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Development of *Phasmarhabditis hermaphrodita* (and members of the *Phasmarhabditis* genus) as new genetic model nematodes to study the genetic basis of parasitism

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Running head: *P. hermaphrodita* - A model nematode to study parasitism

Abstract

The genetic mechanisms of how free-living nematodes evolved into parasites are unknown. Current genetic model nematodes (e.g. *Caenorhabditis elegans*) are not well suited to answer this and mammalian parasites are expensive and logistically difficult to maintain. Here we propose the terrestrial gastropod parasite *Phasmarhabditis hermaphrodita* as a new alternative to study the evolution of parasitism and outline the methodology of how to keep *P. hermaphrodita* in the lab for genetic experiments. We show that *P. hermaphrodita* (and several other *Phasmarhabditis* species) are easy to isolate and identify from slugs and snails from around the U.K. We outline how to make isogenic lines using 'semi-natural' conditions to reduce in lab evolution and how to optimise growth using NGM agar and naturally isolated bacteria. We show that *P. hermaphrodita* is amenable to forward genetics and that *unc* and *sma* mutants can be generated using formaldehyde mutagenesis. We also detail the procedures needed to carry out genetic crosses. Furthermore, we show natural variation within our *Phasmarhabditis* collection with isolates displaying differences in survival when exposed to high temperatures and pH, which facilitates micro and macro evolutionary studies. In summary, we believe that this genetically amenable parasite that shares many attributes with *C. elegans* as well as being in Clade 5 which contains many animal, plant and arthropod parasites, could be an excellent model to understand the genetic basis of parasitism in the Nematoda.

Keywords: *C. elegans*, *P. pacificus*, genetic model, parasitism, evolution

Introduction

Nematodes have evolved to parasitise arthropods, plants and mammals (Blaxter et al., 1998) but the genetic mechanisms of how parasitism evolved are unknown. Pre-adaptations that are thought to be key for this evolutionary transition include close associations with arthropod hosts (Blaxter & Koutsovoulos, 2015) and the ability to arrest development which can aid with coping with stressful conditions such as host enzymes (Poulin, 1998; Weischer & Brown, 2000). There are several parasitic species that have been developed as genetic models but are unsuitable to answer this question as they require intermediate and/or definitive mammal hosts (e.g. *Brugia* sp.) to complete their lifecycle, which can be financially and logistically prohibitive and laborious (Lok & Unnasch, 2013). Due to these problems genetic experiments can be difficult. More developed genetic model nematodes are associated with invertebrates. *Caenorhabditis elegans* is thought to have a loose association with slugs and snails (Petersen et al., 2015; Schulenburg & Félix, 2017) and the diplogastrid nematode *Pristionchus pacificus* has a necromenic relationship (coined by Schulte, 1989) with scarab beetles where it infects hosts waits for them to die and reproduces on their cadaver (Morgan et al., 2012). However, both species are not parasitic (Rae & Sommer, 2011; Herrmann et al., 2006); therefore provide little information about the underlying evolution of genetic mechanisms that are used to infect, parasitise and even kill their hosts. They are however formidable at unravelling genes involved with a plethora of biological important and ecologically important traits (The *C. elegans* Research Community, 2005; Sommer, 2015). Both of these species are successful as nematode genetic models as they can be isolated easily, can be kept in culture and can be grown in large amounts (on Nematode Growing Media (NGM) plates fed *Escherichia coli* OP50), they can be mutagenized and can be mated

easily (Brenner, 1974; Sommer et al., 2000). Furthermore, as well as full genome sequence (*C. elegans* Sequencing Consortium, 1998; Dieterich et al., 2008) post genomic tools such as reverse genetic techniques; first RNAi (Fire et al., 1998; Cinkornpumin & Hong, 2011) now CRISPR-Cas9 (Lo et al., 2013; Witte et al., 2015) can be carried out in both species to understand gene function as well as transgenic techniques facilitating the analysis of gene expression (Chalfie et al., 1994; Schlager et al., 2009). Similar techniques can be carried out in mammalian parasites e.g. *Brugia malayi*, *Nippostrongylus brailiensis* and *Ascaris suum* but the efficiency is variable and only a selection of genes can be inhibited (Geldhof et al., 2006; 2007). A promising genetic model to study nematode parasitism would combine the ease of keeping and growing *C. elegans* and *P. pacificus* en masse in the lab with the ability to collect different species and strains easily to facilitate micro and macro evolutionary studies. Also it would be closely related to other parasitic and necromenic species that would allow genomic comparison of the evolution of potential parasitism genes from different parasitic lifestyles. Furthermore, it would be able to be genetically manipulated that would allow an in-depth analysis of gene function.

A nematode that theoretically meets all these criteria is the terrestrial gastropod parasite *Phasmarhabditis hermaphrodita* (Fig 1A**).** *P. hermaphrodita* can complete its life cycle in several ways. First, it can infect and kill several susceptible slug species (e.g. *Deroceras reticulatum*) (Wilson et al., 1993; Rae et al., 2009). Second, it can infect and remain inside larger slug and snail species waiting for the host to die, where it reproduces on the decaying cadaver when the host dies (termed 'necromeny') (Rae et al., 2009). Third, it can reproduce on decomposing organic matter such as leaf litter, dead earthworms and slug faeces (Macmillan et al., 2009;

Tan and Grewal, 2001a). Therefore, it is not an obligate parasite that requires a host to survive but a bacterivorous nematode that can be grown in the lab without slugs but is still able to retain is pathogenicity towards slugs. Due to its pathogenic potential it has been formulated into a biological control agent (Nemaslug® from BASF-Agricultural Specialities) for farmers and gardeners to control slugs and snails (Rae et al., 2007). Nematodes are applied to soil where they actively seek out slugs and infect and kill them 4-21 days later (Wilson et al., 1993; Tan and Grewal, 2001a). *P. hermaphrodita* has been shown to provide protection against slug damage in many agriculturally important crops (Wilson & Rae, 2015). Although, *P. hermaphrodita* has received considerable attention as an agricultural biopesticide it is interesting from a fundamental evolutionary perspective and has been proposed as an excellent candidate as a genetic model to elucidate how parasitism has arisen in free-living species (Rae, 2017; Wilson et al., 2015). It was even a potential candidate as Sydney Brenner's nematode of choice instead of *C. elegans* (Cold Spring Harbour Laboratory Archives, 2017; http://libgallery.cshl.edu/items/show/75709). *P. hermaphrodita* is the only nematode from an estimated 1 million (Lambshead, 1993) that has evolved to parasitise and kill gastropods. Also there are over 108 species of nematodes that parasitise molluscs and four out of five clades of the Nematoda have members that parasitise gastropods (Grewal et al., 2003; Blaxter et al., 1998). Therefore, parasitism of gastropods is a very important lifestyle choice amongst nematodes; but the genes involved to infect and survive in these hosts are unknown.

The majority of research on *P. hermaphrodita* has focused on optimizing application techniques in the field (see Rae et al., 2007), host range studies (Wilson et al., 1993; Grewal et al., 2003; Rae et al., 2009), taxonomic descriptions and surveys charting abundance and diversity of *Phasmarhabditis* in different countries

(Tandingan De Ley et al., 2014, 2016; Wilson et al., 2012; Nermut et al., 2016a,b,c; Ross et al., 2012; 2015). There are few details and no methods about how to keep this nematode under lab conditions like *C. elegans* and whether it could be amenable to genetic manipulation. There is little information about life history traits and how they change with bacterial diet or temperature. Also there have been few experiments looking at natural variation in the genus *Phasmarhabditis* in any ecological traits as all studies so far have focused on using the commercial strain of *P. hermaphrodita* (DMG0001) that has been in culture for over 20 years (Rae et al., 2007).

Here we provide information on how to grow, maintain, mutagenize and mate not only *P. hermaphrodita* but also several species of the *Phasmarhabditis* genus under lab conditions. We also provide information on how to isolate, identify and make isogenic lines of *P. hermaphrodita*. Taken together, these results show that many of these species can be easily maintained under lab conditions and could make excellent candidates as genetic models to understand the evolution of parasitism in the Nematoda.

Materials and methods

Terrestrial gastropod survey and molecular identification of parasitic nematodes

Slugs (*Deroceras panormitanum, D. reticulatum* (Fig 1B), *Arion subfuscus, A. ater, A. hortensis, Limax flavus, L. maximus, Lehmannia valentiana, Milax budapestensis* and *M. sowerbyi*) and snails (*Cepaea nemoralis, Cornu aspersum* and *Oxychilus draparnaudi*) were collected from several locations in Liverpool, U.K including: Priory wood near St Michael's station (Grid reference number

SJ3673586862) (n = 107), Sefton Park (SJ3787187058) (n = 195) and Otterspool (SJ3707686321) (n = 57). Slugs were also collected from the Cruickshank Botanic gardens at the University of Aberdeen (NJ9376008556) (n = 48) and from Dale, Wales (SM809057) (n = 19). Once collected they were transported back to the lab where they were chopped in half and placed in a 5 cm Petri dish with a few drops of distilled water and stored at room temperature. Over 4 days the slugs were examined for presence of nematodes. Any nematodes that morphologically resembled *Phasmarhabditis*-like nematodes (Fig 1) e.g. over 1 mm hermaphrodites, females or males were individually transferred to modified White traps (White, 1927) (see below for description) to make isogenic lines. After 21 days when the food was exhausted and the nematodes had reached the dauer stage they were present in the surrounding water and were removed and centrifuged at 16,000 rpm to concentrate and their DNA was extracted using a DNA extraction kit (Qiagen). Using PCR three genes were then amplified (ITS1, 18SrRNA and the D2-D3 domain of LSU rDNA) (Blaxter et al. 1998; Tandingan De Ley et al., 2014) and sequenced in both directions for species identification. For the ITS1 gene the primers used were N93 (5'-TTGAACCGGGTAAAAGTCG-3') and N94 (5-TTAGTTTCTTTTCCTCCGCT-3'). The 18SrRNA gene was amplified using 18A (5'-AAAGATTAAGCCATGCATG-3') and 26R (5'-CATTCTTGGCAAATGCTTTCG-3'). The D2-D3 LSU rDNA primers were D2 (5'-AGCGGAGGAAAAGAAACTAA-3') and D3 (5'-TCGGAAGGAACCAGCTACTA-3'). As well as these three genes *P. hermaphrodita* specific primers were also used which were based on 150-200 bp of the Cytochrome Oxidase I gene created by Read et al. (2006), which consisted of Ph-F-1754 (5-TGGGTGCCCCTGATATAAGAT-3) and Ph-R-1887 (5-CGGATGACCAAGGGTACTTAAT-3). These primers were used to examine if they

could provide a rapid and cheap method for identifying *P. hermaphrodita* without DNA sequencing as they have been used previously to determine if *P. hermaphrodita* was present in mites and collembolans (Read et al. 2006). PCR cycling conditions consisted of the following: 3 mins at 95°C followed by 35 cycles of 15 secs at 95°C, 30 secs at 55°C, 1.5 in at 72°C and a final step of 8 mins at 72°C. The PCR products were then purified and sequenced in both forward and reverse directions for each gene (ITS1, 18SrRNA and the D2-D3 domain of LSU rDNA). Gene sequences of nematodes were then compared with NCBI database sequences using BLASTN searches using similarly matches of 99%. For PCR using primers designed by Read et al. (2006) the 200 bp product was visualised after gel electrophoresis where a positive band indicates presence of *P. hermaphrodita* and no sequencing was carried out.

Semi-natural conditions for growth of *Phasmarhabditis* species to make isogenic lines and dauer juveniles

Any potential *Phasmarhabditis* like nematodes observed growing on the collected rotting slugs and snails were transferred to modified White traps (White, 1927) for maintenance, to make isogenic lines and to quantify the number of dauers that were produced per individual hermaphrodite mother. Modified White traps consisted of a 10 cm Petri dish half filled with tap water. A 5 cm lid of a Petri dish was then placed on top of the water, which had a 4.5 cm diameter piece of pre-moistened Whatman number 1 filter paper inside. To each White trap a 3 mm slice of *L. flavus* was added as a food source. *L. flavus* were collected from LJMU greenhouses and frozen at -80°C for 1 hour to kill any nematodes present. *L. flavus* was used for *Phasmarhabditis* food for several reasons including: 1. It is a common

slug species easily collected 2. It is a large slug, which allows many White traps to be made at one time 3. From preliminary experiments it consistently produces large numbers of nematodes. Also it should be stressed that a previous study has shown that feeding on this slug species in White traps produces consistently virulent *P. hermaphrodita* (Rae et al., 2010). A single hermaphrodite mother was then transferred via a worm pick to each White trap and then sealed with Parafilm® and stored at 20°C for 21 days. After which dauer stage nematodes were found in the surrounding water. This procedure was repeated for all of the *Phasmarhabditis* collection to make isogenic lines.

In a separate experiment the numbers of dauer juveniles produced by one self-fertilising hermaphrodite mother was quantified for two strains of *P. hermaphrodita* (commercial strain DMG0001 and the naturally isolated strain DMG0007) and 1 strain of *P. californica* (DMG0017). These strains were chosen to understand if there was a difference between the growth of the commercial strain (*P. hermaphrodita* DMG0001; Hooper et al., 1999) and natural strains of *P. hermaphrodita* as the commercial strain has been in culture for over 20 years and fed solely on the monoxenic diet consisting of the bacterium *Moraxella osloensis* (Wilson et al., 1995a,b). In total the number of dauer juveniles produced by a single hermaphrodite mother of *P. hermaphrodita* DMG0001, DMG0007 or *P. californica* (DMG0017) was quantified by making 5 White traps containing either 0.025 g or 0.25 g of *L. flavus* and left for 21 days at 20°C (Fig 1D). The experiment was repeated twice.

Brood size of *Phasmarhabditis* species exposed to lab and naturally isolated bacteria

We investigated whether *Phasmarhabditis* nematodes could grow on several different bacteria including *Escherichia coli* OP50 (the food of *C. elegans* and *P. pacificus*, Brenner, 1974; Sommer et al., 2000) and *E. coli* BR (a strain used for cloning in molecular biology and easily available) and two naturally isolated bacterial species found associated with *Pristionchus entomophagus* called *Pseudomonas* sp. 1 and *Bacillus* sp. 1 (Rae et al. 2008) (Fig 1C). Bacteria were grown in nutrient broth at 30°C overnight. The following morning 100µl of each bacterium was spread onto five NGM (Nematode Growing Media; Hope, 1999) plates (5 cm) and were then incubated at 30°C overnight. An individual dauer stage nematode was transferred to each plate via a worm pick and incubated at 20°C. The numbers of offspring were then recorded per plate after 6 days. The experiment consisted of using *P. hermaphrodita* DMG0001 and DMG0007 and *P. californica* DMG0017. This experiment was repeated three times with all four bacteria and with all three nematode isolates.

In order to understand the feeding behaviour of *Phasmarhabditis* nematodes in more detail we also recorded the pharyngeal pumping rate whilst they were eating. This has been observed easily in both *C. elegans* and *P. pacificus* (Kroetz et al., 2012) but never for any *Phasmarhabditis* species. The pharyngeal pumping rate of an individual of three *Phasmarhabditis* species (*P. hermaphrodita* DMG0007, *P. neopapillosa* DMG0012 and DMG0016; and *P. californica* DMG0017) was counted for 60 secs and repeated 10 times with separate worms.

Investigating the effect of temperature on the brood size of *Phasmarhabditis* species

To examine what the optimum temperature for growth of *Phasmarhabditis* nematodes was under laboratory conditions fifteen 5 cm NGM plates were seeded with 100µl of *Pseudomonas* sp. 1 and then incubated at 30°C overnight. *Pseudomonas* sp. 1 was chosen as out of all 4 bacterial species tested *Phasmarhabditis* nematodes produced a large number of offspring, which were easy to see in the bacterial lawn due to its translucent nature. A single L4 hermaphrodite was placed onto each NGM plate and groups of 5 plates were incubated at either 10°C, 15°C or 20°C for 6 days. On day 3 and 6 the numbers of offspring were then recorded. This experiment was repeated three times using the same nematode species and strains as above.

Heat shocking and spontaneous male rate of several *Phasmarhabditis* species

It is imperative that a genetic model nematode can be mated under laboratory conditions and it is unknown how common males are produced in *P. hermaphrodita* collected from the wild. Five NGM plates (5 cm) were spread with 50 µl of *Pseudomonas* sp. 1 and incubated overnight at 30°C. The following morning dauer juveniles (between 1,000 to 7,000 per strain) were added to each plate and they were incubated at 20°C for 2-3 days. The numbers of males present was then recorded. The species and strains used (and numbers of dauer observed) were: *P. hermaphrodita* DMG0001 (n = 4040), DMG0002 (n = 6771), DMG0010 (n = 4581), DMG0009 (n = 3108), DMG0003 (n =2503) and DMG0007 (n = 3572); *P. californica* DMG0017 (n = 1098) and DMG0019 (n = 1127) and *P. neopapillosa* (DMG0012, DMG0015 and DMG0016; n = 750 for each strain).

In *C. elegans* the number of males can be increased by exposing hermaphrodite mothers to 30°C for 4 hours (Hope et al., 1999). We examined whether the same was true for *P. hermaphrodita* (DMG0001, DMG0007 and DMG0009). Fifteen to twenty L4 hermaphrodites were added to 5 separate 5 cm NGM plates seeded with *Pseudomonas* sp. 1 and placed in a 30°C incubator for 1, 3, 4.5, 5 and 6 hours. After which the plates were placed at 20°C to recover and the number of males in the offspring was recorded after 4 days. The experiment was repeated 3 times.

Genetic crosses of *Phasmarhabditis* species under lab conditions

Some parasitic nematodes are difficult to mate under lab conditions using agar plates e.g. the free-living generation of *Strongyloides ratti* (Nemetschke et al., 2010) and it is unknown if *P. hermaphrodita* or any other *Phasmarhabditis* species can be mated which is essential to monitor the inheritance of mutations and to facilitate mapping of mutated genes. Therefore, we used methods that are commonly used to mate *C. elegans*. Specifically, five 5 cm NGM plates with 50 µl of *Pseudomonas* sp. 1 and incubated at 30°C overnight. One L4 hermaphrodite was added to each plate with 2 young males and the plates were incubated at 20°C for 6 days. After two days of mating the males were removed with a worm pick and killed. After 6 days the sex and number of offspring were recorded. We used *P. neopapillosa* (DMG0012 and DMG0016) a gonochoristic species which produces almost 50% males, as we had difficulties in finding males from *P. hermaphrodita* even after heat shocking. The experiment was repeated three times.

Natural genetic variation in thermotolerance and pH tolerance of *Phasmarhabditis* species

To assess whether there was natural genetic variation in the ability of the collection of *Phasmarhabditis* nematodes to cope with extreme pH and temperatures the following experiments were carried out. For the thermotolerance experiment three 1.5ml Eppendorf tubes containing between 4,500-6,000 nematodes per 1ml were placed into a heat block set at 33°C, 37°C or 41°C for 15 mins. At time 0 min and after 15 mins the numbers of nematodes were quantified. The Eppendorf containing the nematodes was vortexed every 2.5 minutes to avoid clumping. Eppendorfs containing the same numbers of nematodes but kept at room temperature were used as the control. The experiment was repeated three times for each temperature. The following species and strains were used: *P. hermaphrodita* (DMG0001, DMG0007, DMG0010, DMG0006 and DMG0008), *P. californica* (DMG0017 and DMG0019) and *P. neopapillosa* (DMG0013, DMG0015 and DMG0016).

To assess natural variation of pH tolerance 10 individual dauer stage nematodes were added to eighty wells containing 60µl of water adjusted to pH 4, 5, 6, 7, 8, 9 and 10 as well as a control of distilled water. The correct pH was obtained by addition of either 1 M NaOH or 1 M HCl and confirmed a pH meter and indicator paper. There were 10 wells per pH and the whole experiment was repeated twice. The 96 well plate was then incubated at 20°C and survival was recorded observed daily for 4 days. The same *Phasmarhabditis* species and strains were used as in the thermotolerance experiment.

Formaldehyde mutagenesis of *P. hermaphrodita* DMG0001

To investigate if *P. hermaphrodita* can be mutated using formaldehyde mutagenesis (like *C. elegans* and *P. pacificus*) we used similar methods developed by Johnson & Baillie (1988) for *C. elegans*. *P. hermaphrodita* DMG0001 (L4 and young adult stage) were grown on several NGM plates with *Pseudomonas* sp. 1 for 4 days. They were then washed in distilled water and concentrated to a pellet and exposed to 0.1% formaldehyde for 4 hours. After which the P0's were washed several times in water to remove any residual formaldehyde and 100 individual mothers were separated out and placed on individual NGM plates seeded with *Pseudomonas* sp. 1. They were stored at 20°C for 3-4 days and allowed to produce offspring and then 300 F1's were separated out (3 individuals were randomly picked from each plate of P0 mothers) and the F2's were screened for any morphological abnormalities after 5-7 days.

Data analysis

The difference between the numbers of dauers produced by *P. hermaphrodita* DMG0001, DMG0007 and *P. californica* DMG0017 grown on 0.025 g and 0.25 g of *L. flavus* was analysed using a One Way ANOVA with Tukey's post hoc test. These tests were also used to analyse the data on pumping rate, number of offspring produced on different bacteria and at different temperatures, as well as the numbers of surviving nematodes exposed to 33, 37 and 41°C and pH 4 to 10. The body length of *P. hermaphrodita* DMG0001 WT and *sma* mutants was compared using a Student's t test. SPSS version 23 was used for analysing data.

Results

Phasmarhabditis species can be easily isolated and identified from gastropods

From 426 slugs and snails collected from around the U.K. we found 12 isolates of *P. hermaphrodita*, 3 isolates of *P. californica* and 5 isolates of *P. neopapillosa* (Table 1). These isolates were all from separate slugs apart from *P. californica* where 3 isolates were found in a single *O. draparnaudi*. We had initially identified many of these *Phasmarhabditis* species as *P. hermaphrodita* by using species-specific primers developed by Read et al. (2006) (Supplementary Fig 1). However, we found that these primers do not just amplify *P. hermaphrodita* but also other members of the *Phasmarhabditis* genus and even diverse insect associated and free-living species such as *Steinernema feltiae, Panagrellus redivivus, Aphelenchus avenae, Pelodera teres* and *Pristionchus entomophagus* (Supplementary Fig 1). Hence, they are not suitable for identification of *P. hermaphrodita* specifically and should be used with caution. We therefore amplified and sequenced three genes (ITS1, 18SrRNA and the D2-D3 domain of LSU rDNA) for species identification. These *P. hermaphrodita* strains and *Phasmarhabditis* species are the start of an on-going effort to make a collection of *P. hermaphrodita* strains and *Phasmarhabditis* species to study the genetic evolution of parasitism and we have categorised them using *C. elegans* nomenclature (Table 1).

Growth of *Phasmarhabditis* species using semi-natural conditions

We made isogenic lines by growing single hermaphrodites of *P. hermaphrodita* DMG0001, DMG0007 and *P. californica* DMG0017 on 0.025 g (Fig 2A) and 0.25 g of *L. flavus* (Fig 2B). The numbers of dauer juveniles that were produced by one *P. hermaphrodita* DMG0001, DMG0007 and *P. californica* DMG0017 on 0.025 g of *L. flavus* after 21 days ranged from 43 to 6,166 dauers per White trap (Fig 2A) and did not differ significantly (F (2, 36) = 1.369; p = 0.268). The numbers of dauer juveniles that were produced by single mothers of *P. hermaphrodita* DMG0001, DMG0007 and *P. californica* DMG0017 fed on 0.25 g of *L. flavus* after 21 days ranged from 417 to 27,750 dauers per plate (Fig 2B) and also did not differ significantly (F (2, 38) = 2.832; p = 0.072). Therefore, *Phasmarhabditis* sp. can be easily grown under semi-natural conditions using *L. flavus* White traps and in large numbers for experiments.

Growth of *Phasmarhabditis* species on different bacteria at different temperatures

P. hermaphrodita DMG0001, DMG0007 and *P. californica* DMG0017 were fed two nematode associated bacteria (*Pseudomonas* sp. 1 and *Bacillus* sp. 1) and two strains of *E. coli* that have been used historically in *C. elegans* culture and molecular biology (*E. coli* OP50 and *E. coli* BR, respectively). Over 6 days both nematode species were able to grow on each of these bacteria and laid F1 eggs that developed to offspring and were quantified (Fig 3). There was a significant difference between the numbers of offspring produced by *P. hermaphrodita* DMG0001 when fed *Pseudomonas* sp. 1; *Bacillus* sp. 1; *E. coli* OP50 and *E. coli* BR (F (3, 29) = 11.101; p = 0.000). Specifically, the numbers of offspring produced by *P. hermaphrodita*

DMG0001 was highest on *Pseudomonas* sp. 1, *E. coli* OP50 and *E. coli* BR but produced the least number of offspring on *Bacillus* sp. 1 ($p < 0.05$). This was also the case for *P. hermaphrodita* DMG0007 and *P. californica* DMG0017. Therefore, the laboratory bacteria (*E. coli* OP50 and *E. coli* BR) and naturally isolated *Pseudomonas* sp. 1 can be used for growing *Phasmarhabditis* species.

When grown at specific temperatures (10°C, 15°C and 20°C) and fed *Pseudomonas* sp. 1 to investigate the optimum conditions for growth and brood size of *P. hermaphrodita* DMG0001, *P. hermaphrodita* DMG0007 and *P. californica* DMG0017 it was found that 20°C was best for growth for both species (Fig 4). There was no significant difference between the numbers of offspring of both species when fed *Pseudomonas* sp. 1 at 20°C after 3 days (F (2,35) = 0.917; p = 0.41). However, after 6 days at 20°C *P. hermaphrodita* DMG0007 produced significantly more offspring than *P. hermaphrodita* DMG0001 and *P. californica* DMG0017 (F (2,31) = 5.067; p = 0.013). Production of offspring of both species was low at 10°C after 6 days. Eggs were laid in small numbers but they did not develop into live offspring. There was no significant difference between the numbers of viable offspring produced by both species after 6 days at 15°C (F (2,28) = 1.649; p = 0.212). When grown at temperatures higher than this (25°C) the hermaphrodite mothers died rapidly (Andrus and Rae, unpublished observation) so this seems to represent the upper limit for growth of these natural strains.

When feeding on *Pseudomonas* sp. 1 there was a significant difference between the pumping rates of *P. hermaphrodita* DMG0001, *P. hermaphrodita* DMG0007, *P. neopapillosa* DMG0012 and DMG0016 and *P. californica* DMG0017 (F (4, 19) = 18.577; p = 0.000) (Fig 5). Specifically, there was no difference between the pumping rate of *P. hermaphrodita* DMG0001 and DMG0007, *P. neopapillosa*

DMG0012 and DMG0016 ($p > 0.05$) but all differed significantly with *P. californica* DMG0017, which had the lowest number of pumps per minute ($p < 0.05$) (Fig 5).

Natural variation in thermotolerance of *Phasmarhabditis*

There was a significant difference between the survival of the *Phasmarhabditis* isolates when exposed to 30°C (F (10, 98) = 18.389; $p < 0.001$) (Fig 6A). Specifically, the survival of the commercial strain *P. hermaphrodita* (DMG0001) was significantly lower than *P. hermaphrodita* DMG0007 and DMG0008 ($p < 0.001$) but not *P. hermaphrodita* DMG0010 and DMG0006 ($p > 0.05$). When the survival of *P. hermaphrodita* (DMG0001) was compared to other species of *Phasmarhabditis* there was a significant difference between *P. neopapillosa* DMG0015 and DMG0016 ($p < 0.001$) but not DMG0013 ($p > 0.05$). Also there was a significant difference between the survival of *P. hermaphrodita* (DMG0001) compared to *P. californica* DMG0019 ($p < 0.05$) but not DMG0017 ($p > 0.05$).

When the *Phasmarhabditis* isolates were exposed to 37°C there was a significant difference in their survival (F (10, 98) = 24.017; $p = 0.000$) (Fig 6B). The survival of *P. hermaphrodita* (DMG0001) was significantly lower than *P. hermaphrodita* DMG0007 but significantly higher than DMG0010 ($p < 0.05$) but not any other *P. hermaphrodita* strain. The survival of *P. hermaphrodita* DMG0001 differed from *P. neopapillosa* DMG0015 ($p < 0.05$) but no other species or isolate.

When the *Phasmarhabditis* isolates were exposed to 41°C there was a significant difference in their survival (F (10, 98) = 19.546; $p = 0.000$) (Fig 6C). The

survival of *P. hermaphrodita* (DMG0001) was significantly greater than all other species and isolates ($p < 0.05$).

Natural variation in pH resistance in *Phasmarhabditis*

When the *Phasmarhabditis* species and strains were exposed to pH 4 there was a significant difference in survival (F (9, 29) = 6.060; p = 0.000) (Table 2) with the numbers of surviving *P. hermaphrodita* (DMG0001) being significantly greater than that of *P. neopapillosa* DMG0015 and DMG0016 ($p < 0.05$).

When exposed to pH 5 the survival of *Phasmarhabditis* species and strains was significantly different (F (9, 29) = 2.706; p = 0.031) (Table 2) with the survival of *P. hermaphrodita* DMG0007 being significantly lower than *P. californica* DMG0017 ($p < 0.05$). There was no significant difference between the survival of *Phasmarhabditis* species and strains exposed to pH 6 (F (9, 29) = 1.937; p = 0.105) or pH 8 (F (9, 29) = 1.956; p = 0.101).

The survival of *Phasmarhabditis* also differed at pH 7 (F (9, 29) = 3.778; p = 0.006) with the numbers of alive *P. hermaphrodita* DMG0007 and *P. neopapillosa* DMG0015 being significantly lower than *P. californica* DMG0019 ($p < 0.05$).

At pH 9 (F (9, 29) = 3.378; p = 0.011) and pH 10 (F (9, 29) = 5.481; p = 0.001) survival was significantly different. In both cases there the survival of *P. neopapillosa* DMG0015 was significantly lower than that of *P. californica* DMG0017 and DMG0019 ($p < 0.05$). Also the survival of *P. hermaphrodita* DMG0007 was also significantly lower than *P. californica* DMG0017 and DMG0019 when exposed to pH 10 ($p < 0.05$).

Spontaneous male rate, heat shocking and genetic crosses using *Phasmarhabditis* species

We observed the numbers of *Phasmarhabditis* dauer juveniles that developed into males when grown on NGM agar and fed *Pseudomonas* sp. 1 for 4 days. From 6 strains of *P. hermaphrodita* (DMG0001, DMG0002, DMG0010, DMG0009, DMG0003 and DMG0007) and 2 strains of *P. californica* (DMG0017 and DMG0019) no males were observed and only hermaphrodites were produced. All gonochoristic species produced males in varying amounts *P. neopapillosa* DMG0012 (50% males to 50% females) and DMG0015 (25% males to 75% females), and DMG0016 (46% males to 54% females).

We investigated if the number of males could be increased by heat shocking *P. hermaphrodita* hermaphrodites (DMG0001, DMG0007 and DMG0009) for 1, 3, 4.5, 5 and 6 hours at 30°C. No offspring were produced by hermaphrodites that had been exposed to 30°C for 5 and 6 hours and the number of offspring produced was low for heat treatment for 4.5 hours (11 to 30 animals); 3 hours (11 to 33 animals) but increased when exposed for 1 hour (16 to 115 animals). However, no males were observed in any of the offspring. Therefore, it is problematic to find *P. hermaphrodita* males when grown under these conditions.

To understand whether *Phasmarhabditis* species could be mated under lab conditions we concentrated on using the gonochoristic species *P. neopapillosa* DMG0012 and DMG0016. Both strains were crossed using standard procedures based on *C. elegans* and were fed *Pseudomonas* sp. 1. Under these conditions we could show that two males to one females placed together using *P. neopapillosa* DMG0016 and DMG0012 resulted in between 47.3 ± 6.7 *P. neopapillosa* DMG0016

and 83.9 ± 5.6 *P. neopapillosa* DMG0012 offspring 6 days later. Therefore, successful crossing of these two strains could be carried out to demonstrate inheritance of recessive and dominant mutations and to aid mapping of mutations.

Formaldehyde mutagenesis of *Phasmarhabditis* species

From 300 F1 *P. hermaphrodita* DMG0001 hermaphrodite mothers (Fig 7A) several mutants were isolated. Specifically, 2 small (*sma*) mutants (Fig 7B,C) and 3 uncoordinated (*unc*) mutants (Fig 7D,E) were found. *sma* mutants were significantly smaller that *P. hermaphrodita* WT ($p < 0.05$) and *unc* mutants strongly resembled *C. elegans unc* phenotype as they were lethargic, stationary and the underlying body wall muscle produced a constant twitch (Waterson et al., 1980). Therefore, *P. hermaphrodita* can be mutagenized using formaldehyde permitting forward genetic screens to be carried out.

Discussion

The genus *Phasmarhabditis* contains eleven species including *P. hermaphrodita*, *P. apuliae*, *P. papillosa*, *P. neopapillosa*, *P. valida*, *P. nidrosiensis*, *P. californica*, *P. tawfiki*, *P. bonaquaense*, *P. bohemica* and *P. huizhouensis* (Andrássy, 1983; Azzam, 2003; Hooper et al., 1999; Huang et al., 2015; Tandingan De Ley et al. 2014; 2016; Nermut et al., 2016a,b,c). *P. hermaphrodita* and other *Phasmarhabditis* species have been isolated from slugs from around the world including the U.K. (Wilson et al., 1993; this study), Germany (Schneider, 1859; Mengert, 1953), France (Coupland, 1995; Maupas, 1900), 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), South Africa (Ross et al., 2012; Pieterse et al., 2017a,b). China (Huang et al., 2015), Japan (Waki, 2017), Italy (Nermut et al., 2016a), Czechoslovakia (Nermut et al., 2010; 2016b,c) and *P. californica* has been found in the USA (Tandingan De Ley et al., 2016), Ireland (Carnaghi et al., 2017) and Wales (this study). From these studies it is apparent that members of the *Phasmarhabditis* genus have a cosmopolitan distribution and are very easy to isolate from gastropod hosts. We found 20 separate *Phasmarhabditis* isolates from 426 slugs and snails from three locations around the U.K. From our survey the success rate of collecting *Phasmarhabditis* (4.7%) seems small yet we have discovered a wealth of different species including *P. californica* (which had never been identified in the U.K. before) and several strains of *P. neopapillosa*. Therefore, it seems that the U.K. has an underappreciated diversity of *Phasmarhabditis* species.

Although *P. hermaphrodita* is a parasite we have shown that it can be maintained and cultured under laboratory conditions using a combination of NGM agar and naturally isolated bacteria (*Pseudomonas* sp. 1). For laboratory genetic model nematodes to be used successfully their bacterial food must be nutritious enough to facilitate the growth of hundreds of offspring; transparent (so nematodes are visible); easy to grow and does not grow too much when added to the NGM plate. This was why *E. coli* OP50 was selected to grow and maintain *C. elegans* (Brenner, 1974). However, regular passage of hundreds of thousands of generations of nematodes using the same culture conditions (constant temperature, lots of food and same species of food) with no interaction or variation in the environment can severely affect genetic make up (Huey & Rosenzweig, 2009). For example, *C. elegans* N2 (wild type) has lost the ability to perform thermoregulatory behaviour when exposed

to a temperature gradient due to being reared at the same temperature for over 40 years (Anderson et al., 2007). We propose to use *Pseudomonas sp.* 1. - a bacterium found in the intestine of *P. entomophagus* from Tuebingen, Germany (Rae et al., 2008) and not an unusual food source like *E. coli* OP50. Yet continual culturing on NGM plates is an unnatural culture method for these nematodes as *Phasmarhabditis* are necromenic and parasitic nematodes used to reproducing on rotting cadavers of molluscs and not agar plates (Wilson et al., 1993; Rae et al., 2009). To this end we propose to grow *Phasmarhabditis* on decaying slugs in 'semi-natural' conditions using White traps, which is a more realistic environment. This method means that they can be stored at 10 to 15°C for months as dauers (Grewal & Grewal, 2003), and cultured infrequently (every 4-5 months) and therefore the effect of accumulating any deleterious mutations will be reduced. Furthermore, future research will focus on the development of cryopreservation techniques for *Phasmarhabditis* which will allow access to a library of 'un-evolved' strains and species as well as mutants.

Once *P. hermaphrodita* has killed a slug it feeds on the bacteria growing on the rotting cadaver and this is depleted it turns to the dauer stage and searches for more slugs in the soil. These dauers associate with a rich diversity of bacteria that are carried in their intestine (Rae et al., 2010). Previous studies have shown that the bacteria isolated from the intestine of *P. hermaphrodita*, xenic cultures of *P. hermaphrodita* and from swabs of slugs that died from infection of *P. hermaphrodita* can affect the number of dauers produced as well as their virulence towards slugs (Wilson et al., 1995a,b). These studies showed that *P. hermaphrodita* can grow on an array of bacterial species such as *Pseudomonas fluorescens, Sphingobacterium spintovorum, M. osloensis, Serratia proteamaculans, Aeromonas* sp. and *Providencia rettgeri* (Wilson et al., 1995b), and *P. hermaphrodita* grown on bacteria such as *P.*

fluorescens, *M. osloensis* and *P. rettgeri* can produce high yields of pathogenic nematodes that kill slugs (Wilson et al., 1995a). However, the commercial isolate of *P. hermaphrodita* DMG0001 is grown on *M. osloensis* as it can produce consistent high yields of highly pathogenic nematodes (Tan and Grewal, 2001b; Wilson et al., 1995a,b). It has been shown that when introduced into the shell cavity of *D. reticulatum*, *M. osloensis* produces a lipopolysaccharide (LPS) that acts an endotoxin, causing rapid mortality (Tan and Grewal, 2002). By utilizing this collection of naturally isolated *P. hermaphrodita* and *Phasmarhabditis* species the co-evolution of these tri-trophic interactions between bacteria (such as *M. osloensis*), nematodes and slug hosts could be analysed at the molecular level.

As well as established genetic model nematodes (*C. elegans* and *P. pacificus*) there are several other nematodes that have been proposed including *Poikilolaimus oxycercus* (Hong et al., 2005), *Oscheius tipulae* (Félix, 2006) and *Meloidogyne hapla* (to study plant parasitism) (Opperman et al., 2008). In order for these nematodes (as well as *P. pacificus*) to be used under laboratory conditions information is needed about appropriate bacterial food as well as methods to allow genetic crosses, mutagenesis and long term storage. We have shown that logistically and financially, nematodes such as *Phasmarhabditis* are not difficult to keep and are easy to maintain. There is little difference in the equipment needed to keep *C. elegans* (Stiernagle, 2006) e.g. simple reagents and microbiological media, incubators and freezers for growth and long term storage. As well as these factors, another important point about model nematodes is the ability to be isolated easily can allow tens if not hundreds of strains to be studied to investigate natural phenotypic variation, which can lead to an understanding of the underlying genotype using approaches such as RAD seq (Restriction site Associated DNA Sequencing) (Davey & Blaxter, 2010) and GWAS

(Genome Wide Association Studies) (Cook et al., 2016). In *C. elegans* global sampling efforts have collected several hundred *C. elegans* strains and 26 *Caenorhabditis* species (Frezal & Félix, 2015); which are available from the *Caenorhabditis* Genetic Stock Centre, US. Studying natural variation has been successful in understanding genes involved with foraging behaviour, thermal tolerance and outcrossing (De Bono & Bargmann, 1998; Harvey & Viney, 2007; Teotonio et al., 2006). A similar approach utilising natural strains and investigating variation in behaviour, cold tolerance and dauer formation in *P. pacificus* has been taken (Hong et al., 2008; Mayer & Sommer, 2011; McGaughran & Sommer, 2014). In total there are 28 species of *Pristionchus* (Ragsdale et al., 2015) and hundreds of strains of *P. pacificus* that are available from the Sommer lab, Tuebingen, Germany (Morgan et al., 2012). We have shown that, like both *C. elegans* and *P. pacificus*, *P. hermaphrodita* and a selection of *Phasmarhabditis* species can be isolated and maintained in the lab easily. We have shown that there is natural variation within *P. hermaphrodita* and *Phasmarhabditis* species surviving different temperatures and pHs. This means that with the development of appropriate sequencing and genomic techniques (e.g. RAD seq) macro-evolutionary and micro-evolutionary processes could potentially be unravelled at the genetic level.

The isolation of mutants via forward genetic screens using mutagenesis is a powerful technique that can identify genes responsible for specific phenotypes. The first step of any proposed genetic model nematode is to show it can be mutagenized. Here we showed *P. hermaphrodita unc* and *sma* mutants could be isolated by using similar protocols as *C. elegans* (Johnson & Baillie, 1988). If *P. hermaphrodita* can be mutated then there is no reason why full-unbiased forward genetic screens could not be carried out to investigate an array of evolutionary and ecologically important traits.

These include finding mutants that are defective in killing slugs, inducing slug avoidance (Wilson et al., 1999, Wynne et al., 2016) or failing to chemotax towards host cues such as slug mucus (Rae et al., 2006; 2009). As *P. hermaphrodita* is one of the candidates for the 959 nematode genome project (Kumar et al., 2012) and several species are currently undergoing full genome sequencing, this will allow genomic comparison to closely related free-living nematodes, arthropod and mammalian parasites present in Clade 5.

We found that *P. neopapillosa* could be mated under lab conditions using similar procedures as *C. elegans*. However, generating enough males for genetic crosses with *P. hermaphrodita* proved difficult due to scarcity. This is not unusual for hermaphroditic nematodes that are able to spontaneously produce males. *C. elegans* only produces 0.1-0.2% of males in culture (Hodgkin & Doniach, 1997). Maupas (1900) noted that from 14,888 *P. hermaphrodita* only 1 male was found. Our strains under lab conditions seem to not produce males, even when heat shocked for 1-4 hours at 30°C. Strangely, when these strains were first isolated one was found with males present (DMG007) (Rae, unpublished) but when grown on rotting slug and NGM plates this ability seemed to diminish rapidly over time. Future research will focus on methods used to generate males including exposing hermaphrodites to ethanol (Lyons & Hecht, 1997) or isolating a mutant (using forward genetics) that has a high incidence of males (*him* mutants) (Hodgkin et al., 1979).

In conclusion, we have outlined the methods used to work with *P. hermaphrodita* and *Phasmarhabditis* species under laboratory conditions. We believe that *P. hermaphrodita* (and other *Phasmarhabditis* species) could be used to identify genes that are essential for pathogenicity towards slugs. The most logical way to achieve this would be to take a natural variation approach to isolate as many *P.*

hermaphrodita strains as possible and grow them on rotting slugs (as we have outlined here which does not affect their virulence, Rae et al. (2010) and assess their pathogenic potential towards slugs. The main aim would be to identify a strain that is more or less virulent that DMG0001 (an approach that is currently on going, Rae, unpublished). Through genome sequencing potential parasitism genes could be identified and confirmed by reverse genetics e.g. RNAi and/or CRISPR-Cas9. This information may allow in depth insight into the evolution of parasitism in other Clade 5 animal, plant and invertebrate nematode parasites as well as comparison to *C. elegans* and *P. pacificus*.

Figure legends

Figure 1: The slug parasitic nematode *P. hermaphrodita* (A) is a parasite of several slug species such as *D. reticulatum* (B). It can also be kept under lab conditions on NGM agar feeding on *Pseudomonas* sp. 1 (C) and on rotting *L. flavus* in a White trap (D). Scale bars represent 100 μm in A, 1 cm in B and 1 mm in C and D.

Figure 2: The mean number of offspring that were produced by *P. hermaphrodita* DMG0001, *P. hermaphrodita* DMG0007 and *P. californica* DMG0017 when fed 0.025 g (A) and 0.25 g (B) of *L. flavus*. Bars represent ± one standard error.

Figure 3: The mean number of offspring that were produced by *P. hermaphrodita* DMG0001 (black bars), *P. hermaphrodita* DMG0007 (white) and *P. californica* DMG0017 (grey) when fed *Pseudomonas* sp. 1, *Bacillus* sp. 1, *E. coli* OP50 and *E. coli* BR at 20°C. Bars represent ± one standard error.

Figure 4: The mean number of offspring that were produced by *P. hermaphrodita* DMG0001 (black bars), *P. hermaphrodita* DMG0007 (white) and *P. californica* DMG0017 (grey) at 10°C (A), 15°C (B) and 20°C (C). Bars represent ± one standard error.

Figure 5: The mean pumping rate over 60 seconds of *P. hermaphrodita* (DMG0001 and DMG0007), *P. californica* DMG0017 and *P. neopapillosa* (DMG0012 and DMG0016) fed *Pseudomonas* sp. 1 at 20°C. Bars represent ± one standard error.

Figure 6: The number of alive *P. hermaphrodita* (DMG0001, DMG0007, DMG0010, DMG0006 and DMG0008); *P. californica* (DMG0018 and DMG0019) and *P. neopapillosa* (DMG0013, DMG0015 and DMG0016) exposed to 31°C (A), 37°C (B) and 41°C (C). Bars represent ± one standard error.

Figure 7: *P. hermaphrodita* DMG0001 (A) were mutagenized with 0.1% formaldehyde and *sma* (small) mutants (B, C) and *unc* (uncoordinated) mutants (D, E) were found in the F2 generation. Scale bar represents 100 μm.

Supplementary Figures

Supplementary Figure 1: PCR product of COI gene based on primers by Read et al. (2006) from DNA that was extracted and amplified from *P. hermaphrodita* DMG0001 (lane 1), DMG0002 (2), B30 (3), DMG0003 (4), DMG0004 (5), DMG0005 (6), DMG0006 (7), DMG0007 (8), DMG0008 (9), DMG0009 (10),

DMG0010 (11), DMG0011 (12), *P. neopapillosa* DMG0012 (13), DMG0013 (14), *P. hermaphrodita* AB46 (15), *P. neopapillosa* DMG0014 (16), DMG0015 (17), DMG0016 (18), *P. californica* DMG0017 (19), DMG0018 (20), DMG0019 (21), *C. elegans* N2 (22), *Acrobeloides* sp. (23), *Cervidellus vexilliger* (24), *Aphelenchus avenae* (25), *Pelodera teres* (26), *Pristionchus entomophagus* (27), *Pristionchus uniformis* (28), *Steinernema feltiae* (29), *Steinernema carpocapsae* (30), *Panagrellus redivivus* (31), *Heterorhabditis bacteriophora* (32), negative control (water as template) (33) and positive control (*P. hermaphrodita* DMG0001 DNA) (34). L = 1 kb DNA Ladder.

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Code	*Phasmarhabditis* species	Slug/snail species isolated from	Location	Code
A75	*P. hermaphrodita*	*Arion* spp.	Priory Wood, Liverpool	DMG0002
B30	*P. hermaphrodita* (Deceased)	*Deroceras panormitanum*	Sefton Park, Liverpool	
B33	*P. hermaphrodita*	*Deroceras panormitanum*	Sefton Park, Liverpool	DMG0003
B36	*P. hermaphrodita*	*Deroceras panormitanum*	Sefton Park, Liverpool	DMG0004
B93	*P. hermaphrodita*	*Arion subfuscus*	Sefton Park, Liverpool	DMG0005
B113	*P. hermaphrodita*	*Arion subfuscus*	Sefton Park, Liverpool	DMG0006
B178	*P. hermaphrodita*	*Limax flavus*	Sefton Park, Liverpool	DMG0007
C11	*P. hermaphrodita*	*Deroceras panormitanum*	Festival gardens, Liverpool	DMG0008
C25	*P. hermaphrodita*	*Deroceras panormitanum*	Festival gardens, Liverpool	DMG0009
MG2	*P. hermaphrodita*	*Milax budapestensis*	Festival gardens, Liverpool	DMG0010
MG6	*P. hermaphrodita*	*Milax budapestensis*	Festival gardens, Liverpool	DMG0011
AB9	*P. neopapillosa*	*Deroceras reticulatum*	Cruickshank gardens, University of Aberdeen	DMG0012
AB10	*P. neopapillosa*	*Deroceras reticulatum*	Cruickshank gardens, University of Aberdeen	DMG0013
AB46	*P. hermaphrodita* (Deceased)	*Limax flavus*	Sefton Park, Liverpool	
B170	*P. neopapillosa*	*Limax flavus*	Sefton Park, Liverpool	DMG0014
B190	*P. neopapillosa*	*Limax flavus*	Sefton Park, Liverpool	DMG0015
B175B	*P. neopapillosa*	*Limax flavus*	Sefton Park, Liverpool	DMG0016
P19B	*P. californica*	*Oxychilus draparnaudi*	Dale, Pembrokeshire	DMG0017
P19D	*P. californica*	*Oxychilus draparnaudi*	Dale, Pembrokeshire	DMG0018
P19E	*P. californica*	*Oxychilus draparnaudi*	Dale, Pembrokeshire	DMG0019

Table 1: The *Phasmarhabditis* species that were isolated from slugs and snails collected from Aberdeen, Liverpool and Pembrokeshire and nomenclature of isolated *Phasmarhabditis* species based on *C. elegans* rules.

			pH						
Species	Strain	Control	4	5	6	7	8	9	10
P. hermaphrodita	DMG0001	9.3 ± 0.3	7.7 ± 0.3	7.7 ± 0.3	9.7 ± 0.3	9.3 ± 0.7	7.3 ± 1.2	7.3 ± 0.7	5.7 ± 0.9
	DMG0006	8.7 ± 0.3	6.7 ± 0.3	7.7 ± 0.3	9.3 ± 0.3	8.7 ± 0.3	7.3 ± 0.3	6.7 ± 0.3	5.7 ± 0.3
	DMG0007	8 ± 0.6	6 ± 0.6	6.7 ± 0.9	8.3 ± 0.3	7.7 ± 0.3	7.7 ± 0.3	7.7 ± 0.3	4.3 ± 0.3
	DMG0008	9 ± 0.6	7.3 ± 0.3	8.3 ± 0.3	9 ± 0.3	8.3 ± 0.3	8.3 ± 0.3	7.3 ± 0.3	5.7 ± 0.3
	DMG0010	8.3 ± 0.3	6.7 ± 0.3	8 ± 0	9 ± 0.6	8 ± 0.6	8 ± 0.6	7.3 ± 0.3	5.7 ± 0.3
P. californica	DMG0017	9.7 ± 0.3	8 ± 0	9 ± 0	9.7 ± 0.3	9.3 ± 0.3	9 ± 0	8.7 ± 0.3	7.3 ± 0.3
	DMG0019	9.3 ± 0.3	7.3 ± 0.3	8.3 ± 0.3	9.7 ± 0.3	9.7 ± 0.3	8.7 ± 0.3	8.3 ± 0.3	7 ± 0
P. neopapillosa	DMG0013	8.3 ± 0.3	6.7 ± 0.3	8.3 ± 0.3	8.7 ± 0.3	8 ± 0	7.3 ± 0.3	7.3 ± 0.3	5.3 ± 0.3
	DMG0015	7.3 ± 0.3	5 ± 0.6	7.3 ± 0.3	8.3 ± 0.3	7.7 ± 0.3	6 ± 1	6 ± 0.6	4.3 ± 0.3
	DMG0016	8.7 ± 0.3	5.7 ± 0.3	7.7 ± 0.3	9.7 ± 0.3	9.3 ± 0.3	8 ± 0.6	7.3 ± 0.3	5.7 ± 0.3

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2 Table 2: The mean number of alive *Phasmarhabditis* ± one standard error after 4 days exposure to pH 4 to 10.

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