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

J. Cutler and R. Rae

School of Biological and Environmental Sciences, Liverpool John Moores University, Byrom Street, Liverpool L33AF, UK

Abstract

The gastropod parasitic nematode Phasmarhabditis hermaphrodita has been formulated into a successful biological control agent (Nemaslug®, strain DMG0001) used to kill slugs on farms and gardens. When applied to soil, P. hermaphrodita uses slug mucus and faeces to find potential hosts. However, there is little information on what cues other species of Phasmarhabditis (P. neopapillosa and P. californica) use to find hosts and whether there is natural variation in their ability to chemotax to host cues. Therefore, using chemotaxis assays, we exposed nine wild isolates of P. hermaphrodita, five isolates of P. neopapillosa and three isolates of P. californica to mucus from the pestiferous slug host Deroceras invadens, as well as 1% and 5% hyaluronic acid – a component of slug mucus that is highly attractive to these nematodes. We found P. hermaphrodita (DMG0010) and P. californica (DMG0018) responded significantly more to D. invadens mucus and 1% hyaluronic acid than other strains. Also, P. hermaphrodita (DMG0007), P. neopapillosa (DMG0015) and P. californica (DMG0017) were superior at locating 5% hyaluronic acid compared to other isolates of the same genera. Ultimately, there is natural variation in chemoattraction in Phasmarhabditis nematodes, with some strains responding significantly better to host cues than others.

Introduction

Parasitic nematodes use many diverse cues exuded from hosts to ensure successful infection (Lee, 2002). For example, Howardula aoronymphium parasitizes the mushroom fly (Drosophila fallen) and is attracted to mushroom-derived odorants such as 3-octanone and 1-octen-3-one (Cevallos et al., 2017). The mammalian parasite Heligmosomoides polygyrus is attracted to mouse faeces and compounds such as geranyl acetone and 2-butanone and averted from carbon dioxide (Ruiz et al., 2017). Entomopathogenic nematodes (Steinernema carpocapsae and Heterorhabditis bacteriophora), which are lethal pathogens of insects, are attracted to different host-derived compounds. For example, Hallem et al. (2011) found S. carpocapsae was attracted to propanoic acid and hexanal, 2,3-butanedione (as well as others), yet H. bacteriophora was only attracted to 1-propanol. The relationships between olfaction of parasitic nematodes and hosts are poorly understood (Cevallos et al., 2017); hence, we are attempting to develop a model nematode (Rae, 2017) to elucidate the molecular nature of host location by parasites. The model we propose is the slug and snail parasite Phasmarhabditis hermaphrodita, which has been successfully formulated into a biological control agent (Nemaslug®) to kill pestiferous gastropods on farms and gardens (Rae et al., 2007). Nematodes are mixed with water and sprayed on to soil where they locate and kill slugs in 4–21 days (Wilson et al., 1993; Tan & Grewal, 2001). The host cues P. hermaphrodita uses to find gastropods have been studied in detail. In agar-based chemotaxis assays, the nematodes positively chemotax towards slug and snail mucus and faeces (Rae et al., 2006, 2009; Hapca et al., 2007a, b; Andrus et al., 2018; Andrus & Rae, 2019a). They have also been shown to be attracted to alive and dead slugs in soil-based experiments (MacMillan et al., 2009; Nermut et al., 2012). Mucus from some slug species is more attractive to P. hermaphrodita than others. More specifically, Rae et al. (2009) showed that P. hermaphrodita was strongly attracted to mucus from slugs such as Arionfuscus, but less attracted to Limax marginatus and the snail Cepaea hortensis (for reasons unknown). Small & Bradford (2008) recorded a selection of behaviours when P. hermaphrodita came in contact with mucus from six gastropod species, including forward crawling, head thrusting and head waving, but observed few differences between species. In order to investigate the specific components of slug mucus that the nematodes are attracted to, Andrus et al. (2018) showed that Phasmarhabditis nematodes were strongly attracted to hyaluronic acid – a common component of slug mucus, in a dose-dependent manner.
The majority of chemotaxis experiments using *P. hermaphrodita* have concentrated on studying one strain, designated ‘DMG0001’ by Hooper et al. (1999), which has been used in commercial production by BASF Agricultural Specialities since 1994 and sold as Nemaslug®. There is little information about whether there is natural variation in chemotaxis of *P. hermaphrodita* but any of the 12 other species in the *Phasmarhabditis* genus (*P. bohemicus, P. circassica, P. clausiliae, P. meridonialis, P. apuliae, P. bonaquaense, P. californica, P. safricana, P. huizhouensis, P. neopapillosa, P. papillosa* and *P. tawfiki*) (Tandingan De Ley et al., 2017; Ivanova et al., 2020). One study (Andrus & Rae, 2019a) reported natural variation in the chemoattraction of *P. hermaphrodita, P. californica* and *P. neopapillosa* to certain slug species. For example, *P. hermaphrodita* isolates differed in their preference of slug species but *P. neopapillosa* preferred *Arion* sp. However, there is little information about whether these species differ in their response to mucus from a single slug species or other attractants like hyaluronic acid.

From sampling and dissecting slugs from the UK, we have isolated numerous strains of *P. hermaphrodita, P. californica* and *P. neopapillosa*, which are in culture at Liverpool John Moores University (LJMU) (for further information, see Andrus & Rae, 2019b). To understand whether there is natural variation in the ability of these nematodes to chemotax to slug mucus and associated cues, we exposed nine isolates of *P. hermaphrodita* (including the commercial strain DMG0001), five isolates of *P. neopapillosa* and three isolates of *P. californica* to mucus from the pestiferous slug *Deroceras invadens* (Hutchinson et al., 2014), which is a common host for these nematodes (Andrus & Rae, 2019b), as well as two concentrations of hyaluronic acid (1% and 5%). Ultimately, this will unravel whether behavioural mechanisms responsible for host chemoattraction in the *Phasmarhabditis* genus are under selective pressure.

### Materials and methods

#### Source of invertebrates

*Phasmarhabditis hermaphrodita* (strains DMG0002, DMG0003, DMG0005, DMG0007, DMG0008, DMG0009, DMG010 and DMG011), *P. neopapillosa* (strains DMG012, DMG013, DMG014, DMG015 and DMG016) and *P. californica* (strains DMG017, DMG018 and DMG019) were isolated from slugs from a small survey conducted in 2014 around the UK, and associated cues, we exposed nine isolates of *P. hermaphrodita* from the pestiferous slug *Deroceras invadens* (Hutchinson et al., 2014), which is a common host for these nematodes (Andrus & Rae, 2019b), as well as two concentrations of hyaluronic acid (1% and 5%). Ultimately, this will unravel whether behavioural mechanisms responsible for host chemoattraction in the *Phasmarhabditis* genus are under selective pressure.

#### Chemotaxis assay

A chemotaxis assay was used to assess the ability of *Phasmarhabditis* nematodes to locate slug mucus and hyaluronic acid (Rae et al., 2006, 2009; Andrus et al., 2018). Briefly, 10 cm Petri dishes were half filled with 1.2% technical agar and left to solidify. A 1 cm² piece of Whatman number 1 filter paper was placed 0.5 cm from the edge of the Petri dish and 10 µl of distilled water was added and acted as the control. Another piece of filter paper (same size) was placed equidistant from the control after being used to swab 0.01 g of mucus from adult *D. invadens* (or 10 µl of 1% or 5% hyaluronic acid was added). Approximately 50 dauer larvae of each *Phasmarhabditis* isolate were added to the middle of each Petri dish. They were then sealed with Parafilm® and stored at 20°C overnight. The following morning, the numbers of nematodes that had graduated to the control or treated piece of filter paper were quantified (as well as those that remained in the middle). For each isolate of *Phasmarhabditis*, three Petri dishes were used to test the response to *D. invadens* mucus or hyaluronic acid (1% or 5%) and each experiment was repeated three times (nine dishes per isolate).

### Statistical analysis

The numbers of *Phasmarhabditis* nematodes found in the mucus or hyaluronic acid (1% or 5%) versus water was analysed using a Student’s *t* test. To understand which *Phasmarhabditis* strain responded strongest to mucus or hyaluronic acid (1% or 5%), the numbers of nematodes found in each treatment from each species was compared using a one-way analysis of variance with Tukey’s post-hoc test.

### Results

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

There were significantly more *P. hermaphrodita* (DMG0001, DMG0003, DMG0005, DMG0007, DMG0008, DMG0009, DMG010 and DMG011), *P. neopapillosa* (DMG012, DMG013, DMG014, DMG015 and DMG016) and *P. californica* (DMG017, DMG018 and DMG019) found in the *D. invadens* mucus compared to the water control (fig. 1) (*P < 0.05*). However, this was not the case for *P. hermaphrodita* (DMG0002), as there was no significant difference in the number of nematodes found on each side (fig. 1) (*P > 0.05*).

Of all the *P. hermaphrodita* isolates tested, isolate DMG010 chemotaxed significantly more to *D. invadens* mucus compared to *P. hermaphrodita* (DMG0001, DMG0002, DMG0003, DMG0005, DMG0008, DMG0009 and DMG011) (*P < 0.05*) (fig. 1).

There was no difference in the numbers of *P. neopapillosa* (DMG012, DMG013, DMG014, DMG015 and DMG016) that were found in the *D. invadens* mucus (*P > 0.05*) (fig. 1). Significantly more *P. californica* (DMG018) were found in the *D. invadens* mucus than *P. californica* (DMG019) (*P > 0.05*) (fig. 1), but no difference was found between *P. californica* (DMG017) (*P < 0.05*).

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

*Phasmarhabditis hermaphrodita* (DMG0001, DMG0002, DMG0007, DMG0008, DMG0009 and DMG011) responded positively to 1% hyaluronic acid, with significantly more nematodes found in the treated filter paper compared to the control (*P < 0.05*) (fig. 2a). However, there was no difference between the numbers of *P. hermaphrodita* (DMG0010, DMG0003 and DMG0005) in the 1% hyaluronic acid compared to the water...
control ($P > 0.05$) (fig. 2a). Although there was no significant difference between the numbers of *P. hermaphrodita* (DMG0010) in the mucus and water, this strain reacted more to the 1% hyaluronic acid than *P. hermaphrodita* (DMG0002, DMG0003 and DMG0008) ($P < 0.05$) (fig. 2a). Significantly more *P. neopapillosa* (DMG0012, DMG0013 and DMG0015) were found in the 1% hyaluronic acid compared to the control ($P < 0.05$) (fig. 2a); however, there was no significant difference between the numbers of *P. neopapillosa* (DMG0014 and DMG0016) in the hyaluronic acid or water control ($P > 0.05$) (fig. 2a). Of the *P. neopapillosa* strains tested, significantly more *P. neopapillosa* (DMG012) were found in the 1% hyaluronic acid than *P. neopapillosa* (DMG0016) ($P < 0.05$) (fig. 2a). There was no difference between the numbers of *P. neopapillosa* (DMG0013, DMG0014 and DMG0015) found in 1% hyaluronic acid ($P > 0.05$) (fig. 2a).

Only *P. californica* (DMG0018) significantly chemotaxed to 1% hyaluronic acid compared to water ($P < 0.05$) (fig. 2a). There was no difference between the numbers of *P. californica* (DMG0017 and DMG0019) found on each side ($P > 0.05$) (fig. 2a). There was no difference in the numbers of *P. californica* (DMG0017, DMG0018 and DMG0019) found in the 1% hyaluronic acid ($P > 0.05$) (fig. 2a).

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

When exposed to 5% hyaluronic acid, all isolates of all *Phasmarhabditis* species tested were found significantly more in the hyaluronic acid compared to the water control ($P < 0.05$) (fig. 2b). Significantly more *P. hermaphrodita* (DMG0007) were found in 5% hyaluronic acid compared to *P. hermaphrodita* (DMG0002, DMG0003, DMG0008 and DMG0011) ($P < 0.05$) (fig. 2b). Also, there were significantly more *P. hermaphrodita* (DMG0009) found in the 5% hyaluronic acid than *P. hermaphrodita* (DMG0002 and DMG0011) ($P < 0.05$) (fig. 2b).

From the *P. neopapillosa* isolates tested, strain DMG0015 was found significantly more in the 5% hyaluronic acid compared to the others (DMG0012, DMG0013, DMG0014 and DMG0016) ($P < 0.05$) (fig. 2b). This was also the case with the *P. californica* isolates tested, where one strain (DMG0017) was found significantly more in the 5% hyaluronic acid compared to the others (DMG0018 and DMG0019) ($P < 0.01$) (fig. 2b).

**Discussion**

When exposed to mucus from *D. invadens*, *P. hermaphrodita* (DMG010) and *P. californica* (DMG0018) were found significantly more in *D. invadens* mucus than the other strains tested, suggesting that they are perhaps better at finding hosts. There was also natural variation in chemotaxis observed when we repeated the experiments with hyaluronic acid (1% and 5%), which is a common attractant for other parasites such as the trematode *Acanthostomum brauni* and the protozoan *Plasmodium falciparum* (Haas & Ostrowskide de Núñez, 1988; Beeson et al., 2000). At 1% hyaluronic acid, significantly more *P. hermaphrodita* (DMG0010), *P. neopapillosa* (DMG0012) and *P. californica* (DMG0018) were found in the attractant than the other strains of the same genus. When given the choice of 5%
hyaluronic acid or water, *P. hermaphrodita* (DMG0007), *P. neopapillosa* (DMG0015) and *P. californica* (DMG0017) were found significantly more in the attractant than other strains of the same genus. Natural variation in chemoattraction has been shown in parasitic and genetic model nematodes. For example, Laznik & Trdan (2013) demonstrated differences in attraction

**Fig. 2.** The mean number of *Phasmarhabditis hermaphrodita* (DMG0001, DMG0002, DMG0003, DMG0005, DMG0007, DMG0008, DMG0009, DMG0010 and DMG0011), *P. neopapillosa* (DMG0012, DMG0013, DMG0014, DMG0015 and DMG0016) and *P. californica* (DMG0017, DMG0018 and DMG0019) found in filter paper with water (control) (white bars), in the middle of the agar plate (black bars) or in 1% (A) or 5% (B) hyaluronic acid (grey bars). Significant differences between the number of nematodes found in hyaluronic acid or water are denoted with * for $P < 0.05$ and ** for $P < 0.001$. Different letters denote a significant difference at $P < 0.05$ between the strains of each *Phasmarhabditis* species found in hyaluronic acid. Bars represent ± one standard error.
to damaged maize-derived β-caryophyllene by *H. bacteriophora* strain D54 and *S. carpocapsae* B49, but Steinernema kraussei C46 only showed weak attraction and *Steinernema feltiae* (two strains) exhibited no attraction. Strains of *Caenorhabditis elegans* differ in their evasion response when exposed to the pathogen *Bacillus thuringiensis* (Schulenburg & Müller, 2004). Also, Hong et al. (2008) reported natural variation in chemotaxis of 19 strains of the scarab-beetle-associated nematode *Pristionchus pacificus* exposed to insect pheromones E11-tetradecenyl acetate (EDTA) and Z-7-tetradec-2-one (ZTDO). Similarly, McGaughan et al. (2013) reported significant variation in the chemotraction of 21 *P. pacificus* strains to organic compounds, beetle washes and live beetles from several island locations.

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

The relationships between olfaction of parasitic nematodes and hosts warrants further attention (Cevallos et al., 2017). We propose to address this using our model nematode *P. hermaphroditida*. Although understudied in terms of genetics and genomics, it has a plethora of attributes that make it an excellent model for olfaction of parasitic nematodes. First, it can be isolated easily, facilitating analysis of natural variation at the inter- and intra-species level. Second, it is a facultative parasite yet can be kept in culture on agar plates or under semi-natural conditions. IP address: 15 Dec 2021 at 07:06:25

**Acknowledgements.** We are grateful to Tom Goddard, Jack Shepherd, Craig Wilding and Will Swaney for their discussions.

**Financial support.** This research was funded by BASF Agricultural Specialties.

**Conflicts of interest.** None.

**References**

Andrus P and Rae R (2019a) Natural variation in chemotraction of *Phasmarhabditis hermaphrodita*, *Phasmarhabditis neoapapillosa* and *Phasmarhabditis californica* exposed to slug mucus. *Nematology* 21, 479–488.

Andrus P and Rae R (2019b) Development of *Phasmarhabditis hermaphrodita* (and members of the *Phasmarhabditis* genus) as new genetic model nematodes to study the genetic basis of parasitism. *Journal of Helminthology* 2, 1–13.

Andrus P, Ingle O, Coleman T and Rae R (2018) Gastropod parasitic nematodes (*Phasmarhabditis* sp.) are attracted to hyaluronic acid in snail mucus by cGMP signalling. *Journal of Helminthology* 94, e9.

Bargmann CI, Hartwig E and Horvitz HR (1993) Odorant-selective genes and neurons mediate olfaction in *C. elegans*. *Cell* 74, 515–527.

Beeson JG, Rogerson SJ, Cooke BM, Reeder JC, Chai W, Lawson AM, Molyneux ME and Brown GV (2000) Adhesion of *Plasmodium falciparum*-infected erythrocytes to hyaluronic acid in placental malaria. *Nature Medicine* 6, 86–90.

Cevallos JA, Okubo RP, Perlman SJ and Hallem EA (2017) Olfactory preferences of the parasitic nematode *Nemataella orosorum* and its insect host *Drosophila* fallinii. *Journal of Chemical Ecology* 43, 362–373.

Haas W and Ostrowskide de Núñez M (1988) Chemical signals of fish skin for the attachment response of *Actinostomum brunnia* cercariae. *Parasitological Research* 74, 552–557.

Hallem EA, Dillman AR, Hong AV, Zhang Y, Yano JM, DeMarco SF and Sternberg PW (2011) A sensory code for host seeking in parasitic nematodes. *Current Biology* 21, 377–383.

Hapca S, Budha P, Crawford J and Young I (2007a) Movement of *Phasmarhabditis hermaphrodita* nematode in heterogeneous structure. *Nematology* 95, 731–738.

Hapca S, Crawford J, Rae R, Wilson M and Young I (2007b) Movement of the parasitic nematode *Phasmarhabditis hermaphrodita* in the presence of the slug *Deroceras reticulatum*. *Biological Control* 41, 223–229.

Hong RL, Witte H and Sommer BJ (2008) Natural variation in *Pristionchus pacificus* insect pheromone attraction involves the protein kinase EGL-4. *Proceedings of the National Academy of Sciences of the United States of America* 105, 7779–7784.

Hooper DJ, Wilson MJ, Rowe JA and Glen DM (1999) Some observations on the morphology and protein profiles of the slug parasitic nematodes *Phasmarhabditis hermaphrodita* and *P. neapapillosa* (Nematoda: *Rhabditidae*). *Nematology* 1, 173–182.

Hutchinson J, Reise H and Robinson D (2014) A biography of an invasive terrestrial slug: the spread, distribution and habitat of *Deroceras invadens*. *Neobiota* 23, 17–64.

Ivanova ES, Geraskina AP and Spiridonov SE (2020) Two new species of *Phasmarhabditis* Andrässy, 1976 (Nematoda: Rhabditidae) associated with land snails in Northwest Caucasus, Russian Federation: description and molecular affiliation. *Nematology* 22, 179–197.

Kroetz SM, Srinivasan J, Yaghoobian J, Sternberg PW and Hong RL (2012) The cGMP signalling pathway affects feeding behaviour in the necromenic nematode *Pristionchus pacificus*. *PLoS One* 7, e34464.

Laznik Ž and Trdan S (2013) An investigation on the chemotactic responses of different entomopathogenic nematode strains to mechanically damaged maize root volatile compounds. *Experimental Parasitology* 134, 349–355.

Lee D (2002) Behaviour. pp. 369–388 in Lee D (Ed) *The biology of nematodes*. Boca Raton, CRC Press.

MacMillan K, Haukeland S, Rae RG, Young IM, Crawford JW, Hapca S and Wilson MJ (2009) Dispersal patterns and behaviour of the nematode *Phasmarhabditis hermaphrodita* in mineral soils and organic media. *Soil Biology and Biochemistry* 41, 1483–1490.
McGaughran A, Morgan K and Sommer RJ (2013) Natural variation in chemosensation: lessons from an island nematode. Ecology and Evolution 3, 5209–5224.

Nermut J, Půža V and Mráček Z (2012) The response of Phasmarhabditis hermaphrodita (Nematoda: Rhabditidae) and Steinernema feltiae (Nematoda: Steinernematidae) to different host-associated cues. Biological Control 61, 201–206.

Rae R (2017) Phasmarhabditis hermaphrodita – a new model to study the genetic evolution of parasitism. Nematology 19, 375–387.

Rae RG, Robertson JF and Wilson MJ (2006) The chemotactic response of Phasmarhabditis hermaphrodita (Nematoda: Rhabditidae) to cues of Deroceras reticulatum (Mollusca: Gastropoda). Nematology 8, 197–200.

Rae R, Verdun C, Grewal PS, Robertson JF and Wilson MJ (2007) Biological control of terrestrial molluscs using Phasmarhabditis hermaphrodita: progress and prospects. Pest Management Science 63, 1153–1164.

Rae RG, Robertson JF and Wilson MJ (2009) Chemotaxis and host preference of the gastropod parasitic nematode Phasmarhabditis hermaphrodita. Journal of Parasitology 95, 517–526.

Ruiz F, Castelletto ML, Gang SS and Hallem EA (2017) Experience-dependent olfactory behaviors of the parasitic nematode Heligmosomoides polygyrus. PLoS Pathogens 13, e1006709.

Schulenburg H and Müller S (2004) Natural variation in the response of Caenorhabditis elegans towards Bacillus thuringiensis. Parasitology 128, 433–443.

Small R and Bradford C (2008) Behavioural responses of Phasmarhabditis hermaphrodita (Nematoda: Rhabditidae) to mucus from potential hosts. Nematology 10, 591–598.

Tan L and Grewal PS (2001) Infection behaviour of the rhabditid nematode Phasmarhabditis hermaphrodita to the grey garden slug Deroceras reticulatum. Journal of Parasitology 87, 1349–1354.

Tandingan De Ley I, McDonell R, Paine TD and De Ley P (2017) Phasmarhabditis: the slug and snail parasitic nematodes in North America. pp. 560–578 in Abd-Elgawad MMM, Askary TH and Coupland JC (Eds) Biocontrol agents: entomopathogenic and slug parasitic nematodes. Wallingford, CABI.

White GF (1927) A method for obtaining infective nematode larvae from cultures. Science 66, 302–303.

Williams AJ and Rae R (2015) Susceptibility of the Giant African snail (Achatina fulica) exposed to the gastropod parasitic nematode Phasmarhabditis hermaphrodita. Nematology 17, 122–126.

Wilson MJ, Glen DM and George SK (1993) The rhabditid nematode Phasmarhabditis hermaphrodita as a potential biological control agent for slugs. Biocontrol Science and Technology 3, 503–511.