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

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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 *Arion subfuscus*, 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.

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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 chemoattraction of not just wild strains of *P. hermaphrodita* but any of the 12 other species in the *Phasmarhabditis* genus (*P. bohemica*, *P. circassica*, *P. clausilliae*, *P. meridionalis*, *P. apuliae*, *P. bonaquaense*, *P. californica*, *P. safricana*, *P. huizhouensis*, *P. neopapillosa*, *P. papillosa* 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, DMG0010 and DMG0011), *P. neopapillosa* (strains DMG0012, DMG0013, DMG0014, DMG0015 and DMG0016) and *P. californica* (strains DMG0017, DMG0018 and DMG0019) were isolated from slugs from a small survey conducted in 2014 around the UK, and were positively identified using molecular biology (see Andrus & Rae, 2019b). They have been kept in culture in LJMU fed on dead *Limax flavus* on White traps (White, 1927; Andrus & Rae, 2019b). For the experiments, each nematode isolate was grown on fresh White traps for 21 days until they were at the dauer stage and stored in tissue culture flasks until use. The commercial strain of *P. hermaphrodita* (DMG0001) was purchased from BASF Agricultural Specialities, Littlehampton, UK and stored at 10°C until use. *Deroceras invadens* were collected from Sefton Park in Liverpool and stored in non-airtight plastic boxes lined with moist paper at 15°C until use.

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 cm^2 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, DMG0010 and DMG0011), *P. neopapillosa* (DMG0012, DMG0013, DMG0014, DMG0015 and DMG0016) and *P. californica* (DMG0017, DMG0018 and DMG0019) found in the *D. invadens* mucus compared to the water control (fig. 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 DMG0010 chemotaxed significantly more to *D. invadens* mucus compared to *P. hermaphrodita* (DMG0001, DMG0002, DMG0003, DMG0005, DMG0008, DMG0009 and DMG0011) ($P < 0.05$) (fig. 1).

There was no difference in the numbers of *P. neopapillosa* (DMG0012, DMG0013, DMG0014, DMG0015 and DMG0016) that were found in the *D. invadens* mucus ($P > 0.05$) (fig. 1).

Significantly more *P. californica* (DMG0018) were found in the *D. invadens* mucus than *P. californica* (DMG0019) ($P > 0.05$) (fig. 1), but no difference was found between *P. californica* (DMG0017) ($P < 0.05$).

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

Phasmarhabditis hermaphrodita (DMG0001, DMG0002, DMG0007, DMG0008, DMG0009 and DMG0011) responded positively to 1% hyaluronic acid, with significantly more nematodes found in the treated filter paper compared to the control ($P < 0.05$) (fig. 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

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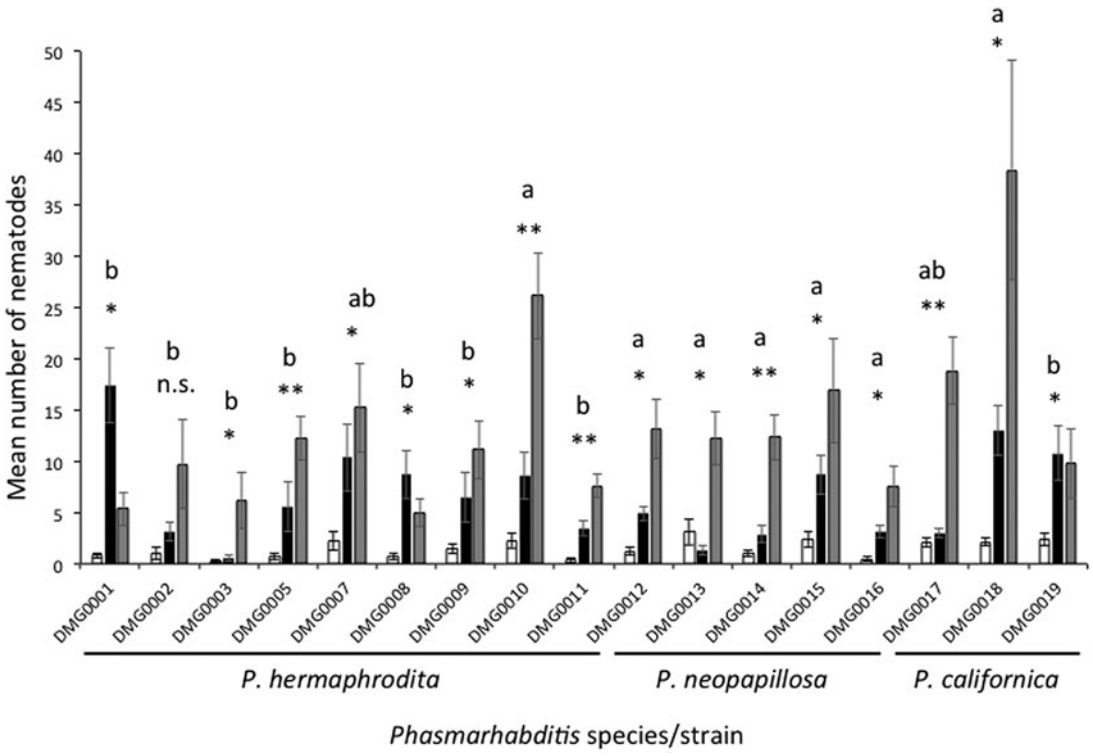

Fig. 1. The mean number of *Phasmarhabditis hermaphrodita* (DMG0001, DMG0002, DMG0003, DMG0005, DMG0007, DMG0008, DMG0009, DMG0010 and DMG0011), *P. neopapillosa* (DMG0012, DMG0013, DMG0014, DMG0015 and DMG0016) and *P. californica* (DMG0017, DMG0018 and DMG0019) found in filter paper with water (control) (white bars), in the middle of the agar plate (black bars) or in filter paper with 0.01 g *Deroceras invadens* mucus (grey bars). Significant differences between the number of nematodes found in the mucus or water are denoted with * for $P < 0.05$ and ** for $P < 0.001$. Different letters denote a significant difference at $P < 0.05$ between the strains of each *Phasmarhabditis* species found in mucus. Bars represent ± one standard error.

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%

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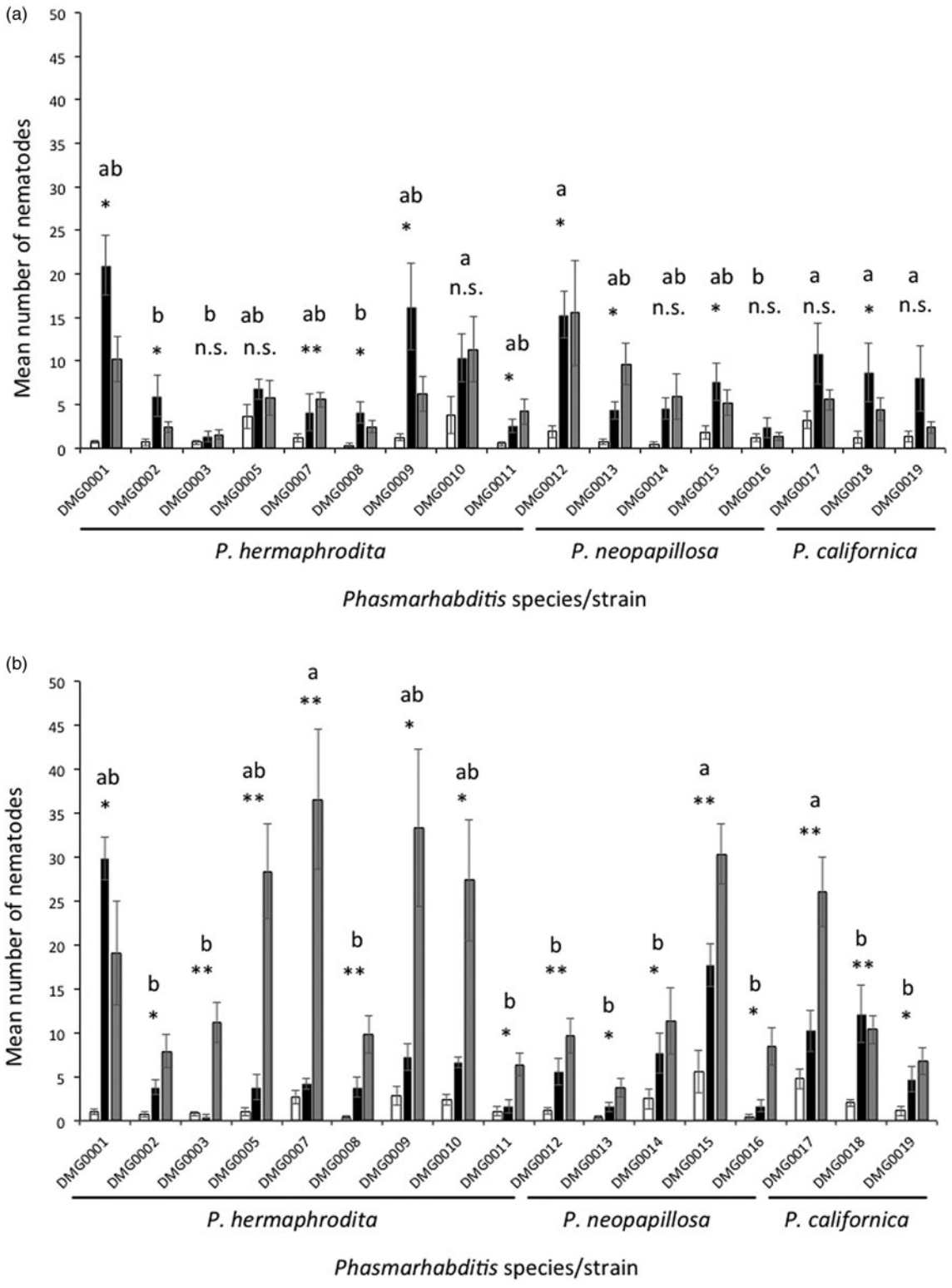

Fig. 2. The mean number of *Phasmarhabditis hermaphrodita* (DMG0001, DMG0002, DMG0003, DMG0005, DMG0007, DMG0008, DMG0009, DMG0010 and DMG0011), *P. neopapillosa* (DMG0012, DMG0013, DMG0014, DMG0015 and DMG0016) and *P. californica* (DMG0017, DMG0018 and DMG0019) found in filter paper with water (control) (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.

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

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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 *E*-11-tetradecenyl acetate (EDTA) and *Z*-7-tetradece-2-one (ZTDO). Similarly, McGaughran *et al.* (2013) reported significant variation in the chemoattraction of 21 *P. pacificus* strains to organic compounds, beetle washes and live beetles from several island locations.

The strain of *P. hermaphrodita* (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. hermaphrodita* has diminished or perhaps the population of *D. invadens* used in our study (collected from Liverpool) were more attractive than those from Aberdeen (for some unknown reason). Nevertheless, by taking our approach and testing chemotaxis behaviour, strains with superior host-finding behaviour could be collected and developed as biological control agents.

The relationships between olfaction of parasitic nematodes and hosts warrants further attention (Cevallos *et al.*, 2017). We propose to address this using our model nematode *P. hermaphrodita*. Although understudied in terms of genetics and genomics, it has a plethora of attributes that make it an excellent model for olfaction of parasitic nematodes. First, it can be isolated easily, 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 fed rotting slug (Andrus & Rae, 2019b). Third, there are multiple studies, inspired by chemoattraction work by *C. elegans* (e.g. Bargmann *et al.*, 1993), examining the olfactory response of *P. hermaphrodita* (and closely related *Phasmarhabditis* species) towards slug mucus (Rae *et al.*, 2006, 2009; Hapca *et al.*, 2007a, b; Andrus & Rae, 2019a) and snail mucus (Andrus *et al.*, 2018) using agar plates and in more realistic soil conditions (Nermut' *et al.*, 2012; MacMillan *et al.*, 2009). Specific components of slug mucus – for example, hyaluronic acid – have been shown to be strong attractants to these nematodes (Andrus *et al.*, 2018; this study), which will allow in-depth analysis of how this compound can affect olfaction at a neurobiological and genomic level. Interestingly, there are similarities in the genetic mechanisms *C. elegans* and *P. pacificus* use to find food and hosts, respectively, as they both rely on the protein kinase EGL-4 (Hong *et al.*, 2008; Kroetz *et al.*, 2012). Chemoattraction in *P. hermaphrodita* towards snail mucus was enhanced by exogenous exposure to cyclic guanosine monophosphate, which activates EGL-4 (Andrus *et al.*, 2018). Therefore, we believe *P. hermaphrodita* (and other *Phasmarhabditis* nematodes) could be used as a parasitic comparison to closely related non-parasitic species such as *C. elegans* and *P. pacificus* to examine the evolution of parasitic behaviours at the molecular and neurobiological level.

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Conflicts of interest. None.

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