Natural variation in chemoattraction in the gastropod parasitic nematodes *Phasmarhabditis hermaphrodita*, *Phasmarhabditis neopapillosa* and *Phasmarhabditis californica* exposed to slug mucus

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Running Head: *Phasmarhabditis* attraction to slug mucus

Abstract

Phasmarhabditis hermaphrodita is a lethal parasite of several slug species and has been formulated into a biological control agent for farmers and gardeners. *P. hermaphrodita* responds to slug faeces, mucus and volatile cues to find hosts in soil. However, these results have only focused on one strain of *P. hermaphrodita* (DMG0001). We exposed four strains of *P. hermaphrodita* (and DMG0001), three strains of *P. neopapillosa* and two strains of *P. californica* to mucus from seven common slug species. Furthermore, we investigated whether there was a relationship between chemoattraction and the numbers of offspring that were produced on each host species. Natural isolates of *P. hermaphrodita* differed in their preference of slug species whereas *P. neopapillosa* tended to prefer *Arion* sp. and strains of *P. californica* displayed striking differences in their responses. The reasons for positive chemoattraction to mucus were not due to higher numbers of offspring produced on these hosts.

Keywords: *Phasmarhabditis*, behaviour, host finding, slugs

Parasitic nematodes have evolved to recognise an array of cues to detect and infect hosts (Lee, 2002). For example, the human parasite *Strongyloides stercoralis* is attracted to human skin and sweat odorants of mammalians hosts (Castelletto et al., 2014). Entomopathogenic nematodes from the genera *Steinernema* and *Heterorhabditis*, which have a symbiotic relationship with the bacteria *Xenorhabdus* and *Photorhabdus* that they vector into insect hosts and kill then in 24-48 hours (Forst et al., 1997) use host odorants, faeces and CO_2 to locate insects (Dillman et al., 2012). Free-living nematodes such as *Caenorhabditis elegans* that feed on bacteria respond to metabolites to find their food (Bargmann et al., 1993) and can even discriminate between pathogenic and non-pathogenic *Serratia marcescens* bacteria (Zhang et al., 2005). The scarab beetle associated nematode *Pristionchus pacificus* responds to insect pheromones to find hosts to latch onto (Hong et al., 2008a). The terrestrial gastropod parasitic nematode *Phasmarhabditis hermaphrodita* is a lethal parasite of several pestiferous slug species (Wilson et al., 1993) and has been formulated into a successful biological control agent by BASF Agricultural Specialities called Nemaslug® (Rae et al., 2007). In order to find slugs in soil *P. hermaphrodita* is attracted to slug mucus, faeces and volatile cues (Hapca et al., 2007a, Nermut et al., 2012, Rae et al., 2009, Rae et al., 2006, Small & Bradford, 2008). These experiments have not only been carried out on agar plates but in more realistic ecological conditions in soil and sand (Hapca et al., 2007b, Nermut et al., 2012). Once a slug has been found the nematodes enter through a pore at the back of the mantle and can kill the host in 4-21 days (Tan & Grewal, 2001, Wilson et al., 1993). *P. hermaphrodita* is strongly attracted to slugs such as *Arion subfuscus* but finds *Limax marginatus* and the snail *Cepaea hortensis* less attractive for reasons unknown (Rae et al., 2009). Previous research (Rae et al., 2009) investigated reasons for these differences in

attraction by focussing on examining whether more attractive slug species were better hosts for producing dauer juveniles. Only weak correlations were found and therefore the exact biological reason for these differences in chemoattraction remain unknown.

Experiments examining how *P. hermaphrodita* responds to host cues (Hapca et al., 2007a, Nermut et al., 2012, Rae et al., 2009, Small & Bradford, 2008) have all focussed on using the commercial strain designated DMG0001 (Hooper et al., 1999), which has been in culture for over 20 years (Wilson et al., 1993). There is no information on how other strains of *P. hermaphrodita* or *Phasmarhabditis* species respond to slug mucus. There are 11 *Phasmarhabditis* species (Rae, 2017) that have been described, yet there are few experiments investigating their biology. Therefore, we sought to understand whether there was natural variation in chemotaxis towards slug mucus by utilising a collection of *Phasmarhabditis* species (*P. hermaphrodita, P. neopapillosa* and *P. californica*) that were collected from around the U.K. and are currently maintained in culture at Liverpool John Moores University (LJMU) (Andrus & Rae, 2018).

Several studies investigating the chemotactic response using *P. hermaphrodita* have focused on studying exposure to mucus from single slug species (*Deroceras reticulatum*) (Hapca et al., 2007a, Hapca et al., 2007b, Rae et al., 2006) however, in nature slugs can be found in mixed populations in close contact with different species crowded together (Cook, 1981, South, 1992). These mixed populations contain individuals that often vary in infection levels of *Phasmarhabditis* nematodes (Andrus & Rae, 2018). How do *Phasmarhabditis* nematodes discriminate between mucus from different slug species in mixed populations? We decided to test this by exposing the nematodes to mucus from several slug species simultaneously. Furthermore, we also examined why these *P. hermaphrodita* strains and *Phasmarhabditis* species

responded differently to slug mucus by investigating whether greater numbers of offspring would be produced on the more attractive slug species. A previous study (Rae et al., 2009) investigated whether increased chemoattraction of *P. hermaphrodita* towards slugs was due to the higher number of dauer juveniles produced on attractive hosts but found no relationship, however we decided to understand whether the total number of offspring produced (rather than just number of dauers) could be the factor for attraction.

Materials and methods

Source of invertebrates

Slugs (*Deroceras invadens* (mean weight 0.21 ± 0.02 g), *D. reticulatum* (mean weight 0.26 ± 0.02 g), *Arion ater* (mean weight 1.27 ± 0.14 g), *A. hortensis* (mean weight 0.47 ± 0.16 g), *Lehmannia valentiana* (mean weight 0.39 ± 0.1 g), *Limax flavus* (mean weight 3.05 ± 0.35 g) and *Milax sowerbyi* (mean weight 1.56 ± 0.08 g) were collected from greenhouses at LJMU and stored in non-airtight plastic boxes lined with moist paper and fed lettuce *ad libitum*. Mean weight of each slug species were used from Rae et al (2009) apart from *A. hortensis* and *L. valentiana* where 10 slugs were weighed. The commercial strain of *P. hermaphrodita* (DMG0001) was purchased from Becker Underwood-BASF Agricultural Specialities and stored at 15°C until use. Natural strains of *P. hermaphrodita* and species of *Phasmarhabditis* were collected previously (Andrus & Rae, 2018). *P. hermaphrodita* (DMG0002, DMG0007, DMG0009 and DMG0010), *P. neopapillosa* (DMG0012, DMG0015 and DMG0016) and *P. californica* (DMG0018 and DMG0019) were

grown on White traps with rotting cellar slug (*L. flavus*) for 21-28 days until they reached the dauer stage (White, 1927).

Chemotaxis of *Phasmarhabditis* species towards slug mucus

In previous experiments investigating the attraction of *P. hermaphrodita* to slug associated cues a standard agar plate assay has been used (Hapca et al., 2007a, Rae et al., 2009, Rae et al., 2006), based on *C. elegans* assays for examining chemoattraction towards chemical odorants (Bargmann et al., 1993). This allows the chemotactic response of the nematodes to be quantified when exposed to just one slug species (compared to a water based control). We sought to understand whether the nematodes would show a preference towards certain species when exposed to mucus from all slug species simultaneously. We developed a set up where we could expose the nematodes to mucus from seven species of slug at the same time (Fig. 1). We swabbed 0.01g of foot mucus with separate pieces of 1 cm^2 piece of Whatman filter paper number 1 from the foot of the seven slug species, and placed each 0.5 cm from the side of a 10 cm Petri dish filled with 1.2% technical agar in a circle. The paper with the mucus from each species was equidistant from the opposite piece. To another piece of filter paper 10 µl of distilled water was added and acted as the control. Dauer stage nematodes of five strains of *P. hermaphrodita* (DMG0001, DMG0002, DMG0007, DMG0009 and DMG0010), two strains of *P. californica* (DMG0018 and DMG0019) and three strains of *P. neopapillosa* (DMG0012, DMG0015 and DMG0016) were added to the middle of each Petri dish and left for 10-20 mins so the water evaporated and then sealed with Parafilm® and incubated at 20°C overnight. The following day the numbers of nematodes that had migrated to each piece of filter

paper were then quantified. Three replicate plates were used for each nematode species/strain and the whole experiment was repeated three times.

The results of chemotaxis assays using nematodes e.g. *C. elegans* tend to be reported as a chemotaxis index (Bargmann et al., 1993), which records the number of nematodes that graduated to the treated and control side. This does not take into account the number of nematodes that remained at the point of application (which can sometimes be large) and the calculation of chemotaxis index can sometimes rely on small number of nematodes which can lead to misleading results. Therefore, we recorded and presented our data as the number of nematodes found on and in the mucus, in the water control and the numbers of nematodes found at the point of application to give a clearer idea of where the nematodes could be found. In addition, chemotaxis experiments with *C. elegans* and *P. pacificus* (Bargmann et al., 1993, Hong & Sommer, 2006) use 1M sodium azide to stop nematode movement immediately when they encounter the control or attractant. We do not use this as when *P. hermaphrodita* dauers encounter mucus they stay there (Hapca et al., 2007a, Rae et al., 2009, Rae et al., 2006).

Examination of the numbers of offspring produced on each host species

The numbers of offspring that were produced on each slug species by each *Phasmarhabditis* species was quantified by using White traps (White, 1927). Briefly, 0.1 g of each slug was added to a 5 cm Petri dish that was lined with a 5 cm piece of pre-moistened Whatman filter paper. Slugs of the seven species were placed into a -20°C freezer for 20 mins to kill any resident nematodes that would interfere with the experiment. The 5 cm Petri dish was then added to a 10 cm Petri dish, which was half

filled with distilled water. A single J2 stage hermaphrodite of each *Phasmarhabditis* species was added to each piece of slug and the plates were sealed with Parafilm® and left for 10 days. After which the numbers of offspring produced was quantified. Five plates were made for each slug species and the whole experiment was repeated twice. We did not test the numbers of offspring produced by *P. neopapillosa* as it is a gonochoristic species and was difficult to mate and grow from two individuals on pieces of rotting slug compared to single hermaphrodites.

Data analysis

The numbers of nematodes found in each piece of filter paper with different slug mucus and the numbers of nematodes that were produced on 0.1 g of each slug species were compared using a One Way ANOVA with Tukey's post hoc test. Fisher's Least Significant Difference (LSD) at 5% ($p < 0.05$) was calculated and presented in figures for chemotaxis data for each *Phasmarhabditis* strain. To test if there was a relationship between chemoattraction and the numbers of nematodes produced on a) 0.1 g of each slug species and b) adult slugs (calculated by using mean weights of each species) regression analysis was used.

Results

Natural variation in the ability of *Phasmarhabditis* species to detect slug mucus

There was no significant difference between the numbers of *P. hermaphrodita* (DMG0001) found in the foot mucus of the seven slug species and the control (F (6,

62) = 1.22, $p = 0.30$) (Fig 2A). Low numbers of nematodes were found in each piece of filter paper (range 2-8 nematodes per slug species) and the majority of nematodes (47 ± 3.8) remained in the centre of the plate where they were applied. In contrast to the commercial strain the recently isolated wild isolate of *P. hermaphrodita* (DMG0002) was significantly attracted to mucus from all seven species compared to the control ($p < 0.001$). Specifically, *P. hermaphrodita* (DMG0002) was significantly more attracted to mucus from *D. reticulatum, L. valentiana, A. hortensis* and *A. ater* than mucus from *M. sowerbyi* and *L. flavus* ($p < 0.05$) (Fig 2B). *P. hermaphrodita* DMG0007 (Fig 2C) was significantly more attracted to mucus from all seven slug species compared to the control ($p < 0.001$). It had no preference of mucus from *D. invadens, D. reticulatum, M. sowerbyi, L. flavus, A. hortensis* or *A. ater* ($p > 0.05$) but significantly less nematodes were found in the mucus of *L. valentiana* than *D. invadens* and *A. hortensis* ($p < 0.05$). Similarly, *P. hermaphrodita* DMG0009 (Fig 2D) found the mucus of all seven slug species significantly more attractive than the control ($p < 0.001$). There was no significant difference between the numbers of *P. hermaphrodita* DMG0009 (Fig 2D) found in mucus of the seven slug species ($p > 0.05$) and only the numbers of nematodes found in mucus from *M. sowerbyi* differed with *A. hortensis* ($p < 0.05$). In contrast to the four other isolates of *P. hermaphrodita* there was no significant difference between the numbers of *P. hermaphrodita* DMG0010 (Fig 2E) found in mucus from *D. reticulatum, L. flavus, L. valentiana, A. hortensis, A. ater* and the control ($p > 0.05$). However, there were significantly more *P. hermaphrodita* DMG0010 found in the mucus from *D. invadens* and *M. sowerbyi* compared to the control ($p < 0.001$).

There was no significant difference between the numbers of *P. californica* DMG0018 exposed to mucus from *D. invadens* and *L. flavus* and the control (Fig 2F;

$p > 0.05$) but significantly more nematodes were found in mucus from *D. reticulatum*, *M. sowerbyi*, *L. valentiana*, *A. ater*, *A. hortensis* than the control ($p < 0.05$). Significantly more *P. californica* DMG0018 were found in the mucus from *D. reticulatum* and *M. sowerbyi* compared to *L. valentiana, L. flavus* and *A. ater* ($p < 0.05$). In contrast to *P. californica* DMG0018, strain DMG0019 (Fig 2G) behaved differently and there was no significant difference between the numbers of *P. californica* DMG0019 found in mucus from *D. invadens, D. reticulatum, L. flavus, L. valentiana, A. hortensis* and the control. However, significantly more *P. californica* DMG0019 were found in the mucus of *M. sowerbyi* and *A. ater* compared to the control ($p < 0.001$).

There was no significant difference between the numbers of *P. neopapillosa* DMG0016 found in mucus from *D. invadens, D. reticulatum, L. flavus, L. valentiana, A. hortensis, A. ater* and the control (Fig 2H) ($p > 0.05$). However, there were significantly more *P. neopapillosa* DMG0016 found in mucus from *M. sowerbyi* compared to the control ($p < 0.05$). There were significantly more *P. neopapillosa* DMG0015 (Fig 2I) found in the mucus from all seven slug species compared to the control ($p <0.05$). *P. neopapillosa* DMG0015 showed a clear preference for *A. hortensis* with significantly more nematodes found in the mucus from this species compared to the other species ($p < 0.05$). Similarly, there were significantly more *P. neopapillosa* DMG0012 (Fig 2J) found in the mucus of six slug species (*D. invadens, D. reticulatum, L. flavus, M. sowerbyi, A. hortensis* and *A. ater*) and the control ($p < 0.05$), but there was no difference between the numbers of nematodes in the control and in the mucus of *L. valentiana* ($p > 0.05$). Of these six slugs species *A. hortensis* and *A. ater* were the most attractive with significantly more *P. neopapillosa* DMG0012 found in their mucus compared to the other four slug species ($p < 0.05$).

The fecundity of *Phasmarhabditis* species fed on each slug species

There was a significant difference between the numbers of offspring produced on the seven slug species by *P. hermaphrodita* DMG0001 (F (6, 66) = 4.195, p = 0.001). The highest numbers of offspring were produced on *L. flavus*, which was significantly greater than *A. hortensis* and *L. valentiana* ($p < 0.05$) (Fig 3A). *P. hermaphrodita* DMG0007 also grew well on all slug species but produced the most amount of offspring on *L. flavus* and *A. ater* which was significantly different than the other species ($p < 0.05$) (Fig 3C). *P. hermaphrodita* DMG0010 also grew best on *L. flavus*, *A. ater* and *M. sowerbyi* which were significantly greater than all slug species ($p < 0.05$) (Fig 3E). There was no difference between the numbers of offspring that were produced by *P. hermaphrodita* DMG0002 or DMG0009 on each slug species (Fig 3B, D) ($p > 0.05$).

The numbers of offspring that were produced by *P. californica* DMG0018 was significantly different depending on slug species (F (6, 64) = 3.349, p = 0.007) (Fig 3F). The largest numbers of offspring were produced on *L. flavus*, which was significantly different from *D. invadens*, *A. hortensis* and *A. ater* ($p < 0.05$). In contrast, there were no significant differences between the numbers of offspring that were produced on any of the slug species when a single *P. californica* DMG0019 was added to the seven slug species (F (6, 63) = 1.218, p = 0.310) (Fig 3G).

The relationship between chemotaxis and number of offspring produced on each slug species

There was no significant relationship between the chemotactic response of *P. hermaphrodita* DMG0001 (r^2 = 0.006, p = 0.43), *P. hermaphrodita* DMG0002 (r^2 = 0.001, p = 0.47), *P. hermaphrodita* DMG0007 (r^2 = 0.014, p = 0.39), *P. hermaphrodita* DMG0009 (r^2 = 0.402, p = 0.06), *P. hermaphrodita* DMG0010 (r^2 = 0.151, p = 0.19), *P. californica* DMG0018 (r^2 = 0.008, p = 0.42) or *P. californica* DMG0019 (r^2 = 0.057, p = 0.30) responding to slug mucus and numbers of offspring that were produced on 0.1 g each slug species. There was also no significant relationship between the chemotactic response of *P. hermaphrodita* DMG0001 (r^2 = 0.01, p = 0.42), *P. hermaphrodita* DMG0002 (r^2 = 0.247, p = 0.13), *P. hermaphrodita* DMG0007 (r^2 = 0.025, p = 0.37), *P. hermaphrodita* DMG0009 (r^2 = 0.17, p = 0.18), *P. hermaphrodita* DMG0010 (r^2 = 0.058, p = 0.3), *P. californica* DMG0018 (r^2 = 0.103, p = 0.24) or *P. californica* DMG0019 (r^2 = 0.027, p = 0.36) and overall numbers of offspring that were calculated to be produced on each slug species at their adult mean weight.

Discussion

Natural variation in behaviour towards host cues or food has been shown in many nematodes. In *C. elegans* there is natural variation in avoidance behaviour of many strains exposed to pathogenic *Bacillus thuringiensis* (Schulenburg & Muller, 2004) and the ability to cope with pathogenic *Serratia marcescens* (Schulenburg & Ewbank, 2004). The scarab beetle associated nematode *P. pacificus* displays natural variation in chemoattraction towards beetle pheromones (Hong et al., 2008b) and pheromones produced to initiate dauer formation (Mayer & Sommer, 2011). We found that *P. hermaphrodita* DMG0010 preferred mucus from *D. invadens* and *M.*

sowerbyi compared to the other five species. Whereas *P. hermaphrodita* DMG0002 disliked *M. sowerbyi* and *L. flavus* and *P. hermaphrodita* DMG0007 disliked mucus from *L. valentiana*. However, some *P. hermaphrodita* strains such as DMG0007 and DMG0009 (and the commercial strain DMG0001) had very little preference for any of the seven slug species. Interestingly, the commercial strain of *P. hermaphrodita* (DMG0001) responded poorly to mucus from all slug species with the majority of nematodes staying at the point of application. This strain has been in industrial production since 1994 fed on a monoxenic diet of *Moraxella osloensis* - a bacterium that was initially chosen as it produced the greatest number of virulent nematodes (Wilson et al., 1994, Wilson et al., 1995). Research using this same strain 6 to 10 years ago reported strong chemoattraction towards *D. reticulatum*, *A. subfuscus* and *D. invadens* (Hapca et al., 2007a, Nermut et al., 2012, Rae et al., 2009, Small & Bradford, 2008), which was not observed in this study. In fact the majority of nematodes remained at the point of application. Perhaps there is in house lab evolution due to the constant culturing conditions that have not changed for decades? Culturing at constant temperatures with the same food source and no interaction with the environment has been shown to increase deleterious mutations in other nematode species (Huey & Rosenzweig, 2009). For example, continued lab cultivation (since 1944) of the Dougherty strain of *Caenorhabditis briggsae* fed on the bacterium *Escherichia coli* and kept at constant temperatures resulted in defects in movement, chemotaxis and the ability to respond to dauer pheromones (Fodor et al., 1983) due to mutations in the G-protein coupled receptor genes *srg-36* and *srg-37* (McGrath et al., 2011). However, it must be noted that even if there was some sort of within lab evolution ongoing with the continued culturing conditions of *P. hermaphrodita* DMG0001 it remains as virulent as 10 years ago (Williams & Rae, 2015).

The commercial strain of *P. hermaphrodita* (DMG0001) can kill *D. invadens*, *D. reticulatum* and *M. sowerbyi* (Rae et al., 2009, Wilson et al., 1993) but it cannot kill *L. valentiana*, *A. hortensis*, *L. flavus* or large *A. ater* (Dankowska, 2006, Grewal et al., 2003, Iglesias & Speiser, 2001, Rae et al., 2008). Those that it cannot kill represent necromenic hosts where they penetrate inside and wait for the hosts to die when they can then reproduce (Schulte, 1989). Necromenic hosts offer the advantage of allowing the nematode protection from the environment and to be transported to new ecological niches. The reasons for chemotactic preferences of the nematodes towards parasitic or necromenic hosts remains elusive and does not seem to be due to the numbers of offspring that can be produced on each species; which has been reported previously (Rae et al., 2009) (although they monitored numbers of dauer juveniles). However, we did not test the fitness of these offspring. Perhaps they were more pathogenic to slugs; had increased longevity or an increased fat content aiding survival in soil?

As well as differences in *P. hermaphrodita* chemoattraction, *P. neopapillosa* and *P. californica* also showed preferences for different slug species. In general, there is little known about the basic biology of *P. neopapillosa* (Hooper et al., 1999) and nothing known about *P. californica*, which has been recently described from north America (Tandingan De Ley et al., 2016) and recently found in Ireland (Carnaghi et al., 2017) and Wales (Andrus & Rae, 2018). We could show that there were striking differences between the chemotaxis responses of two strains of *P. californica*. Both strains were isolated from a single specimen of *Oxychilus draparnaudi* from Dale, Wales yet *P. californica* DMG0018 preferred *M. sowerbyi* and *D. reticulatum* whereas the majority of *P. californica* DMG0019 did not move and remained the

point of application. This shows that closely related strains of the same species can have drastically divergent chemoattraction profiles and preferences for slug hosts.

Studies in *C. elegans* have managed to identify many genes, neurons and neuropeptides that are essential for chemotaxis towards bacteria, associated metabolites and alcohols (Bargmann, 2006). In addition, in more distantly related species (*P. pacificus*) the protein kinase EGL-4 has been implicated in controlling chemoattraction behaviour towards insect pheromones (Hong et al., 2008b). As *P. hermaphrodita* has been proposed as a genetic model (Rae, 2017, Wilson et al., 2015) currently being developed (Andrus & Rae, 2018) the molecular mechanisms of ecologically relevant genes essential for locating hosts could be identified.

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Figure Legends

Fig 1. A diagram of the chemotaxis assay used to test the behaviour of *Phasmarhabditis* nematodes exposed to mucus from seven species (and a control) simultaneously. A 10 cm Petri dish was half filled with 1.2% technical agar and 0.01g of foot mucus was swabbed from *Arion ater* (AA), *M. sowerbyi* (MS), *L. valentiana* (LV), *L. flavus* (LF), *D. invadens* (DP), *D. reticulatum* (DR) and *A. hortensis* (AH) using a 1 cm^2 piece of Whatman filter paper. A control (C) consisted of the filter paper with 10 µl of distilled water. Nematodes were applied to the center of the plate (marked "X").

Fig 2. The mean number of *P. hermaphrodita* DMG0001 (A), DMG0002 (B), DMG0007 (C), DMG0009 (D), DMG0010 (E); *P. californica* DMG0018 (F) and DMG0019 (G), *P. neopapillosa* DMG0016 (H), DMG0015 (I) and DMG0012 (J) exposed to mucus of seven species of slug. Fisher's Least Significant Difference (LSD) at 5% ($p < 0.05$) was calculated for each strain.

Fig 3. The mean number of *P. hermaphrodita* DMG0001 (A), DMG0002 (B), DMG0007 (C), DMG0009 (D), DMG0010 (E); *P. californica* DMG0018 (F) and DMG0019 (G) that were produced on 0.1 g of each of the seven slug species. Bars represent ± one standard error.