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Gastropod parasitic nematodes (*Phasmarhabditis* sp.) are attracted to hyaluronic acid in snail mucus by cGMP signalling

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Running head: Chemoattraction of *Phasmarhabditis* nematodes towards snail mucus

Abstract

Phasmarhabditis hermaphrodita is a terrestrial gastropod parasitic nematode that has been formulated into a biological control agent for farmers and gardeners to kill slugs and snails. In order to locate slugs it is attracted to mucus, faeces and volatile cues however, there is no information about whether these nematodes are attracted to snail cues. It is also unknown how wild isolates of *P. hermaphrodita* or different *Phasmarhabditis* species behave when exposed to gastropod cues. Therefore, we investigated whether *P. hermaphrodita* (commercial and wild isolated strains), *P. neopapillosa* and *P. californica* were attracted to mucus from several common snail species (*Cepaea nemoralis*, *Cepaea hortensis*, *Arianta arbustorum* and *Cornu aspersum*). We also examined whether snails (*C. aspersum*) collected from different locations around the U.K. differed in their attractiveness to wild isolates of *P. hermaphrodita*. Furthermore, we also investigated what properties of snail mucus the nematodes were attracted to including hyaluronic acid and metals ($FeSO_4$, $ZnSO_4$, $CuSO_4$ and $MgSO_4$). We found that the commercial strain of *P. hermaphrodita* responded poorly to snail mucus compared to wild isolated strains and *C. aspersum* collected from different parts of the U.K. differed in their attractiveness to the nematodes. We found that *Phasmarhabditis* nematodes were weakly attracted to all metals tested but were strongly attracted to hyaluronic acid. In a final experiment we also showed that pharmacological application of cyclic GMP increased chemoattraction to snail mucus, suggesting that the protein kinase EGL-4 may be responsible for *Phasmarhabditis* sp. chemoattraction.

Keywords: nematodes, chemotaxis, gastropods, parasites, EGL-4, hyaluronic acid

Introduction

Parasitic nematodes detect and respond to specific cues in order to locate and parasitise hosts (Lee, 2002). For example, entomopathogenic nematodes (*Steinernema* and *Heterorhabditis* sp.) respond to odour blends and carbon dioxide emitted by live insect hosts (Dillman et al., 2012). The human parasite *Strongyloides stercoralis* is attracted to skin and sweat odorants (Castelletto et al., 2014). Similarly, *Heligmosomoides polygyrus* (a parasite of rodents) is attracted to sweat odorants, faeces and carbon dioxide (Ruiz et al., 2017). The terrestrial gastropod parasitic nematode *Phasmarhabditis hermaphrodita* is a lethal parasite of several pestiferous slug species (Wilson et al., 1993) and is attracted to slug faeces, mucus and volatiles (Rae et al., 2006; 2009; Hapca et al., 2007a,b; Nermut et al., 2012, Small & Bradford, 2008). *P. hermaphrodita* has been formulated into a biological control agent (Nemaslug®) used to kill slugs and snails across northern Europe (Rae et al., 2007). Nematodes are applied to soil where they seek out hosts, penetrate through the mantle and kill slugs within 4 to 21 days (Wilson et al., 1993, Tan and Grewal, 2001). The nematodes then reproduce on the decaying cadaver and go in search for more hosts (Rae et al., 2009). *P. hermaphrodita* has been shown to successfully protect crops such as lettuce and oilseed rape against slug damage (Wilson & Rae, 2015).

P. hermaphrodita is able to infect and kill many slug species from the families Arionidae, Milacidae, Limacidae and Vaginulidae (Rae, 2017a) and uses mucus, faeces and volatiles to find slugs (Rae et al., 2006; 2009; Hapca et al., 2007a,b; Nermut et al., 2012; Small & Bradford, 2008). However, all of these behavioural studies have concentrated on studying chemoattraction towards slugs and not snails. *P. hermaphrodita* is able to kill several species of snails including juvenile *Cornu*

aspersum (Glen et al., 1996) and adult *Monacha cantiana* and *Cernuella virgata* (Wilson et al., 2000; Coupland, 1995) however; species such as *Arianta arbustorum* and *Cepaea nemoralis* are resistant (Wilson et al., 2000; Williams & Rae, 2016; Rae, 2018). The reasons for their resistance to *P. hermaphrodita* are unknown but it could be due to the presence of the shell, which has the ability to trap, encase and kill nematodes (Rae, 2017b). We decided to investigate whether *P. hermaphrodita* and other *Phasmarhabditis* species were attracted to snail mucus.

All behavioural studies using *P. hermaphrodita* (Rae et al., 2006; 2009; Hapca et al., 2007a,b; Nermut et al., 2012; Small & Bradford, 2008), have concentrated on using one strain (the commercial isolate, designated "DMG0001" by Hooper et al., 1999), which has been in production for over 20 years. There is no information about how wild strains of *P. hermaphrodita* and other *Phasmarhabditis* species respond to gastropod cues such as mucus. Therefore, we utilised a collection of recently isolated wild strains of *P. hermaphrodita* and *Phasmarhabditis* species (including *P. californica* and *P. neopapillosa*) (Andrus & Rae, 2018) to examine their chemoattraction behaviour to snail mucus to see if it differed from the commercial isolate.

It is unknown what properties of gastropod mucus *P. hermaphrodita* nematodes are specifically attracted to. Mucus is used by gastropods for locomotion, lubrication, adhesion, protection and communication (Ng et al., 2013). It is constantly secreted all over the gastropod body and is composed of mainly water (>80%), proteins (proteoglycans and glycoproteins), carbohydrates (glycosaminoglycans- such as hyaluronic acid), lipids, metals and other molecules (Smith et al., 2009; Kubota et al., 1985; Kim, et al., 1996; Sallam et al., 2009; Werneke et al., 2007; Burton, 1965). Therefore, we exposed *Phasmarhabditis* nematodes to a subset of these properties

including metals ($FeSO_4$, $ZnSO_4$, $CuSO_4$ and $MgSO_4$), hyaluronic acid and examined whether heat treatment of mucus (which denatures large glycoproteins) would alter the chemoattraction of the nematodes.

Nematodes are excellent organisms to study the genetic and neurobiological mechanisms that are responsible for behaviour (Rengarajan & Hallem, 2016). Studies using *Caenorhabditis elegans* have identified genes, neurons and neurotransmitters that are essential for chemotaxis and avoidance behaviour towards alcohols, bacteria and different compounds (Bargmann, 2006). Also research using the necromenic nematode *Pristionchus pacificus* (and other *Pristionchus* species), which is associated with scarab beetles, has shown strong chemoattraction to insect pheromones (Hong et al., 2006) due to activation of the protein kinase EGL-4 (Hong et al., 2008). However, the role this gene plays in chemoattraction in other nematodes remains unknown. Therefore, in a final experiment, we also examined whether *Phasmarhabditis* attraction was regulated by the cGMP-dependent protein kinase EGL-4 through manipulation by pharmacological treatment using 8-bromo-cGMP.

Materials and methods

Source of invertebrates

P. hermaphrodita (commercial strain DMG0001-Nemaslug®) was supplied by BASF-Becker Underwood Agricultural Specialities and stored at 15°C before use. Other nematodes used in this study consisted of wild isolated *P. hermaphrodita* strains (DMG0007 and DMG0008), *P. californica* (DMG0019) and *P. neopapillosa* (DMG0014) that are maintained as isogenic lines at Liverpool John Moores

University and have been described elsewhere (see Andrus & Rae, 2018). Snails (*C. nemoralis* and *C. hortensis*) were collected from sand dunes in Formby, Merseyside. A selection of commonly found *C. nemoralis* morphs were collected including pink (0 and 1 bands) and yellow (1 and 5 bands) snails. Only yellow 5 banded *C. hortensis* were found and used in this study. *Cornu aspersum* were collected from Formby, Halifax, Liverpool, Whitby and Thurso. *Arianta arbustorum* were collected from Thurso. Snails were transported back to the lab and fed lettuce *ad libitum* at 15°C until use.

Chemotaxis assay

To assess the behaviour of *Phasmarhabditis* nematodes exposed to snail mucus an agar plate chemotaxis assay was used as in previous studies (Rae et al., 2006, 2009). Briefly, 10 cm Petri dishes were half filled with 1.2% technical agar and left to dry for 48 hours. Using a 1 cm^2 piece of Whatman number 1 filter paper 0.01 g of snail mucus was gently swabbed from the foot of each snail and placed 0.5 cm from the edge of the plate. On the opposing side of the Petri dish 10 µl of distilled water was added to a 1 cm^2 piece of Whatman number 1 filter paper and acted as the control. Approximately 50 dauer stage *Phasmarhabditis* nematodes were added to the middle of the plate and each plate was sealed with Parafilm® and stored at 20°C. The following morning the numbers of nematodes that had graduated to each piece of filter paper and the numbers that remained in the middle of the plate were recorded. Wild strains of *Phasmarhabditis* were sub-cultured by growing them in White traps (described in Andrus & Rae, 2018) where approximately 100 nematodes were added to a rotting piece of *Limax flavus* and left for 28 days until they grew to the dauer

stage and then used in experiments. For each snail species three replicate plates were used and the experiment was repeated three times.

Usually chemotaxis data using nematodes are presented using a chemotaxis index (Bargmann et al., 1993), however this does not take into account the number of nematodes that remained at the point of application and it is sometimes based on very few numbers of nematodes that graduated to the treatment or control, which can be misleading. Therefore, for each experiment we counted (and presented) the numbers of nematodes that moved to the mucus, the control and also those that remained at the point of application. Also, when studying chemotaxis in *C. elegans* 1M sodium azide is added to the treatment and control to stop nematode movement immediately (Bargmann et al., 1993). However, once *P. hermaphrodita* has found mucus it remains there (Rae et al., 2006; 2009; Hapca et al., 2007a), hence there is no need to immobilise them.

Investigating the properties of snail mucus that *Phasmarhabditis* nematodes are attracted to

We attempted to discover what properties of mucus *Phasmarhabditis* sp. were attracted to. To do this we used the same chemotaxis assay described above with modifications. We added four 1 cm^2 pieces of filter paper to each plate and added different concentrations (0, 10, 50 and 100 μM) of each metal ($FeSO_4$, $ZnSO_4$, $MgSO_4$ and $CuSO_4$) to each piece of filter paper. Approximately 50 dauer stage *P. hermaphrodita* (DMG0007), *P. neopapillosa* (DMG0014) or *P. californica* (DMG0019) were added to three replicate plates and the whole experiment was repeated three times. It should be noted that it is unknown whether the higher salt

concentrations may affect the pH of the solutions added to the filter paper. Also we did not use the commercial strain *P. hermaphrodita* (DMG0001) as in previous experiments it consistently remained at the point of application. We also repeated the same set up but exposed the same set of species of *Phasmarhabditis* (*P. hermaphrodita* DMG0007, *P. neopapillosa* DMG0014 and *P. californica* DMG0019) nematodes to sodium hyaluronate (the sodium salt of hyaluronic acid) at four different concentrations (0%, 1%, 5% and 10%).

We also investigated whether any large (unknown) glycoproteins may be involved in the attraction of *Phasmarhabdits* nematodes to snail mucus. Proteins in snail mucus can be denatured using heat treatment. *C. aspersum* mucus was harvested (as previously described), placed into 1.5 ml Eppendorfs and heated at two temperatures (41°C or 82°C) for 45 mins in a heat block. The first treatment (41°C) was used to destroy smaller proteins (>40,000 kDa) present in the mucus (Branden, 1999). The second treatment (82°C) was used to target large glycoproteins found in mucus (>120,000 kDa) (Kubota et al., 1985). The heat-treated filter paper with mucus was then placed on the agar plate (as previously described) and a control piece of filter paper with water and treated at the same temperatures was placed opposite. Three replicate plates were used for each heat-treatment and the experiment was repeated three times with *P. hermaphrodita* (DMG0007), *P. neopapillosa* (DMG0014) and *P. californica* (DMG0019).

Assessment of behaviour of *Phasmarhabditis* nematodes exposed to mucus after pharmacological treatment using 8-bromo-cGMP

Nematodes (*C. elegans* and *P. pacificus*) use the protein kinase EGL-4 to detect cues, which can be activated by treatment with membrane permeable cyclic guanosine monophosphate (8-bromo-cGMP) (Hong et al., 2008; Kroetz et al., 2012). Therefore, we investigated whether treatment of *Phasmarhabditis* nematodes with 8-bromo-cGMP would increase their host seeking ability. We exposed approximately three hundred dauer or adult stage *P. hermaphrodita* (DMG0001) or *P. hermaphrodita* (DMG0007) to 500 μM 8-bromo-cGMP (Sigma-Aldrich) in a 1.5 ml Eppendorf at 20°C (following Hong et al., 2008). Dauers were exposed to 8-bromo-cGMP for 3 hours and adults just 1 hour. After which we briefly washed the nematodes in buffer and applied them to a chemotaxis plate with 0.01 g *C. aspersum* mucus on one side and a water control on the other (as used in the first experiments). In parallel, nematodes were exposed to water and not 8-bromo-cGMP and used in chemotaxis assays as a control. *P. hermaphrodita* (DMG0001) were used in this experiment to investigate whether we could enhance its weak chemoattraction by increasing the activity of EGL-4. Three plates were used and the entire experiment was repeated three times.

Statistical analysis

The number of nematodes found in the snail mucus compared to the water control was compared using a Mann-Whitney U test. The numbers of nematodes found in the mucus from each snail species (or snail location), and in the increasing concentrations of metals and sodium hyaluronate was compared using a Kruskal Wallis test. Statistical analysis was carried out using SPSS 21.

Results

Phasmarhabditis nematodes are attracted to mucus from several snail species

There was a significant difference between the numbers of *P. hermaphrodita* (DMG0001) found in mucus from pink *C. nemoralis* with zero bands (p = 0.023), yellow *C. nemoralis* with 5 bands (p = 0.0007) and *C. aspersum* (p = 0.0035) compared to the water control (Fig 1A). However, there was no significant difference between the numbers of *P. hermaphrodita* (DMG0001) found in mucus of yellow or pink *C. nemoralis* (1 band), *C. hortensis* or *A. arbustorum* and water (p > 0.05; Fig. 1A). In general, very few nematodes (<5) moved towards the mucus, whereas the majority (23-36) were found still at the point of application. In contrast, the recently isolated strain of *P. hermaphrodita* (DMG0007) was more active and attracted to snail mucus with significantly more nematodes found in mucus from yellow *C. nemoralis* 1 band (p = 0.0052) and 5 band (p = 0.0002); pink *C. nemoralis* (0 bands) (p = 0.046), *C. hortensis* (p = 0.0008), *A. arbustorum* (p = 0.0135) and *C. aspersum* (p = 0.0002) compared to water (Fig 1B). There was no significant difference between the numbers of *P. hermaphrodita* (DMG0007) found in mucus from pink *C. nemoralis* (1 band) and water (p = 0.066; Fig 1B).

The numbers of *P. californica* (DMG0019) found in the mucus from pink *C. nemoralis* 0 band (p = 0.007) and 1 band (p = 0.0002), yellow *C. nemoralis* 1 band (p = 0.005) and 5 bands (p = 0.035), *C. hortensis* (p = 0.015) and *C. aspersum* (p = 0.0002) was significantly greater than the number of nematodes found in water (Fig 1C). However, there was no significant difference between the numbers of *P.*

californica (DMG0019) found in mucus from *A. arbustorum* compared to water (p = 0.43; Fig 1C).

Natural variation in chemoattraction of *Phasmarhabditis* nematodes to *C. aspersum* collected from around the U.K.

There was no significant difference between the numbers of *P. hermaphrodita* (DMG0001) found in mucus from *C. aspersum* collected from Formby, Thurso or Liverpool compared to water (p > 0.05; Fig 2A) however, significantly more nematodes were found in mucus of *C. aspersum* collected from Whitby (p = 0.038) and Halifax (p = 0.006) than water (Fig 2A). The majority of nematodes however, were found at the point of application (similar to the previous experiment). In contrast, significantly more *P. hermaphrodita* (DMG0007) were found in the mucus from *C. aspersum* collected from Formby (p = 0.003), Liverpool (p = 0.0002), Whitby (p = 0.002) and Halifax (p = 0.0002) compared to water (Fig 2B). Mucus collected from *C. aspersum* from Formby, Liverpool and Whitby was significantly more attractive to *P. hermaphrodita* (DMG0007) than from snails from Halifax (p < 0.05). There was no difference in the numbers of *P. hermaphrodita* (DMG0007) found in mucus from *C. aspersum* collected from Thurso and water (p = 0.5; Fig 2B).

P. hermaphrodita (DMG0008) were found significantly more in mucus from *C. aspersum* collected from all locations compared to water (p < 0.05; Fig 2C). There was no significant difference between the numbers of nematodes that were found in mucus from all locations (p > 0.05; Fig 2C). In contrast when *P. californica* (DMG0019) was exposed to mucus from *C. aspersum* collected from Formby, Liverpool, Whitby and Thurso there was no significant difference between the

numbers of nematodes found in the mucus compared to water ($p > 0.05$; Fig 2D). However, *P. californica* (DMG0019) were found significantly more in mucus from *C. aspersum* collected from Halifax than water ($p = 0.003$; Fig 2D).

***Phasmarhabditis* nematodes are weakly attracted to metals found in snail mucus**

Significantly more *P. hermaphrodita* (DMG0007) were found in 10, 50 and 100 mM $FeSO_4$ compared to the 0 mM control ($p < 0.05$; Fig 3A). There was no significant difference between the numbers of nematodes found in 10, 50 or 100 mM $FeSO_4$ ($p > 0.05$). When exposed to a range of concentrations of $ZnSO_4$ there were significantly more *P. hermaphrodita* (DMG0007) found in 10 and 50 mM of $ZnSO_4$ ($p < 0.05$) but not 100 mM of $ZnSO_4$ ($p > 0.05$) compared to 0 mM. There was a significant difference between the numbers of *P. hermaphrodita* (DMG0007) that were found in 0 and 50 or 100 mM of $MgSO_4$ ($p < 0.05$) but not 10 mM ($p < 0.05$). There was no significant difference between the numbers of *P. hermaphrodita* (DMG0007) that were found in 0, 10, 50 or 100 mM of $CuSO_4$ ($p > 0.05$).

There were significantly more *P. neopapillosa* (DMG0014) found in 10, 50 or 100 mM of $FeSO_4$, $ZnSO_4$ and $MgSO_4$ compared to the control (0 mM) ($p < 0.05$; Fig 3B). $CuSO_4$ was also attractive to the nematodes with significantly more found nematodes found in 50 or 100 mM ($p < 0.05$) than the 0 mM control but not at 10 mM ($p > 0.05$; Fig 3B).

The numbers of *P. californica* (DMG0019) found in 10, 50 or 100 mM of $FeSO_4$, $ZnSO_4$ and $CuSO_4$ compared to the 0 mM control was significantly different ($p < 0.05$; Fig 3C). There was no significant difference between the numbers of *P.*

californica (DMG0019) found in 0, 10 and 100 mM $MgSO_4$ ($p > 0.05$) but there was significantly more nematodes found in 50 mM $MgSO_4$ than 0 mM ($p < 0.05$).

Attraction of *Phasmarhabditis* nematodes to mucus is attenuated by heat treatment

As previously reported *P. hermaphrodita* (DMG0007), *P. neopapillosa* (DMG0014) and *P. californica* (DMG0019) were significantly attracted to *C. aspersum* mucus compared to the water control ($p < 0.001$; Fig 4A-C). This was also the case when mucus from *C. aspersum* was treated at 41°C and 82°C with all species ($p < 0.001$; Fig 4A-C). However, the mucus from *C. aspersum* exposed to 41°C and 82°C was significantly less attractive than mucus than was untreated ($p < 0.001$; Fig 4A-C). This implies that a protein (or proteins) present in the mucus is important in attraction towards mucus for *Phasmarhabditis*.

***Phasmarhabditis* nematodes are attracted to sodium hyaluronate**

P. hermaphrodita (DMG0007), *P. neopapillosa* (DMG0014) and *P. californica* (DMG0019) were significantly attracted to sodium hyaluronate at 1%, 5% and 10% compared to the 0% control ($p < 0.001$; Fig 5). There was no significant difference between the numbers of *P. hermaphrodita* (DMG0007), *P. neopapillosa* (DMG0014) or *P. californica* (DMG0019) found at 1% or 5% sodium hyaluronate ($p < 0.05$) but there were significantly more *P. hermaphrodita* (DMG0007) found in 10% sodium hyaluronate than *P. neopapillosa* (DMG0014) or *P. californica* (DMG0019) ($p < 0.0001$).

Assessment of behaviour of *Phasmarhabditis* nematodes exposed to mucus after pharmacological treatment with 8-bromo-cGMP

When dauers of the commercial strain of *P. hermaphrodita* (DMG0001) were exposed to *C. aspersum* mucus, 2.56 ± 0.5 moved to it (compared to 0.56 ± 0.18 to water control) ($p < 0.05$) (data not shown). When *P. hermaphrodita* (DMG0001) dauers were treated with 8-bromo-cGMP 3.33 ± 0.65 moved to the mucus (compared to 0.56 ± 0.24 to water control) ($p < 0.05$). There was no significant difference between the numbers of *P. hermaphrodita* (DMG0001) dauers found in the *C. aspersum* mucus when treated with 8-bromo-cGMP or not ($p < 0.05$) (data not shown).

When *P. hermaphrodita* (DMG0007) dauers were exposed to *C. aspersum* mucus 10.56 ± 0.97 moved to it (compared to 0.67 ± 0.17 to water control) ($p < 0.001$) (data not shown). When *P. hermaphrodita* (DMG0007) dauers were treated with 8-bromo-cGMP 7.78 ± 1.22 nematodes moved to the mucus (compared to 0.78 ± 0.28 to water control) ($p < 0.001$). There was no significant difference between the numbers of *P. hermaphrodita* (DMG0007) dauers found in the mucus when they were treated with 8-bromo-cGMP or not ($p < 0.05$) (data not shown).

We believe that the 8-bromo-cGMP was unable to penetrate the thick cuticle of the dauers. *P. hermaphrodita* dauers are very resistant to treatment with chemicals due to their thick cuticle and can survive prolonged exposure to detergents such as 1% SDS whereas adults die quickly (Rae et al., 2010), which is also the case for *C. elegans* (Cassada and Russell, 1975). It should be noted that we only tested two strains of *P. hermaphrodita* (DMG0001 and DMG0007). It may not be the dauer

stage that is resistant but just these two isolates, therefore other strains or species of *Phasmarhabditis* could be found that are not resistant to 8-bromo-cGMP treatment. Nevertheless, we decided to concentrate on adult *Phasmarhabditis* and exposed them to 8-bromo-cGMP as they do not possess the impenetrable cuticle. *P. hermaphrodita* (DMG0007), *P. neopapillosa* (DMG0014) and *P. californica* (DMG0019) adults were not attracted to *C. aspersum* mucus, with equal numbers found in the mucus and water control ($p > 0.05$; Fig 6, 7A). However, when adults of each species were exposed to 8-bromo-cGMP this had a highly significant effect and increased their attraction to *C. aspersum* mucus ($p < 0.0001$; Fig 6, 7B). The most extreme effect was found with *P. hermaphrodita* (DMG0007) where significantly more nematodes were found in the mucus after treatment of 8-bromo-cGMP than *P. neopapillosa* (DMG0014) or *P. californica* (DMG0019) ($p < 0.05$; Fig 6).

Discussion

Here we have shown that there are striking differences in the chemotactic response of several recently isolated strains and the commercial strain of *P. hermaphrodita* as well as *P. californica* and *P. neopapillosa* when exposed to mucus from several snail species. *P. hermaphrodita* (DMG0001) largely remained at the point of application and showed little evidence of chemoattraction to mucus from all snail species tested. In contrast, recently isolated *P. hermaphrodita* (DMG0007) and *P. californica* (DMG0019) were attracted to the snail mucus from *C. nemoralis*, *C. hortensis* and *A. arbustorum*. Over 10 years ago using the same agar based assay *P. hermaphrodita* (DMG0001) was able to chemotax towards many different slug species and was rarely found at the application point (Rae et al., 2006; 2009). It was

also shown to be attracted to mucus from *C. aspersum* and *C. hortensis*, scoring chemotaxis indices of 0.45 and 0.2, respectively (Rae et al., 2009). As this nematode has been in commercial production for over 20 years this suggests there may be a degree of in lab evolution occurring. This is not uncommon in nematodes commonly used in research. For example, through decades of being propagated under lab conditions using the same monoxenic diet of *Escherichia coli* OP50 and being cultured at the same temperature (20-25°C) *C. elegans* N2 is phenotypically different from wild strains in terms of aggregation behaviour, maturation time, fecundity, body size and many other traits (Sterken et al., 2015). At the genetic level this continued culturing has lead to laboratory derived variation in three genes including *npr-1*, *glb-5* and *nath-10*, which have striking effects on behaviour (Andersen et al. 2014), oxygen sensing (McGrath et al. 2009) and several other life history traits (Duveau & Félix, 2012) compared to wild isolated strains. *P. hermaphrodita* (DMG0001) was initially discovered in a moribund slug (*D. reticulatum*) showing signs of infection from Long Ashton Research Station, U.K. in 1988 (Wilson et al. 1993). Since then it has been under commercial production fed the bacterium *Moraxella osloensis* which was chosen as it produces high yields of nematodes that are consistently virulent (Wilson et al., 1995a.b). It is therefore possible that decades of growth under the same laboratory conditions away from natural conditions and gastropod hosts may have affected chemoattraction in *P. hermaphrodita* (DMG0001). Similar results showing that lack of chemotactic ability towards several slug species have been reported (Andrus and Rae, submitted). However, it should be noted that even if a potentially deleterious mutation may have hindered the ability of this nematode to respond to snail mucus it remains highly pathogenic to slugs (Williams & Rae, 2015).

We also observed striking intra and interspecies differences in chemotaxis in *Phasmarhabditis* nematodes. When exposed to mucus from *C. aspersum* collected from five different locations from around the U.K. the two recently isolated strains of *P. hermaphrodita* (DMG0007 and DMG0008) were significantly attracted to mucus from snails from all locations (unlike the commercial strain DMG0001). In contrast, *P. californica* (DMG0019) did not find *C. aspersum* mucus attractive apart from those collected from Halifax. Presumably, this strain of *C. aspersum* produces some sort of attractive compound in greater quantity than the others that is detected by *P. californica* (DMG0019). *P. californica* was first discovered in California (Tandingan de Ley et al. 2016), and has since been found in Ireland (Carnaghi et al., 2017) and Wales (Andrus & Rae, 2018). Our strain was isolated from a snail (*Oxychilus draparnaudi*) collected from Pembrokeshire, Wales (Andrus & Rae, 2018). Research into *P. californica* has concentrated on its recent description (Tandingan de Ley et al. 2016) but there is little information about its biology. It seems curious that this species displays such limited attraction to snail mucus from *C. aspersum* yet was found parasitising *O. draparnaudi*.

We have gained some insight into the properties that *Phasmarhabditis* nematodes use to detect mucus from snails. Mucus is mainly made of water and a plethora of compounds including glycoproteins, carbohydrates, metals and hyaluronic acid (Smith et al., 2009; Kubota et al., 1985; Kim, et al., 1996; Sallam et al., 2009; Werneke et al., 2007; Burton, 1965). We have shown that *Phasmarhabditis* nematodes are weakly attracted to several metals that are abundant in terrestrial gastropod mucus. Werneke et al. (2007) found zinc concentrations ranging from 70-340 p.p.m and levels of iron, manganese and copper ranging from 2-7 p.p.m in mucus from individual slugs (*Arion hortensis*). We also showed that heat treatment of mucus

significantly reduced the attraction of snail mucus to the nematodes, which suggests that there are large (unknown) glycoproteins that the nematodes detect. However, our data strongly points towards hyaluronic acid as a significant source of nematode attraction in mucus. We found that recently isolated *P. hermaphrodita* (DMG0007), *P. neopapillosa* (DMG0014) and *P. californica* (DMG0019) were significantly attracted to increasing amounts of sodium hyaluronate (the sodium salt of hyaluronic acid). Hyaluronic acid has been shown to be an attractive cue for a diverse range of parasites. For example, cercariae of *Acanthostomum brauni* are attracted to hyaluronic acid from fish (Haas & Ostrowskide de Núñez, 1988). Also the malarial parasite *Plasmodium falciparum* adheres to hyaluronic acid in cells in the placenta of infected pregnant mothers and is responsible for their aggregation (Beeson et al., 2000).

In a final experiment we investigated what genetic mechanism was used by *Phasmarhabditis* nematodes to detect snail mucus. We exposed *P. hermaphrodita* (DMG0007), *P. neopapillosa* (DMG0014) and *P. californica* (DMG0019) to exogenous 8-bromo-cGMP, which increases the activity of the protein kinase EGL-4 in other nematodes (Hong et al., 2008; Kroetz et al., 2012). EGL-4 has been implicated in regulating behaviour in an array of different organisms from nematodes (*C. elegans* and *P. pacificus*) to fruit flies (Osbourne et al. 1997) and honeybees (Ben-Shahar et al., 2002). We did not see an increase in chemotaxis behaviour when dauer stage nematodes were exposed to the compound, presumably because the compound cannot get through the rigid cuticle (Rae et al., 2010). Future research will focus on trying to remove the second stage cuticle via chemical exposure to maximise the uptake of 8-bromo-cGMP. We concentrated on using adult stage nematodes. This is not the host seeking stage in *P. hermaphrodita* (Tan & Grewal, 2001) and will not chemotax towards mucus, however, after pharmacological application we found that

the adults began chemotaxing to the snail mucus. This strongly implicates cGMP signalling and the role of EGL-4 in chemotaxis towards snail mucus in *Phasmarhabditis* nematodes. As this nematode is being developed as a genetic model to study the evolution of parasitism (Andrus & Rae, 2018), this approach can be used to further investigate and genetically dissect the mechanisms responsible for behaviour used to find hosts – the first stage of parasitism. Also, these results emphasise the importance of the cGMP pathway and EGL-4 and its evolutionary conserved role as a modulator of host seeking in nematodes from the Diplogastridae (*P. pacificus*) and Rhabditidae (*C. elegans* and *P. hermaphrodita*), which were thought to have diverged 250-400 MYA (Dieterich et al., 2008).

In summary, we have shown that there is interspecific and intraspecific variation in chemotaxis behaviour of *P. hermaphrodita* and *Phasmarhabditis* nematodes when exposed to snail mucus. We have shown that the commercial strain seems to have a reduced chemotactic response towards snail mucus perhaps due to artificial selection due to mass production but this has had little effect on its pathogenic potential towards pestiferous slugs (Williams & Rae, 2015). We have also determined that one of the compounds used by *Phasmarhabditis* nematodes to detect snail mucus is hyaluronic acid and that the genetic mechanism used by these nematodes to detect snail mucus is the evolutionary conserved cGMP signalling pathway activated by the protein kinase EGL-4.

Figure legends

Fig 1. The mean numbers of *P. hermaphrodita* (DMG0001) (A), *P. hermaphrodita* (DMG0007) (B) and *P. californica* (DMG0019) (C) that were found in mucus of pink

C. nemoralis (0 and 1 bands), yellow *C. nemoralis* (1 and 5 bands), *A. arbustorum*, *C. hortensis* and *C. aspersum* or the control (water) or the application point. Significant differences between the numbers of nematodes found in mucus and the control at $p < 0.05$ are denoted by * and at $p < 0.001$ denoted by **, n.s. means non-significant ($p > 0.05$). Bars represent ± one standard error.

Fig 2. The mean numbers of *P. hermaphrodita* (DMG0001) (A), *P. hermaphrodita* (DMG0007) (B), *P. hermaphrodita* (DMG0008) (C) and *P. californica* (DMG0019) (D) that were found in mucus of *C. aspersum* collected from Formby, Liverpool, Thurso, Whitby and Halifax or the control (water) or the application point. Significant differences between the numbers of nematodes found in mucus and the control at $p < 0.05$ are denoted by * and at $p < 0.001$ denoted by **, n.s. means non-significant ($p > 0.05$). Bars represent ± one standard error.

Fig 3. The mean numbers of *P. hermaphrodita* (DMG0007) (A), *P. neopapillosa* (DMG0014) (B) and *P. californica* (DMG0019) (C) that were found in 0, 10, 50 and 100 mM of $FeSO_4$ (black bars), $ZnSO_4$ (white bars), $MgSO_4$ (dark grey bars) and $CuSO_4$ (light grey bars). Significant differences between the numbers of nematodes found in 0 and 10, 50 or 100 mM are denoted by * at $p < 0.05$, n.s. means non-significant ($p > 0.05$). Bars represent ± one standard error.

Fig 4. The mean numbers of *P. hermaphrodita* (DMG0007) (A), *P. neopapillosa* (DMG0014) (B) and *P. californica* (DMG0019) (C) that were found in mucus from *C. aspersum* exposed to 41°C (black bars) and 82°C (white bars). Significant differences between the numbers of nematodes found in untreated mucus and heat

treated mucus at $p < 0.05$ are denoted by * and at $p < 0.001$ denoted by **, n.s. means non-significant ($p > 0.05$). Bars represent ± one standard error.

Fig 5. The mean numbers of *P. hermaphrodita* (DMG0007) (black bars), *P. neopapillosa* (DMG0014) (white bars) and *P. californica* (DMG0019) (grey bars) found in 0, 1, 5 and 10% sodium hyaluronate. Significant differences between the numbers of nematodes found at each concentration of sodium hyaluronate vs. the control (0%) at $p < 0.05$ are denoted by * and at $p < 0.001$ denoted by **, n.s. means non-significant ($p > 0.05$). Bars represent ± one standard error.

Fig 6. The mean numbers of untreated or treated adult *P. hermaphrodita* (DMG0007) (black bars), *P. neopapillosa* (DMG0014) (white bars) and *P. californica* (DMG0019) (grey bars) found in mucus from *C. aspersum*. Pharmacological treatment consisted of 1-hour exposure to 500 μM 8-bromo-cGMP. Significant differences between the numbers of nematodes found in mucus and the control at $p < 0.05$ are denoted by * and at $p < 0.001$ denoted by **, n.s. means non-significant ($p > 0.05$). Bars represent ± one standard error.

Fig 7. After 12-16 hours of being added to the chemotaxis plate testing the behavioural response of adult *P. hermaphrodita* (DMG0007) to *C. aspersum* mucus the majority remain at the point of application at the centre of the plate (A). However, if treated for 1 hour with 500 μM 8-bromo-cGMP then added to the plate adult *P. hermaphrodita* (DMG0007) disperse over the agar plate searching for snail mucus (B). Scale bar represents 1 cm.

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