Forum article

Phasmarhabditis hermaphrodita - a new model to study the genetic evolution of parasitism

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Summary - The evolutionary genetic mechanisms that are responsible for the transition of free-living nematodes to parasites are unknown and current nematode models used to study this have limitations. The gastropod parasite *Phasmarhabditis hermaphrodita* could be used as a new model to dissect the molecular mechanisms involved in the evolution of parasitism. *Phasmarhabditis hermaphrodita* is a facultative parasite of slugs and snails that can also be maintained easily under laboratory conditions like *Caenorhabditis elegans* and *Pristionchus pacificus*. *Phasmarhabditis hermaphrodita* and *Phasmarhabditis* species are easy to isolate from the wild and have been found around the world. The phylogenetic position of *Phasmarhabditis* is ideal for genomic comparison with other Clade 9 species such as *C. elegans* and *P. pacificus*, as well as mammalian and insect parasites. These attributes could make *P. hermaphrodita* an excellent choice of model to study the evolutionary emergence of parasitism.

Keywords - *Caenorhabditis elegans*, gastropods, genetic model, parasites, *Pristionchus pacificus.*

Nematodes have evolved to parasitise plants, mammals and arthropods multiple times (Blaxter *et al.*, 1998); however, the genetic mechanisms of how a free-living nematode evolves into a parasite are largely unknown. It has been proposed that several factors must occur, for example, nematodes must have evolved close relationships with arthropods as mammalian parasitic nematodes are thought to have arisen from insect parasitic ancestors (Blaxter & Koutsovoulos, 2015). These relationships can be loosely classified as necromeny or phoresy. Necromeny ('waiting for the cadaver'), was proposed by Schulte (1989) and arises when nematodes infect an invertebrate, wait in the body until it dies and then reproduces on the decaying cadaver. Phoresy is where nematodes use hosts as a means of transport and has been documented in many species. These sorts of associations require pre-adaptations, such as the formation of dauer juveniles, which can tolerate stressful conditions and host enzymes *e.g.*, proteases (Poulin, 1998; Weischer & Brown, 2000). In order to understand the molecular mechanisms that are involved in these evolutionary transitions there are few genetic nematode models that can be used. This is because a suitable model would have to exhibit both a necromenic and parasitic lifestyle. It would also have to be easily culturable under laboratory conditions, preferably using Nematode Growth Media (NGM) agar plates seeded with a bacterial food source where it can be grown in large amounts quickly (to allow forward genetic approaches and genetic crosses) and not require growth or maintenance in mammalian hosts as they are logistically and financially prohibitive. Strains and species of the nematode should also be able to be isolated easily from the wild to facilitate micro and macro-evolutionary studies. It should (ideally) be a self-fertilising hermaphrodite that can produce males in low numbers for genetic crosses and that can be used to make isogenic and inbred lines. These are all prerequisites that were used in the selection

and development of *Caenorhabditis elegans* and *Pristionchus pacificus* making them formidable nematode genetic model organisms (Brenner, 1974; Sommer *et al*., 2000). As well as being able to maintain and easily culture a proposed parasitic model nematode in the laboratory, the evolutionary position of an ideal model for parasitism would need to be a species that was closely related to other model nematodes and was related to a plethora of other necromenic and phoretic nematodes that were associated with gastropods or arthropods and mammalian parasites. A nematode that meets all of these previously listed prerequisites is the terrestrial gastropod parasitic nematode, *Phasmarhabditis hermaphrodita.* I believe, like Wilson *et al*. (2015), that it would be an excellent model to answer the most pertinent biological questions about the evolutionary emergence of parasitism, including which traits and which genomic features are associated with parasitism? What selective forces maintain them and how do these change through the on going struggle between host and parasite? (Blaxter & Koutsovoulos, 2015). Here I detail how and why *P. hermaphrodita* (and other *Phasmarhabditis* species) would be excellent candidates for answering these fundamental questions about the molecular mechanisms involved in the evolutionary emergence of parasitism.

Phasmarhabditis hermaphrodita **was developed as a weapon**

Phasmarhabditis hermaphrodita is largely known as a biological control agent (Nemaslug®), which is used by farmers and gardeners to kill several pestiferous slug species from the families Milacidae, Limacidae and Vagnulidae (Rae *et al*., 2007; Wilson et al., 1993a). *Phasmarhabditis hermaphrodita* is broadcast at a rate of 3×10^9 nematodes ha^{-1} and has been successfully used to reduce slug damage in

oilseed rape, winter wheat, strawberries, asparagus, Brussels sprouts, orchids and hostas (Wilson *et al*., 1994, 1995a; Glen *et al*., 2000; Grewal *et al*., 2001; Ester *et al*., 2003a, b, c). *Phasmarhabditis hermaphrodita* is sold in the U.K., Ireland, France, The Netherlands, Belgium, Germany, Denmark, Norway, Finland, Poland, Spain, the Czech Republic, Italy and Switzerland (Rae *et al*., 2007). *Phasmarhabditis hermaphrodita* (strain DDTM1) is also used further afield in agriculture in Kenya as Slugtech® (Talwana *et al*., 2016). Previous to its development as a biological control it was isolated and studied by the great nematologists of the latter 19th and early to mid 20th centuries such as Schneider in 1859, Emile Maupas in 1900 and Herta Mengert in 1953. It was first isolated in 1859 by Schneider from decaying terrestrial molluscs and named *Pelodytes hermaphroditus*. It was then studied by Maupas in 1900 who proposed it had a necromenic lifestyle (although did not use this term). At this time he also studied several other nematodes including *C. elegans* (Maupas, 1900). Decades later these two nematodes were also collected together by Sydney Brenner and his team in the 1960s and kept in culture before he finally decided on using *C. elegans*. *Phasmarhabditis hermaphrodita* could have easily been chosen instead of *C. elegans* as it showed the same advantageous characteristics in culture and Brenner had actually collected more strains of *P. hermaphrodita* than *C. elegans*! (see Cold Spring Harbour Laboratory Archives, 2017; http://libgallery.cshl.edu/items/show/75709). Over a hundred years after its initial description by Schneider it was isolated and studied intensively by Mike Wilson and co-workers at Long Ashton Research Station (Glen *et al*., 1996). After an extensive body of work investigating culture conditions (Wilson *et al*., 1993b~~a~~, 1995b, c), host range (Wilson *et al*., 1993a~~b~~) and conducting field trials (Wilson *et al*., 1994) it

showed huge promise as biological control agent for slugs and has been on the market since 1994.

Phasmarhabditis hermaphrodita is Clade 9 nematode (Van Megen *et al*., 2009), which contains many necromenic and parasitic species of insects, gastropods and mammals. For example, *Oscheius tipulae* is associated with *Tipula paludosa* (Sudhaus, 1993), *C. elegans* and *C. briggsae* use slugs and snails as phoretic and necromenic hosts (Kiontke & Sudhaus, 2006; Petersen *et al*., 2015), and *Heterorhabditidoides chongmingensis* is entomopathogenic (Zhang *et al*., 2008). More distantly related families include insect pathogens (Heterorhabditidae) as well as mammalian pathogens from families such as Strongylidae, Ancylostomatidae and Trichostrongylidae and Heligmonellidae (Kiontke *et al*., 2007; van Megen *et al*., 2009). Thus, *P. hermaphrodita* is in an excellent phylogenetic position for comparative genomics with these other parasitic and necromenic species, as well as the model nematodes *C. elegans* and *P. pacificus*.

Phasmarhabditis hermaphrodita is part of the *Phasmarhabditis* genus that contains *P. apuliae, P. papillosa, P. neopapillosa, P. valida, P. nidrosiensis, P. californica, P. tawfiki, P. bonaquaense*, *P. bohemica* and *P. huizhouensis* (Andrássy, 1983; Hooper *et al*., 1999; Azzam, 2003; Tandingan De Ley *et al*., 2014, 2016; Huang *et al*., 2015; Nermuť *et al*., 2016a, b). There is also a '*Phasmarhabditis* sp. EM434' from the east coast of North America but it is poorly characterised and only several DNA sequences seem to exist on the National Center for Biotechnology Information database (NCBI) (Kiontke *et al*., 2007). Also there are two un-described South African species (*Phasmarhabditis* sp. SA1 and SA2) (Ross *et al*., 2012). The *Phasmarhabditis* genus is closely related to other gastropod parasites, such as *Agfa flexilis* and *Angiostoma limacis*, although morphologically they are very different

(Ross *et al*., 2010; Tandingan De Ley *et al*., 2016). Most *Phasmarhabditis* species are found in terrestrial environments, although *P. nidrosiensis* and *P. valida* are found in marine and littoral habitats. The infection behaviour of many of these species is unknown and only *P. hermaphrodita* and *P. neopapillosa* have been shown to kill slugs (~~Wilson *et al*., 1993b;~~ Glen *et al*., 1996). Like *C. elegans*, *P. hermaphrodita* is a self-fertilising hermaphrodite that produces males in low numbers (Maupas, 1900). It is a facultative parasite and able to grow on rotting slug or vegetation (Tan & Grewal, 2001) and does not need a slug host to reproduce.

Current nematode genetic model organisms pose problems when studying the evolution of animal parasitism

Caenorhabditis elegans and *P. pacificus* are excellent at unravelling the genetic mechanisms of different traits but are not ideal to understand the evolutionary emergence of parasitism. The wild type strain of *C. elegans* (strain N2) was isolated in 1956 and has since undergone hundreds of thousands of generations fed on the laboratory food, *Escherichia coli* OP50. Its natural ecological niche is rotting vegetation, such as apples, where it lives a quiet life eating bacteria and eukaryotes like yeast (Frezal & Felix, 2015) but can also be found on or in slugs and snails (Petersen *et al*., 2015). There is little evidence of parasitism across the *Caenorhabditis* genus (Kiontke & Sudhaus, 2006), although it has been suggested that *C. briggsae* can become entomopathogenic when fed certain bacteria such as *Serratia marcescens* (Abebe *et al*., 2011); however, this has been disputed (Rae & Sommer, 2011). Hence, the study of *C. elegans* to study the evolution of parasitism would be severely limited.

Pristionchus nematodes from the Diplogastridae are associated with beetles where they can be easily isolated (Morgan *et al*., 2012). They are necromenic nematodes (Herrmann *et al*., 2006) and there is little evidence to suggest they are parasitic. Undoubtedly, a full genome sequence, genetic techniques such as forward and reverse genetic tools and transgenic techniques (Sommer, 2015) make *Pristionchus* a formidable genetic nematode but not one to answer fundamental questions about parasitology, as it is not actually a parasite. That is not to say that they may never evolve to become parasitic as necromeny is thought to be a stepping-stone to true parasitism (Dieterich *et al*., 2008).

Other nematodes that have been proposed as models to study parasitism include mammalian parasites *e.g*., *Strongyloides* sp. However, the major problems with many of these mammalian parasites are associated with culturing techniques, which are labour intensive as they have long lifecycles that require mammalian hosts and they can be difficult for genetic studies. For example, *Strongyloides stercoralis* is a pathogen of humans, representing a biohazard risk and must be maintained in dogs (Lok, 2007). *Strongyloides ratti* must be maintained in rats (Viney & Lok, 2007) and infective stages need to be collected from faeces. *Trichnella spiralis* is a pathogen of humans (Mitreva & Jasmer, 2006) and *Brugia malayi* requires two hosts to complete its lifecycle (*Aedes* mosquitos and a mammalian host), hence making it time consuming to culture in the laboratory (Lok & Unnasch, 2013). These difficulties make doing standard genetic experiments like forward genetic screens difficult, but by no means impossible (Viney *et al*., 2002). Similarly, reverse genetics approaches utilising RNA interference (RNAi) have been shown to work in mammalian parasites such as *B. malayi* (Aboobaker & Blaxter, 2003), *Nippostrongylus brailiensis* (Hussein *et al*., 2002) and *Ascaris suum* (Islam *et al*., 2005) but there are questions about its

efficacy, repeatability and whether only a selection of genes can be inhibited (Geldhof *et al*., 2006, 2007). Far superior to RNAi in terms of efficacy and efficiency is CRISPR-Cas genome editing technology, which has been developed for *C. elegans*, *Caenorhabditis* sp. 9 and *P. pacificus* (Lo *et al*., 2013; Witte *et al*., 2015), but has not been shown to work in parasitic species as yet. Unlike mammalian parasites, the facultative parasite *P. hermaphrodita* is a saprobic microbivorous nematode that can reproduce on slug faeces, dead earthworms, insects and leaf litter quickly and in great numbers (Tan & Grewal, 2001; MacMillen *et al*., 2009, Rae *et al*., 2009; Nermuť *et al*., 2014). It does not need a terrestrial gastropod to complete its lifecycle and has been grown under laboratory conditions for over 20 years (Wilson *et al*. 1993a) and initial research outlined the optimum bacteria and growth conditions that are needed to grow *P. hermaphrodita* en masse (Wilson *et al*., 1995b, c). Another advantage of using *P. hermaphrodita* is that it can also be grown easily *in vivo* in slug hosts following protocols by Wilson (2012).

Entomopathogenic nematodes (*Steinernema* and *Heterorhabditis* spp.) have been proposed as genetic models to understand the genetics of bacterial symbiosis and parasitism. Recently, the genomes and transcriptomes of several *Steinernema* species including *S. carpocapsae, S. scapterisci*, *S. monticolum*, *S. feltiae* and *S. glaseri* have been sequenced and unravelled an abundance of protease genes that are thought to responsible for causing death to insects (Dillman *et al*., 2015). Also the genome of *Heterorhabditis bacteriophora* has been sequenced and has revealed that approximately 50% of putative protein coding genes had no homology to other sequenced nematodes (Bai *et al*., 2013). The sister group of the Heterorhabditidae is the Strongylomorpha (Blaxter *et al*., 1998), thus making their phylogenetic position very exciting in comparative genomic studies. Undoubtedly, both of these nematodes,

coupled with genetic tools such as RNAi (which has been shown to work in *H. bacteriophora*; Ratnappan *et al*., 2016) hold huge potential for identifying genes involved with insect pathogenicity due to the evolution of bacterial symbiosis. However, *P. hermaphrodita* is different. It does not have a strict symbiotic relationship with bacteria and associates with a vast array of many different species (Rae *et al*., 2010). It is true that *P. hermaphrodita* is grown on *Moraxella osloensis* under factory conditions and that large quantities, when injected, will kill slugs such as *Deroceras reticulatum* (Tan & Grewal, 2001), but the bacterium is not vertically transmitted to further nematode offspring (which are pathogenic to slugs) (Rae *et al*., 2010) and *P. hermaphrodita* can kill slugs without *M. osloensis* and when grown on lots of different bacterial species (Wilson *et al*., 1995b, c). Hence, *M. osloensis* is unnecessary for *P. hermaphrodita* to kill slugs (see Wilson & Rae, 2015). Therefore, *P. hermaphrodita* could be used as a genetic model to understand the evolutionary emergence of parasitism and not the evolution of parasitism due to bacterial symbiosis.

Phasmarhabditis spp. are easy to isolate from the wild

For any burgeoning nematode genetic model it is absolutely essential that it can be collected and isolated easily from the wild. This is true for current nematode models. Global sampling efforts have isolated several hundred *C. elegans* strains and 26 *Caenorhabditis* species from six continents (Frezal & Felix, 2015), which are available from the *Caenorhabditis* Genetic Stock Centre (USA). Similarly, sampling efforts by *Pristionchus* researchers have collected 28 species of *Pristionchus* (Ragsdale *et al*., 2015) and hundreds of strains of *P. pacificus* are available from the

Sommer laboratory, Tübingen, Germany (Morgan *et al*., 2012). These natural isolates have shown natural genetic variation in behaviour, cold tolerance and dauer formation in *P. pacificus* (Hong *et al*., 2008; Mayer & Sommer, 2011; McGaughran & Sommer, 2014) and in *C. elegans* natural variation approaches have been successful in understanding genes involved with hybrid incompatibility, copulatory plugging, foraging behaviour and thermal tolerance (De Bono & Bargmann, 1998; Harvey & Viney, 2007; Rockman & Kruglyak, 2009). These collections, whether of *C. elegans* or *P. pacificus*, allow for in depth analysis of traits at the macro- and micro-evolutionary level and *Phasmarhabditis* and *P. hermaphrodita* do not have to be any different. *Phasmarhabditis* spp. are easy to isolate (Wilson *et al*., 2016), can be cultured on rotting slug or agar plates (Wilson, 2012) and can be identified easily with species-specific PCR primers (Read *et al*., 2006). There are many studies over the last 20 years that have dissected, chopped, killed and collected slugs and snails looking for *Phasmarhabditis* spp. For example, 956 slugs were collected by Tandingan De Ley *et al*. (2014), which yielded 10 isolates of *Phasmarhabditis* spp, including four *P. hermaphrodita* from California, USA. While this is a very low return, it is in stark contrast to a survey conducted by Morand *et al*. (2004) who found that 18-64% of slugs were infected with *P. hermaphrodita* and 33-100% of slugs were infected by *P. neopapillosa*. By taking similar approaches *P. hermaphrodita* and *Phasmarhabditis* species have been isolated around the world, including UK (Wilson *et al*., 1993~~ab~~), Germany (Schneider, 1859; Mengert, 1953), France (Coupland, 1995; Maupas, 1900), Czech Republic (Nermuť *et al*., 2010, 2016a), Iran (Karimi *et al*., 2003), Egypt (Azzam, 2003; Genena *et al*., 2011), Norway (Ross *et al*., 2015), Chile (France & Gerding, 2000), New Zealand (Wilson *et al*., 2012) and South Africa (Ross *et al*., 2012). Recently, new species of *Phasmarhabditis* have been isolated in China (*P.*

huizhouensis) (Huang *et al*., 2015), Italy (*P. apuliae*) (Nermuť *et al*., 2016a) and in Czechoslovakia (*P. bonaquaense* and *P. bohemica*) (Nermuť *et al*., 2016b, c). Also a new species of *Phasmarhabditis* (*P. californica*) has been isolated and described from the USA (Tandingan De Ley *et al*., 2016). Interestingly, this species has also been found in New Zealand and recently it has been found parasitising slugs (*Geomalacus maculosus*) in Ireland (Carnaghi *et al*., 2017) and snails in Wales (Rae, unpublished). As many *Phasmarhabditis* species and *P. hermaphrodita* have been isolated around the world this opens up collaborative efforts to understand the genetic diversity of these species using population genomics as well as looking at natural genetic variation in virulence towards slugs. By screening through hundreds of species or strains of *P. hermaphrodita*, if natural variation in virulence was observed, strains showing extreme phenotypes could be mated and Genome Wide Association Studies (GWAS) could be carried out to discover genes essential for pathogencity towards slugs and their evolution across the genus. From an applied perspective the discovery of these strains and species from around the world could enhance the use of *Phasmarhabditis* as a biological agent to control not just slugs but also snails that are vectors of human disease. For example, it was recently shown that *P. hermaphrodita* can kill *Biomphalaria* spp., which are vectors of *Schistosoma mansoni* in Africa (Okonjo *et al*., 2015).

Phasmarhabditis hermaphrodita **as a model to understand the genetics of host interactions**

Four out of five clades of Nematoda (Blaxter *et al*., 1998) include slug parasitic nematodes, which suggest there are multiply origins of slug parasitism (Ross

et al., 2010). These include seven families of nematodes (Agfidae, Alloionematidae, Angiostomatidae, Cosmocercidae, Diplogasteridae, Mermithidae and Rhabditidae) and 108 described species of nematode (Grewal *et al*., 2003a). These nematodes use slugs and snails as paratenic, definitive and intermediate hosts. Of the 61 nematode species that use molluscs as intermediate hosts, 49 of these are from the Metastrongyloidea (Grewal *et al*., 2003a). Of the 47 species that use molluscs as definitive hosts, 33 belong to the Rhabditida (Grewal *et al*., 2003a). Of the 108 species of nematodes that use molluscs as hosts the only species that has evolved to be pathogenic towards them is *P. hermaphrodita*. It is a gastropod-specific parasite and does not affect other organisms such as earthworms, flatworms, acarids, collembolans or insects (Grewal & Grewal, 2003; Iglesias *et al*., 2003; DeNardo *et al*., 2004; Rae *et al*., 2005). Thirty-six slug and snail species have been tested for susceptibility to *P. hermaphrodita* (strain *P. hermaphrodita* DMG0001) (Table 1). The conclusions from these experiments should be taken with some caution. This is because these tests have always been carried out with the commercial strain of *P. hermaphrodita* (designated DMG0001), which was isolated over 20 years ago, and these experiments have never been repeated with any other naturally isolated *Phasmarhabditis* species. It is therefore unknown if natural strains differ in their pathogenicity towards slugs. The only study looking at this was by Wilson *et al*. (2012) who showed that a strain of *P. hermaphrodita* isolated from slugs in New Zealand was pathogenic to *D. reticulatum.*

It is clear that *P. hermaphrodita* is able to parasitise and kill many different pestiferous slug species including *D. reticulatum*, *D. panormitanum*, *Arion ater*, *Milax budapestensis* and *M. sowerbyi* (Wilson *et al*., 1993a~~b~~; Rae *et al*., 2009). There are however, some slug species that are resistant to *P. hermaphrodita* such as *Limax*

pseudoflavus and *Limax maximus* (Rae *et al*., 2008; Grewal *et al*., 2003b). In general all slugs from the Limacidae tested are resistant, whereas all Agriolimacidae are highly susceptible. The reasons for this are unknown. Some slug species are only susceptible as juveniles *e.g*., *A. ater* and *A. lusitanicus* (Wilson *et al*. 1993~~ab~~). Snails, like slugs, differ in their susceptibility to *P. hermaphrodita*. For example, *Helix aspersa* (young stages) and *Cernuella virgata* are susceptible to *P. hermaphrodita* but some species such as *Cepaea nemoralis* and *Discus rotundatus* are resistant (Coupland, 1995; Wilson *et al*., 2000). Interestingly, some of these infection studies have obtained different results. For example, *C. hortensis* and *L. stagnalis* have been shown to be susceptible to *P. hermaphrodita* in some studies (Wilson *et al*., 2000; Morley & Morrit, 2006), whilst resistant in others (Rae *et al*., 2009; Whitaker & Rae, 2015). Perhaps there is natural genetic variation in host immunity towards these nematodes from snails collected from different areas?

How slugs and snails combat infection by *P. hermaphrodita* has not been investigated in any great detail. One study (Scheil *et al*., 2014) investigated if phenoloxidase (PO) activity was altered in infected snails but found there was no effect. However, recently, snails such as *C. nemoralis* and *Achatina fulica* were shown to have the ability to trap, encase and kill parasitic nematodes in their shell (Williams and Rae, 2015; 2016). Although to investigate this further more extensive molecular analysis will have to be carried out to profile the genes that are responsible for producing the shell, such as calcite and aragonite as well as glycoproteins and polysaccharides (Marin & Luquet, 2004).

***Phasmarhabditis hermaphrodita* as a genetic model that can be used to understand parasite behaviour and how parasites manipulate host behaviour**

As well as virulence *P. hermaphrodita* could be used to study the genetics of parasite behaviour. In order to find hosts *P. hermaphrodita* responds to slug mucus and faeces, dead slugs and host volatiles (Rae *et al*., 2006, 2009; Hapca *et al*., 2007a, b; Nermuť *et al*., 2012). These experiments were based on a modified assay that is commonly used by *C. elegans* researchers, *e.g*. Bargmann *et al*. (1993), to examine genes and neurons involved with behaviour. An important point to note about the use of *P. hermaphrodita* to study behaviour is that it can be observed not only on agar plates (Rae *et al*., 2006, 2009; Hapca *et al*., 2007a) but also in more realistic ecologically relevant substrates such as sand and soil (Hapca *et al*., 2007b; Macmillan *et al*., 2009; Nermuť *et al*., 2012). Therefore it should be possible to use forward genetics and mutagenise nematodes and look for mutants that are defective in attraction to slug mucus (or showing increased attraction), which may reveal ecologically important genes essential for chemotactic behaviour in soil. Another important point is that researchers using *C. elegans* and *P. pacificus* in chemotaxis assays concentrate on the adult stage of the lifecycle (Bargmann *et al*., 1993; Hong *et al*., 2008). In a parasitic nematode species, such as *P. hermaphrodita* (and all rhabditid nematodes parasites), this approach would not be ecologically relevant as it is the dauer stage that is used to find and penetrate into hosts. Dauer stage *P. hermaphrodita* have been used in all chemotaxis experiments (Rae *et al*., 2006, 2009; Hapca *et al*., 2007a, b; Nermuť *et al*., 2012) and not adults and other stages as they cannot penetrate into slugs (Tan & Grewal, 2001) as their main purpose is to feed on bacteria and reproduce. In summary, by using *P. hermaphrodita* ecologically relevant genes and neurons responsible for finding gastropod hosts in *P. hermaphrodita* could

be identified and compared with *C. elegans* behaviour regulatory networks which could provide fascinating insight into the evolution of host seeking behaviour.

Once a slug is infected by *P. hermaphrodita* it can severely affect the behaviour of its host. For example, infection by *P. hermaphrodita* makes slugs slower (Bailey *et al*., 2003), stop feeding (Glen *et al*., 2000), die and be avoided by predators (Foltan & Puza, 2009), move down into soil to die (Pechova & Foltan, 2008), more likely to be found under refuge traps (Wilson *et al*., 1994), and freshwater snails (*Lymnaea stagnalis*) are more likely to be found outside water (Morley & Morrit, 2006). The advantages of controlling slug behaviour means that the host can be positioned in a place that is better for the growth and reproduction of the nematodes and its offspring *e.g*., deeper down in soil or away from freshwater. *In vivo* genetic analysis of *P. hermaphrodita* when infecting slugs or snails using transcriptomics and RNA-Seq could provide an unparalleled opportunity to unravel novel genes that are responsible for manipulating the behaviour of hosts.

Uninfected slugs can detect and avoid areas where *P. hermaphrodita* has been applied (Wilson *et al*., 1999; Wynne *et al*., 2016). This is interesting, not only from an agricultural application and financial point of view as less nematodes could be applied to crops deterring slugs from those areas (Hass *et al*., 1999), but also from an evolutionary and genetic perspective. This means that over time slugs have evolved closely with *P. hermaphrodita* and are aware that these nematodes have the ability to cause ill health. This poses questions such as: what are the nematodes producing that the slugs are detecting? How do slugs detect nematodes? Are there strains of *P. hermaphrodita* that are not detected by slugs? Ultimately, the answers to these questions, and many others, could be answered by analysis of the secretome of *P. hermaphrodita*. This approach successfully identified small molecules, such as

ascarosides, which are exuded from *C. elegans* (and other nematodes such as *P. pacificus*) and are regulators of a vast array of processes such as dauer formation and olfaction (Ludewig & Schroeder, 2013).

Conclusions

Currently, there are no forward, reverse or transgenic techniques that have been developed for *P. hermaphrodita* but the genomes and transcriptomes of *P. hermaphrodita* (and several other *Phasmarhabditis* species) are currently being sequenced and are part of the 959 Nematode Genomes initiative (Kumar *et al*., 2012). To unravel genes involved in the evolution of virulence the ideal analysis would involve comparing the genome of *P. hermaphrodita* to *C. elegans* or *C. briggsae*. Could *C. elegans* become pathogenic to slugs and snails if these potential virulence genes were transferred from *P. hermaphrodita*? Coupled with comparisons of the genomes of *C. elegans* and *P. hermaphrodita*, transcriptomics and RNA-Seq could be used to profile the genes that are being expressed by *P. hermaphrodita* when infecting slugs. The analysis of the genome of *P. hermaphrodita* and development of genetic tools could unravel genes involved in an array of processes and it could enhance the use of *P. hermaphrodita* or *Phasmarhabditis* spp. as a biological control agents of slugs and snails that are of agricultural and health importance not just in northern Europe but worldwide.

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Commented [r4]: I altered the order and designation of the Wilson et al, 1993 references so that 1993a becomes the first citation in th text. However, there are only two 1993 reference; should his one be deleted? (Your original 1993c becomes a and your original 1993a becomes b, with the references swapped round)

1993C has been deleted and I choose Wilson et al 1993A (The rhabditid nematode etc.....) as the first one and put it in the text earlier (as it was the first paper).

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