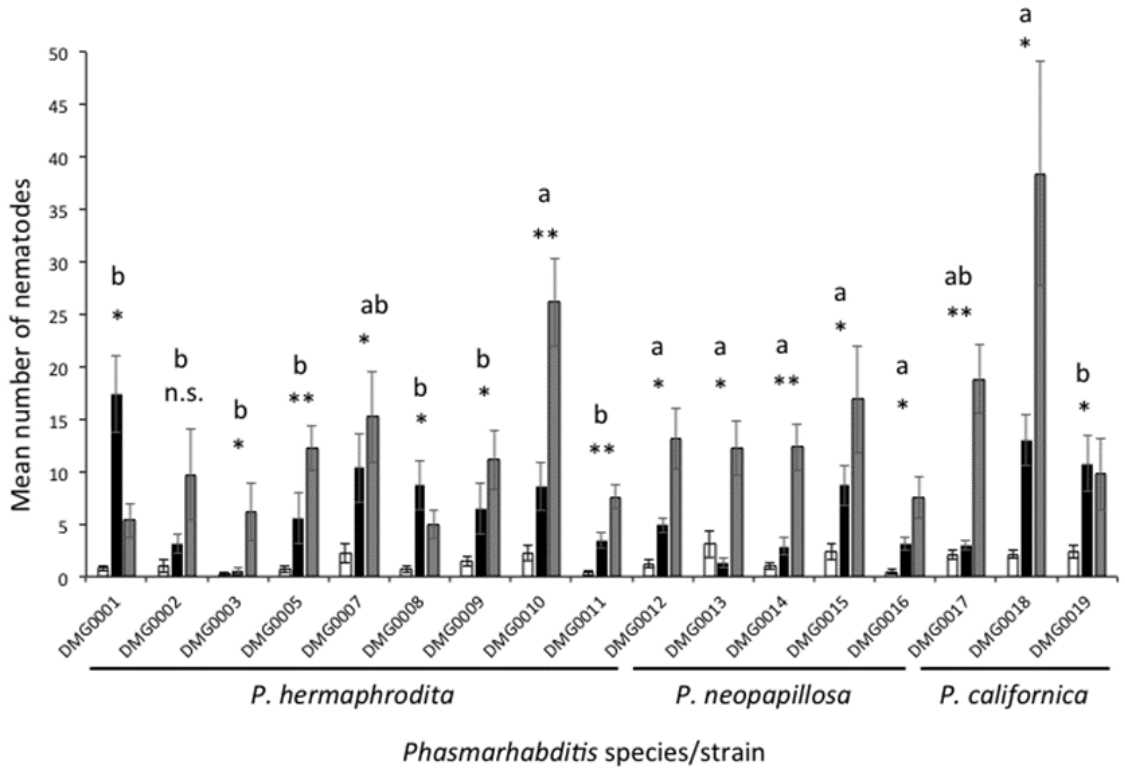

Figure 5.1: The mean number of *Phasmarhabditis hermaphrodita* (DMG0001, DMG0002, DMG0003, DMG0005, DMG0007, DMG0008, DMG0009, DMG0010 and DMG0011), *P. neopapillosa* (DMG0012, DMG0013, DMG0014, DMG0015 and DMG0016) and *P. californica* (DMG0017, DMG0018 and DMG0019) found in filter paper with water (control) (white bars), in the middle of the agar plate (black bars) or in filter paper with 0.01 g *Deroceras invadens* mucus (grey bars). Significant differences between the number of nematodes found in the mucus or water are denoted with * for $P<0.05$ and ** for $P<0.001$. Different letters (a or b) denote a significant difference at $P<0.05$ between the strains of each *Phasmarhabditis* species found in mucus. Bars represent ± one standard error.

5.3.2. Natural variation in the ability of *Phasmarhabditis* nematodes to chemotax to 1% hyaluronic acid

Phasmarhabditis hermaphrodita (DMG0001, DMG0002, DMG0007, DMG0008, DMG0009 and DMG0011) responded positively to 1% hyaluronic acid with significantly more nematodes found in the treated filter paper compared to the control ($P<0.05$) (Fig. 5.2a). However, there was no difference between the numbers of *P. hermaphrodita* (DMG0010, DMG0003 and DMG0005) in the 1% hyaluronic acid compared to the water control ($P>0.05$) (Fig. 5.2a). Although there was no significant difference between the numbers of *P. hermaphrodita* (DMG0010) in the mucus and water this strain reacted more to the 1% hyaluronic acid than *P. hermaphrodita* (DMG0002, DMG0003 and DMG0008) ($P<0.05$) (Fig. 5.2a).

Significantly more *P. neopapillosa* (DMG0012, DMG0013 and DMG0015) were found in the 1% hyaluronic acid compared to the control ($P<0.05$) (Fig. 5.2a) however, there was no significant difference between the numbers of *P. neopapillosa* (DMG0014 and DMG0016) in the hyaluronic acid or water control ($P>0.05$) (Fig. 5.2a). Of the *P. neopapillosa* strains tested, significantly more *P. neopapillosa* (DMG0012) were found in the 1% hyaluronic acid than *P. neopapillosa* (DMG0016) ($P <0.05$) (Fig. 5.2a). There was no difference between the numbers of *P. neopapillosa* (DMG0013, DMG0014 and DMG0015) found in 1% hyaluronic acid ($P>0.05$) (Fig. 5.2a).

Only *P. californica* (DMG0018) significantly chemotaxed to 1% hyaluronic acid compared to water ($P<0.05$) (Fig. 5.2a). There was no difference between the numbers of *P. californica* (DMG0017 and DMG0019) found on each side ($P>0.05$) (Fig. 5.2a). There was no difference in the numbers of *P. californica* (DMG0017, DMG0018 and DMG0019) found in the 1% hyaluronic acid ($P>0.05$) (Fig. 5.2a).

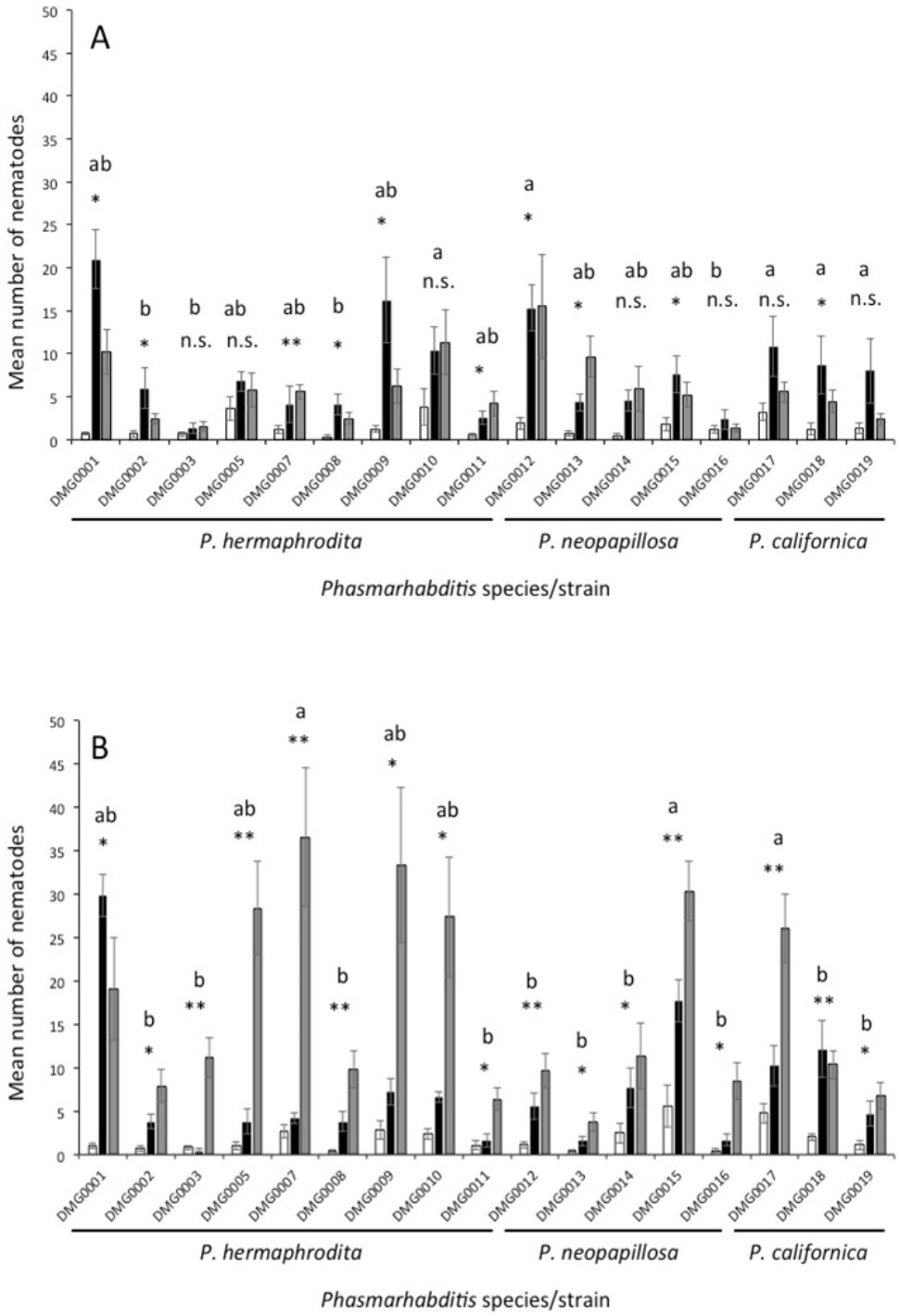

Figure 5.2: The mean number of Phasmarhabditis hermaphrodita (DMG0001, DMG0002, DMG0003, DMG0005, DMG0007, DMG0008, DMG0009, DMG0010 and DMG0011), P. neopapillosa (DMG0012, DMG0013, DMG0014, DMG0015 and DMG0016) and P. californica (DMG0017, DMG0018 and DMG0019) found in filter paper with water (control) (white bars), in the middle of the agar plate (black bars) or in 1% (a) or 5% (b) hyaluronic acid (grey bars). Significant differences between the number of nematodes found in hyaluronic acid or water are denoted with * for $P<0.05$ and ** for $P<0.001$. Different letters (a or b) denote a significant difference at $P<0.05$ between the strains of each *Phasmarhabditis* species found in hyaluronic acid. Bars represent ± one standard error.

5.3.3. Natural variation in the ability of *Phasmarhabditis* nematodes to chemotax to 5% hyaluronic acid

When exposed to 5% hyaluronic acid all isolates of all *Phasmarhabditis* species tested were found significantly more in the hyaluronic acid compared to the water control ($P<0.05$) (Fig. 5.2b).

Significantly more *P. hermaphrodita* (DMG0007) were found in 5% hyaluronic acid compared to *P. hermaphrodita* (DMG0002, DMG0003, DMG0008 and DMG0011) ($P<0.05$) (Fig. 5.2b). Also, there were significantly more *P. hermaphrodita* (DMG0009) found in the 5% hyaluronic acid than *P. hermaphrodita* (DMG0002 and DMG0011) ($P<0.05$) (Fig. 5.2b).

From the *P. neopapillosa* isolates tested strain DMG0015 was found significantly more in the 5% hyaluronic acid compared to the others (DMG0012, DMG0013, DMG0014 and DMG0016) ($P<0.05$) (Fig. 5.2b).

This was also the case with the *P. californica* isolates tested where one strain (DMG0017) was found significantly more in the 5% hyaluronic acid compared to the others (DMG0018 and DMG0019) ($P<0.01$) (Fig. 5.2b).

5.4. Discussion

When exposed to mucus from *D. invadens, P. hermaphrodita* (DMG0010) and *P. californica* (DMG0018) were found significantly more in *D. invadens* mucus than the other strains tested, suggesting they are perhaps better at finding hosts. There was also natural variation in chemotaxis observed when we repeated the experiments with hyaluronic acid (1% and 5%), which is a common attractant for other parasites such as the trematode *Acanthostomum brauni* Mañé-Garzón & Gil, 1961 and the protozoan *Plasmodium falciparum* Welch, 1897 (Haas & Ostrowskide de Núñez, 1988; Beeson et al., 2000). At 1% hyaluronic acid significantly more *P. hermaphrodita* (DMG0010), *P. neopapillosa* (DMG0012) and *P. californica* (DMG0018) were found in the attractant than the other strains of the same genus. When given the choice of 5% hyaluronic acid or water, *P. hermaphrodita* (DMG0007), *P. neopapillosa* (DMG0015) and *P. californica* (DMG0017) were found significantly more in

the attractant than other strains of the same genus. Natural variation in chemoattraction has been shown in parasitic and genetic model nematodes. For example, Laznik & Trdan (2013) demonstrated differences in attraction to damaged maize derived β -caryophyllene by *H. bacteriophora* strain D54 and *S. carpocapsae* B49 but *S. kraussei* C46 only showed weak attraction and *S. feltiae* (two strains) exhibited no attraction. Strains of *C. elegans* differ in their evasion response when exposed to the pathogen *Bacillus thuringiensis* (Schulenburg & Müller, 2004). Also, Hong et al. (2008) reported natural variation in chemotaxis of 19 strains of the scarab beetle associated nematode *Pristionchus pacificus* Sommer, Carta, Kim & Sternberg, 1996, exposed to insect pheromones *E*-11-tetradecenyl acetate (EDTA) and *Z*-7-tetradece-2-one (ZTDO). Similarly, McGaughran et al. (2013) reported significant variation in chemoattraction of 21 *P. pacificus* strains to organic compounds, beetle washes and live beetles from several island locations.

Phasmarhabditis hermaphrodita (DMG0001) has been commercially produced since 1994, fed on a diet of the bacterium *M. osloensis* (Rae et al., 2007). This strain is lethal to pestiferous gastropods such as *D. invadens* (Williams & Rae, 2015) yet in these experiments it did not respond as well as some wild isolates to slug mucus. This is in contrast to previous research (Rae et al., 2009a) that used the same assay with the same strain and slug species and found that approximately 40% of the nematodes graduated towards the mucus. Perhaps over 10 years of being grown in industrial fermenters the chemotactic response of *P. hermaphrodita* has diminished or perhaps the population of *D. invadens* used in this study (collected from Liverpool) were less attractive than those from Aberdeen (for some unknown reason)? Nevertheless, by taking our approach and testing chemotaxis behaviour, strains with superior host finding behaviour could be collected and developed as biological control agents.

The relationships between olfaction of parasitic nematodes and hosts warrants further attention (Cevallos et al., 2017). This could be addressed using the proposed model nematode *P. hermaphrodita*. Although understudied in terms of genetics and genomics it has a plethora of attributes that make it an excellent model for olfaction of parasitic nematodes. First, it can be isolated easily facilitat-

ing analysis of natural variation at the inter and intra species level. Second, it is a facultative parasite yet can be kept in culture on agar plates or under 'semi-natural' conditions fed on rotting slug (Andrus & Rae, 2018). Third, there are multiple studies, inspired by chemoattraction work by *C. elegans* (e.g. Bargmann et al., 1993), examining the olfactory response of *P. hermaphrodita* (and closely related *Phasmarhabditis* species) towards slug mucus (Rae et al., 2006; 2009a; Hapca et al., 2007a,b; Andrus & Rae, 2019) and snail mucus (Andrus et al., 2018) using agar plates and in more realistic soil conditions (Nermut' et al., 2012a; MacMillan et al., 2009). Specific components of slug mucus, e.g. hyaluronic acid, have been shown to be strong attractants to these nematodes (Andrus et al., 2018; this study), which will allow in-depth analysis of how this compound can affect olfaction at a neurobiological and genomic level. Interestingly, there are similarities in the genetic mechanisms *C. elegans* and *P. pacificus* use to find food and hosts, respectively as they both rely on the protein kinase EGL-4 (Hong et al., 2008; Kroetz et al., 2012). Chemoattraction in *P. hermaphrodita* towards snail mucus was enhanced by exogenous exposure to cyclic guanosine monophosphate (GMP), which activates EGL-4 (Andrus et al., 2018). Therefore, we believe *P. hermaphrodita* (and other *Phasmarhabditis* nematodes) could be used as a parasitic comparison to closely related non-parasitic species such as *C. elegans* and *P. pacificus* to examine the evolution of parasitic behaviours at the molecular and neurobiological level.

6. The effect of sertraline, haloperidol and apomorphine on the behavioural manipulation of slugs (*D. invadens*) by the nematode *P. hermaphrodita*

6.1. Introduction

The ability to manipulate the behaviour of hosts by parasites is common across the tree of life (Moore, 2002; Hughes et al., 2012). How parasites change the behaviour of hosts can be broadly split into two main types: those that coerce intermediate hosts to move into areas where they are more likely to come in contact with their definitive host, and those that compel their host to migrate to a habitat that will increase dispersal of offspring (Hughes & Libersat, 2019). The trematode *Leucochloridium paradoxum* Carus, 1835, is a good example of the former. *L. paradoxum* infects snails (*Succinea* spp.) and makes them less photophobic (as well as producing multi-coloured, pulsating tentacles) and more likely to be eaten by their definitive host - birds. The trematode then reproduces in the bird's stomach and eggs are expelled with faeces and eaten by more snails (Wesolowska & Wesolowski, 2013). In contrast, the gypsy moth multicapsid nuclear polyhedrosis virus infects gypsy moths (*Lymantria dispar* Linnaeus, 1758) and makes them climb to the top of trees where they will die and the virus replicates and spreads viral particles across the forest floor to infect more caterpillars (Hoover et al., 2011).

Although there are many other examples of viruses, trematodes, protozoa and fungi that influence host behaviour (Hughes et al., 2012; Moore, 2002) there are only a handful of other examples of metazoans such as members of the Nematoda that can alter the behaviour of hosts. Recently, it was shown *P. hermaphrodita* has an unusual ability to change the behaviour of their slug hosts (Morris et al., 2018).

Specifically, many slug species (*D. reticulatum*, *D. invadens*, *A. ater*, *A. subfuscus* and *A. hortensis*) avoid areas where *P. hermaphrodita* has been applied (Wilson et al., 1999; Wynne et al., 2016; Morris et al., 2018); however, when infection by the nematode does occur it alters slug behaviour in numerous ways. Infected slugs are slower (Bailey et al., 2003), their feeding is inhibited (Glen et al., 2000), they are unattractive to predatory beetles (Foltan & Puza, 2009) and they move down into soil to die (Pechova & Foltan, 2008). The most striking example of host behavioural manipulation by *P. hermaphrodita*, which cannot be explained simply as a symptom of malaise, is the phenomenon where infected slugs of several species (*D. invadens, A. hortensis* and *A. subfuscus*) are attracted to areas where *P. hermaphrodita* has been applied (Morris et al., 2018). The adaptive reason for this behavioural manipulation is unknown but it is speculated that by moving the slug towards areas with more nematodes this increases the chances of the slug dying and therefore providing food for the nematode to complete its life cycle. This study also showed that uninfected *D. invadens* fed fluoxetine (which increases serotonin levels) were not averted by the nematodes, and were recorded more often on areas with *P. hermaphrodita*. Conversely, slugs infected with *P. hermaphrodita* and fed cyproheptadine (reduces serotonin levels) were no longer attracted to the side with *P. hermaphrodita*. This research showed manipulation of biogenic amines in slugs can produce similar behavioural phenotypes as those caused by *P. hermaphrodita* infection (Morris et al., 2018). We sought to continue this research by examining further the effects of drugs that affect serotonin and dopamine signalling would have on the behavioural manipulation of *D. invadens* when infected with *P. hermaphrodita*. Ultimately, this approach will allow an insight into the molecular mechanism these nematodes have evolved to control gastropod behaviour through pharmacological manipulation.

6.2. Materials and Methods

6.2.1. Source and maintenance of invertebrates

Deroceras invadens were collected from greenhouses at LJMU and placed in non-airtight plastic boxes lined with moist tissue paper, fed lettuce *ad libitum* and stored at 15°C. Slugs were kept for a minimum of 7 days before use to ensure the slugs were not naturally infected with *P. hermaphrodita*. *P. hermaphrodita* (Nemaslug®) was purchased from BASF-Agricultural Specialties and stored at 15°C until use.

6.2.2. Assessing the behaviour of infected and uninfected *D. invadens*

The behaviour of *D. invadens* when exposed to *P. hermaphrodita* was monitored using a standard soil-based assay (Wilson et al., 1999; Wynne et al., 2016; Morris et al., 2018). Briefly, 100 grams of sterile loam soil (21% water content) was added to separate plastic boxes (24 x 9 x 6 cm). Copper tape was placed around the side of each box to ensure the slugs remained on the soil. To one side (12 x 9 cm) 5 ml of water was evenly applied over the soil surface and acted as the control. To the other side, 120 *P. hermaphrodita* per cm^2 were added in 5 ml of water. *Phasmarhabditis hermaphrodita* was applied to the treatment side at 120 nematodes per cm^2 as this was the dose that slugs consistently avoided in previous studies (Wilson et al., 1999; Wynne et al., 2016; Morris et al., 2018). Two 4.5 cm diameter pieces of bread were placed on either side of the box. Three millilitres of 10 µM sertraline, 10 µM apomorphine or 10 µM haloperidol (drugs obtained from Sigma-Aldrich, UK, and dissolved in distilled water) were applied to each piece of bread. The concentration of 10 µM for the drugs was used based on studies by Morris et al. (2018). Five *D. invadens* (mean weight ± SE = 0.21 ± 0.004 g, n = 270) were added to the middle of the box, sealed and they were stored at 20°C. Every 24 hours for 4 days the numbers of *D. invadens* were recorded on the nematode and water side and were then placed back in the middle of the box. The experiment consisted of three boxes and the entire experiment was repeated three times (n = 9 replicate boxes; n = 45 slugs). This experiment was also repeated with *D. invadens* previously infected with *P. hermaphrodita*. To infect the slugs, 10 *D. invadens* were

added to five separate plastic boxes (24 x 9 x 6 cm) filled with 100 grams of soil. *Phasmarhabditis hermaphrodita* was applied to the entire soil surface at the standard field application rate of 30 nematodes per cm^2 (Wilson et al., 1994) and slugs were exposed for 5 days at 15°C, which has been shown to be a suitable time and number of nematodes to ensure infection (Wilson et al., 1993; Tan & Grewal, 2001a; Morris et al., 2018). After 5 days the slugs were used in the same experimental set-up outlined above.

6.2.3. Data analysis

A two-way repeated measures analysis of variance (ANOVA) was used to compare the numbers of slugs found on the control and nematode side on days 1, 2, 3 and 4.

6.3. Results

When fed sertraline and apomorphine *D. invadens* did not avoid *P. hermaphrodita* and were found significantly more on the nematode side ($P<0.05$) (Fig. 6.1). In contrast, ingestion of haloperidol made slugs avoid *P. hermaphrodita* with significantly more slugs found on the water side compared to the nematode side ($P<0.05$)(Fig. 6.1).

Deroceras invadens previously infected with *P. hermaphrodita* fed sertraline and apomorphine were found significantly more on the nematode side ($P<0.05$)(Fig. 6.1). However, when *D. invadens* infected with *P. hermaphrodita* were fed haloperidol there was no significant difference between the numbers of slugs recorded on the nematode and control sides ($P>0.05$) (Fig. 6.1).

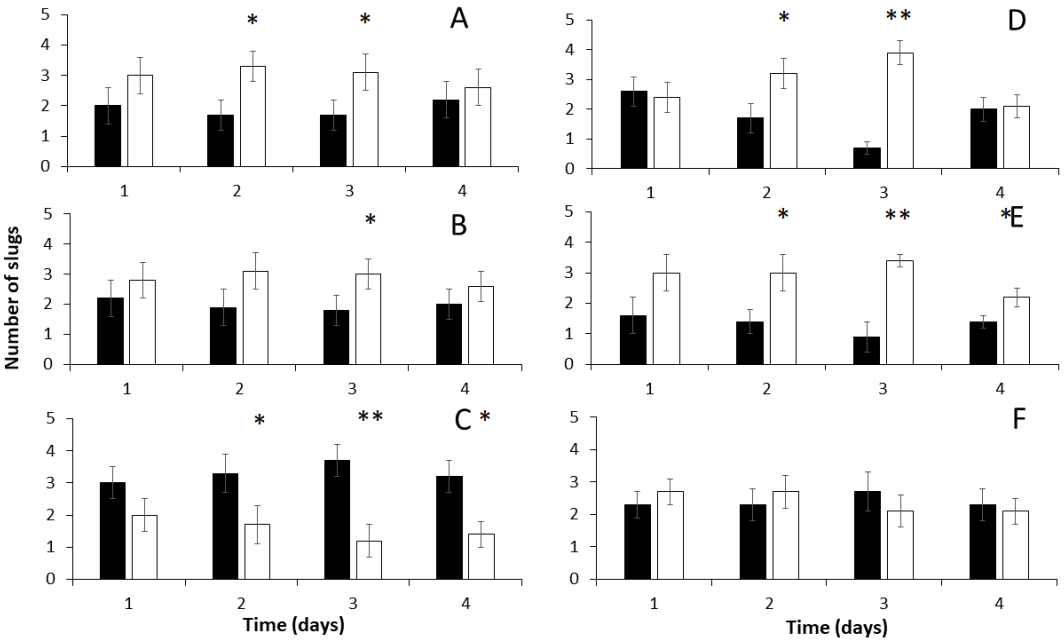

Figure 6.1: The mean number of uninfected slugs (*Deroceras invadens*) found on the control (water) side (black bars) and the side with *Phasmarhabditis hermaphrodita* (white bars) on days 1, 2, 3 and 4. Slugs were fed 10 µM sertraline (a), 10 µM apomorphine (b) or 10 µM haloperidol (c). Slugs pre-infected with *P. hermaphrodita* were fed 10 µM sertraline (d), 10 µM apomorphine (e) or 10 µM haloperidol (f). Significant differences between the number of nematodes on the control and treatment side at $p < 0.05$ are denoted by * and at $p < 0.001$ denoted by **. Bars represent ± one st. error.

6.4. Discussion

Influencing levels of biogenic amines to manipulate the behaviour of hosts is a successful mechanism used by several parasites. For example, the protozoan parasite *Toxoplasma gondii* Nicolle & Manceaux, 1908, which can change the behaviour of rodents making them less photophobic and reducing their fear of cats (their definitive hosts) (Webster, 1994), has the genetic machinery to make dopamine (Gaskell et al., 2009). Inhibition of dopamine through application of haloperidol in infected rats reduces their parasite-induced behaviours (Webster et al., 2006). Similarly, acanthocephalan worms (*Pomphorhynchus* spp.) that infect amphipods makes them congregate at the surface of water to increase the likelihood of being eaten by birds (their definitive host) by altering serotonin levels (Jacquin et al., 2014; Tain et al., 2006). Injection of serotonin in uninfected amphipods makes them move to the water surface - similar to their parasite induced behaviour, whereas untreated amphipods avoid the water surface (Tain et al., 2006). It would be interesting

if similar mechanisms of host manipulation occur between *P. hermaphrodita* and slug hosts, with *P. hermaphrodita* altering slug behaviour to cause death, allowing nematodes to use the cadaver as an immediate source of food and somewhere to reproduce (rather than remaining inside the slug and witing for it to die – termed 'necromeny'). We believe that this manipulation behaviour is strongly influenced by levels of serotonin and dopamine. The reasons are four-fold. First, in accordance with Morris et al. (2018) who showed uninfected *D. invadens* fed fluoxetine (which prevents re-uptake of serotonin in cells) were found more on the *P. hermaphrodita* side; we showed that sertraline (another Selective Serotonin Reuptake Inhibitor) produces the same nematode attraction behaviour. Second, uninfected *D. invadens* fed apomorphine (which activates dopamine receptors) no longer were averted from *P. hermaphrodita* and were found in similar numbers on each side - and were even attracted to the nematode side. Third, application of haloperidol to infected slugs (which should graduate to the nematode side) were no longer attracted to the nematodes, presumably as dopamine signalling was antagonised. Fourth, feeding infected slugs (which will move to the side with *P. hermaphrodita*) with cyproheptadine (which antagonises serotonin signalling) makes them no longer attracted to the nematodes. Therefore, we believe the results of this study and that of Morris et al. (2018) strongly implicate the influence of biogenic amines in the behavioural manipulation of *D. invadens* by *P. hermaphrodita*.

7. Conclusions

The results from this thesis have clearly demonstrated there is ample opportunity for developing and formulating other members of the *Phasmarhabditis* genus as biological agents to control slugs in a superior manner to *P. hermaphrodita* DMG0001. From Chapter 2, several wild strains of *P. hermaphrodita* were shown to be more pathogenic than *P. hermaphrodita* (DMG0001) to slugs, and this was also shown for the closely related species *P. neopapillosa* and *P. californica* (Chapter 3). As well as monitoring pathogenicity, it was shown from these studies that there are strains of *Phasmarhabditis* that are more efficient at responding to host cues such as *D. invadens* mucus and 1% and 5% hyaluronic acid (Chapter 5) than *P. hermaphrodita* (DMG0001) – these strains could also be used as superior biological control agents, posing no risk to non-target organisms such as earthworms and insects (Chapter 4) and they have the unusual ability to affect the behaviour of slugs (Chapter 6).

Ultimately, as metaldehyde is being banned for use in the UK by 2022, the only alternative control method of slugs are nematodes from the genus *Phasmarhabditis*. As the current strain has become progressively poorer at controlling slugs, new strains and species are needed in order to control slug damage. This thesis lays a blueprint for which species show the most potential, not just in terms of pathogenic ability but also in host seeking. At the time of writing, BASF (who co-fund this project) plan on releasing "Nemaslug 2" in spring 2022. This new product utilises *P. californica* based on results obtained from this work.

SUPPLEMENTARY TABLE 1. HOST RANGE OF *Phasmarhabditis hermaphrodita*.

Table 7.1: Host range of *Phasmarhabditis hermaphrodita*.

Gastropod	Species	Susceptible to *P.hermaphrodita*?	Relationship	Reference
Slug	*Deroceras reticulatum*	Yes	Parasitic	Wilson et al. (1993); Rae et al. (2009)
	Deroceras panormitanum	Yes	Parasitic	Wilson et al. (1993); Rae et al. (2009)
	Deroceras laeve	Yes	Parasitic	Grewal et al. (2003b)
	Limax maximus	No	Necromenic	Grewal et al. (2003b); Rae et al. 2009
	Limax pseudoflavus	No	Necromenic	Rae et al. (2008)
	Luimax marginatus	No	Necromenic	Rae et al. (2009a)
	Ambigolimax valentianus	No	Necromenic	Dankowska (2006); Ester et al. (2003c)
	Arion ater	Only juveniles	Parasitic/Necromenic	Wilson et al. (1993); Rae et al. (2009a)
	Arion silvaticus	Yes	Parasitic	Wilson et al. (1993)
	Arion intermedus	Yes	Parasitic	Wilson et al. (1993)
	Arion distinctus	Yes	Parasitic	Wilson et al. (1993); Iglesias and Speiser (2001)
	Arion lustanicus	Only juveniles	Parasitic/Necromenic	Speiser et al. (2001); Grimm (2002); Glen et al. (2000)
	Arion subfuscus	No	Necromenic	Grewal et al. (2003b); Rae et al. (2009b)
	Arion hortensis	No	Necromenic	Iglesias and Speiser (2001); Grewal et al. (2003b)
	Arion fasciatus	Yes	Parasitic	Antzée-Hyliseth et al. (2020)
	Arion vulgaris	No	Necromenic	Antzée-Hyliseth et al. (2020)
	Geomalacus maculosus	No	Necromenic	Carnaghi et al. (2017)
	Tandonia sowerbyi	Yes	Parasitic	Wilson et al. (1993); Rae et al. (2009)
	Tandonia budapestensis	Yes	Parasitic	Wilson et al. (1993)
	Milax gagates	Yes	Parasitic	Rae et al. (2008, 2009a)
	Leidyula floridana	Yes	Parasitic	Grewal et al. (2003b)
Snail	*Cornu aspersum*	Only juveniles	Parasitic/Necromenic	Glen et al. (1996); Rae et al. (2009a)
	Monacha cantiana	Yes	Parasitic	Wilson et al. (2000)
	Cepaea hortensis	Yes/No	Parasitic/Necromenic	Wilson et al. (2000); Rae et al. (2009a)
	Cepaea nemoralis	No	Necromenic	Wilson et al. (2000); Williams and Rae (2016)
	Theba pisana	Yes	Parasitic	Coupland (1995); Tandingan De Ley et al. (2020)
	Cochlicella acuta	Yes	Parasitic	Coupland (1995)
	Cernuella virgata	Yes	Parasitic	Coupland (1995)
	Arianta arbustorum	No	Necromenic	Rae (2018);Antzée-Hyliseth et al. (2020)
	Lymnaea stagnalis	Yes/No	Parasitic/Necromenic	Morley and Morrit (2006); Whitaker and Rae (2015)
	Physa fontinalis	No	Necromenic	Morley and Morrit (2006)
	Pomatias elegans	No	Necromenic	Wilson et al. (2000)
	Oxychilus helveticus	No	Necromenic	Wilson et al. (2000)
	Clausilia bidentata	No	Necromenic	Wilson et al. (2000)
	Discus rotundatus	No	Necromenic	Wilson et al. (2000)
	Lissachatina fulica	No	Parasitic/Necromenic	Williams and Rae (2015); Mc Donnell et al. (2018a)
	Bithynia tentaculata	No	Necromenic	Whitaker and Rae (2015)
	Planorbarius corneus	No	Necromenic	Whitaker and Rae (2015)
	Biomphalaria pfeifferi	Yes	Parasitic	Okonjo et al. (2015)
	Biomphalaria alexandrina	Yes	Parasitic	Abou-Elnour et al. (2015)
	Pomacea canaliculata	Yes	Parasitic/Necromenic	Montanari et al. (2000)

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