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

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**Research Paper**

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## Abstract

*Phasmarhabditis hermaphrodita* is a parasitic nematode of terrestrial gastropods 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 UK 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 metal salts ($FeSO_4$, $ZnSO_4$, $CuSO_4$ 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 UK 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 manipulation of cyclic guanosine monophosphate (cGMP) increased chemoattraction to snail mucus, suggesting that the protein kinase EGL-4 may be responsible for *Phasmarhabditis* sp. chemoattraction.

## Introduction

Parasitic nematodes detect and respond to specific cues in order to locate and parasitize hosts (Lee, 2002). For example, entomopathogenic nematodes (*Steinernema* and *Heterorhabditis* spp.) 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; Small and Bradford, 2008; Nermut *et al.*, 2012). *Phasmarhabditis 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 4–21 days (Wilson *et al.*, 1993; Tan and Grewal, 2001). The nematodes then reproduce on the decaying cadaver and go in search of more hosts (Rae *et al.*, 2009). *Phasmarhabditis hermaphrodita* has been shown to successfully protect crops such as lettuce and oilseed rape against slug damage (Wilson and Rae, 2015).

*Phasmarhabditis 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; Small and Bradford, 2008; Nermut *et al.*, 2012). However, all of these behavioural studies have concentrated on studying chemoattraction towards slugs and not snails. *Phasmarhabditis hermaphrodita* is able to kill several species of snails, including juvenile *Cornu aspersum* (Glen *et al.*, 1996) and adult *Monacha cantiana* and *Cernuella virgata* (Coupland, 1995; Wilson *et al.*, 2000); however, some species, including *Arianta arbustorum* and *Cepaea nemoralis*, are resistant (Wilson *et al.*, 2000; Williams and 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.



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All behavioural studies using *P. hermaphrodita* (Rae *et al.*, 2006, 2009; Hapca *et al.*, 2007a,b; Small and Bradford, 2008; Nermut *et al.*, 2012), 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 utilized a collection of recently isolated wild strains of *P. hermaphrodita* and *Phasmarhabditis* species (including *P. californica* and *P. neopapillosa*) (Andrus and Rae, 2018a) 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 mainly of water (> 80%), proteins (proteoglycans and glycoproteins), carbohydrates (glycosaminoglycans, such as hyaluronic acid), lipids, metals and other molecules (Burton, 1965; Kubota *et al.*, 1985; Kim *et al.*, 1996; Werneke *et al.*, 2007; Sallam *et al.*, 2009; Smith *et al.*, 2009). Therefore, we exposed *Phasmarhabditis* nematodes to a subset of these properties, including metal salts ($FeSO_4$, $ZnSO_4$, $CuSO_4$ and $MgSO_4$) and 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 responsible for behaviour (Rengarajan and Hallem, 2016). Studies using *Caenorhabditis elegans* have identified genes, neurons and neurotransmitters that are essential for chemotaxis and avoidance behaviour towards alcohols, bacteria and various 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 and Sommer, 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 cyclic guanosine monophosphate (cGMP)-dependent protein kinase EGL-4 through manipulation by pharmacological treatment using 8-bromo-cGMP.

## Materials and methods

### Source of invertebrates

*Phasmarhabditis hermaphrodita* (commercial strain DMG0001-Nemaslug®) was supplied by BASF 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 and Rae, 2018a). Snails (*C. nemoralis* and *C. hortensis*) were collected from sand dunes in Formby, Merseyside, UK. 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 five-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 laboratory 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 swabbed gently from the foot of each snail and placed 0.5 cm from the edge of the plate. On the opposite 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 and Rae, 2018a), where *c.* 100 nematodes were added to a rotting piece of *Limax flavus* and left for 28 days until they grew to the dauer stage, and were 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*, 1 M sodium azide is added to the treatment and control to stop nematode movement immediately (Bargmann *et al.*, 1993). However, once *P. hermaphrodita* nematodes find mucus they remain there (Rae *et al.*, 2006, 2009; Hapca *et al.*, 2007a), hence there is no need to immobilize them.

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

We attempted to discover what properties of mucus *Phasmarhabditis* spp. 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 salt ($FeSO_4$, $ZnSO_4$, $MgSO_4$ 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 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 remained consistently 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%).

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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. *Cornu aspersum* mucus was harvested (as described previously), placed into 1.5 ml Eppendorfs and heated at 41°C or 82°C for 45 minutes in a heat block. The first treatment (41°C) was used to destroy smaller proteins (> 40,000 kDa) present in the mucus (Branden and Tooze, 1999). The second treatment (82°C) was used to target large glycoproteins (> 120,000 kDa) (Kubota *et al.*, 1985). The heat-treated filter paper with mucus was then placed on the agar plate (as described previously) 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 *c.* 300 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 for just 1 hour, after which we washed the nematodes briefly 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. *Phasmarhabditis hermaphrodita* (DMG0001) was 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 was compared to the number in the water control 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 were compared using a Kruskal–Wallis test. Statistical analysis was carried out using SPSS 21 (IBM, Armonk, USA).

## 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 five 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, $P = 0.0052$; 5 bands, $P = 0.0002$); 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 bands, $P = 0.007$; 1 band, $P = 0.0002$), yellow *C. nemoralis* (1 band, $P = 0.005$; 5 bands, $P = 0.035$), *C. hortensis* ($P = 0.015$) and *C. aspersum* ($P = 0.0002$) were 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 UK*

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 that 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).

*Phasmarhabditis 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 the different 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 metal salts 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 ($P < 0.05$;

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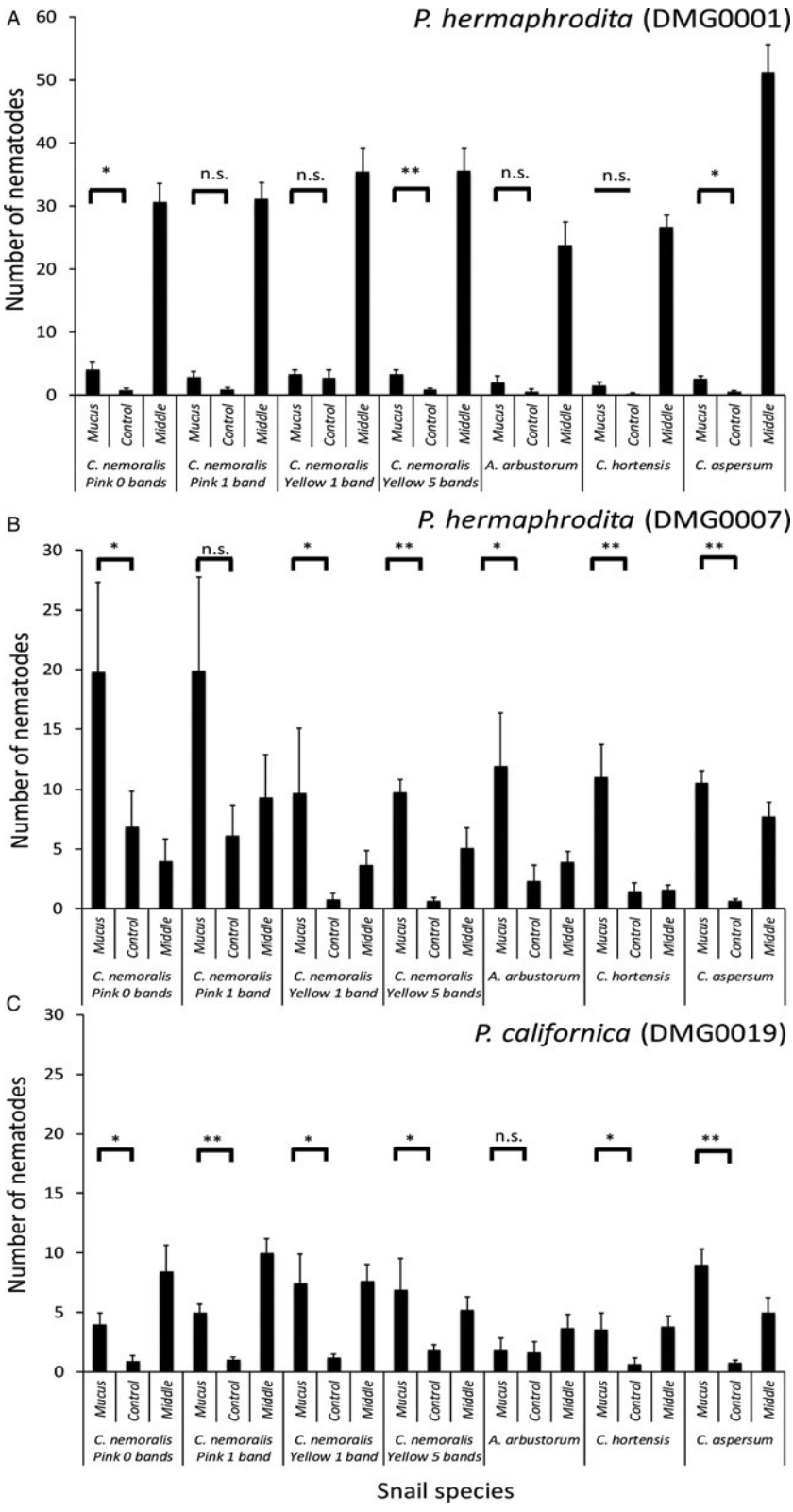


**Fig. 1.** The mean numbers of *P. hermaphrodita* (DMG0001) (A), *P. hermaphrodita* (DMG0007) (B) and *P. californica* (DMG0019) (C) found in mucus of pink *C. nemoralis* (0 and 1 bands), yellow *C. nemoralis* (1 and 5 bands), *A. arbustorum*, *C. hortensis* and *C. aspersum*, in the control (water), or at 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. = non-significant ($P > 0.05$). Bars represent ± one standard error.

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 $CuSO_4$ ($P > 0.05$).

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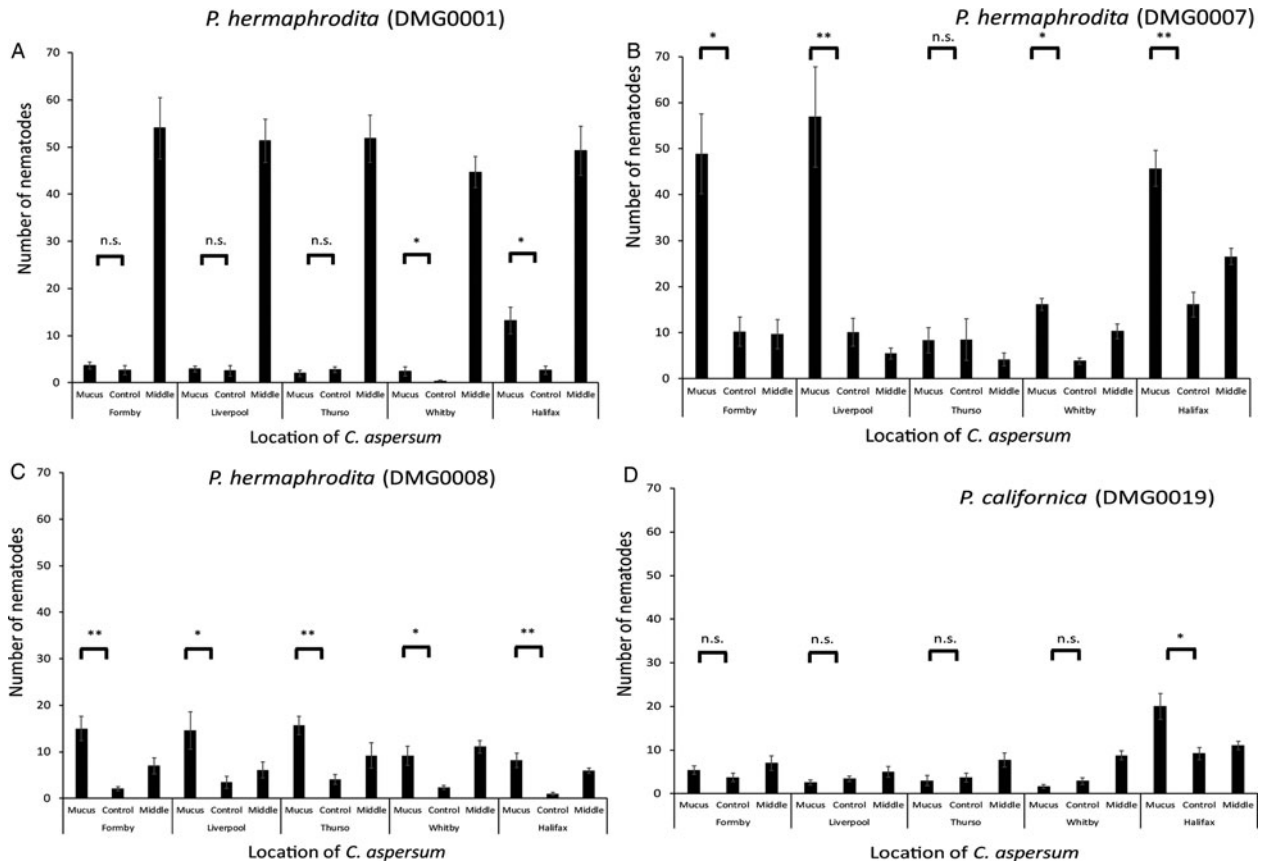


**Fig. 2.** The mean numbers of *P. hermaphrodita* (DMG0001) (A), *P. hermaphrodita* (DMG0007) (B), *P. hermaphrodita* (DMG0008) (C) and *P. californica* (DMG0019) (D) found in mucus of *C. aspersum* collected from Formby, Liverpool, Thurso, Whitby and Halifax, in the control (water), or at the application 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. = non-significant ($P > 0.05$). Bars represent ± one standard error.

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 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 were 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 significantly more nematodes were found in 50 mM $MgSO_4$ than in 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 for all species when mucus from *C. aspersum* was treated at 41°C and 82°C ($P < 0.001$; fig. 4A–C). However, the mucus from *C. aspersum* exposed to 41°C and 82°C was significantly less attractive than mucus that 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*

*Phasmarhabditis 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) than *P. neopapillosa* (DMG0014) or *P. californica* (DMG0019) found in 10% sodium hyaluronate ($P < 0.0001$).

### *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 the 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 the water control) ($P < 0.05$). There was no significant difference between the numbers of *P. hermaphrodita* (DMG0001) dauers found in the *C. aspersum*

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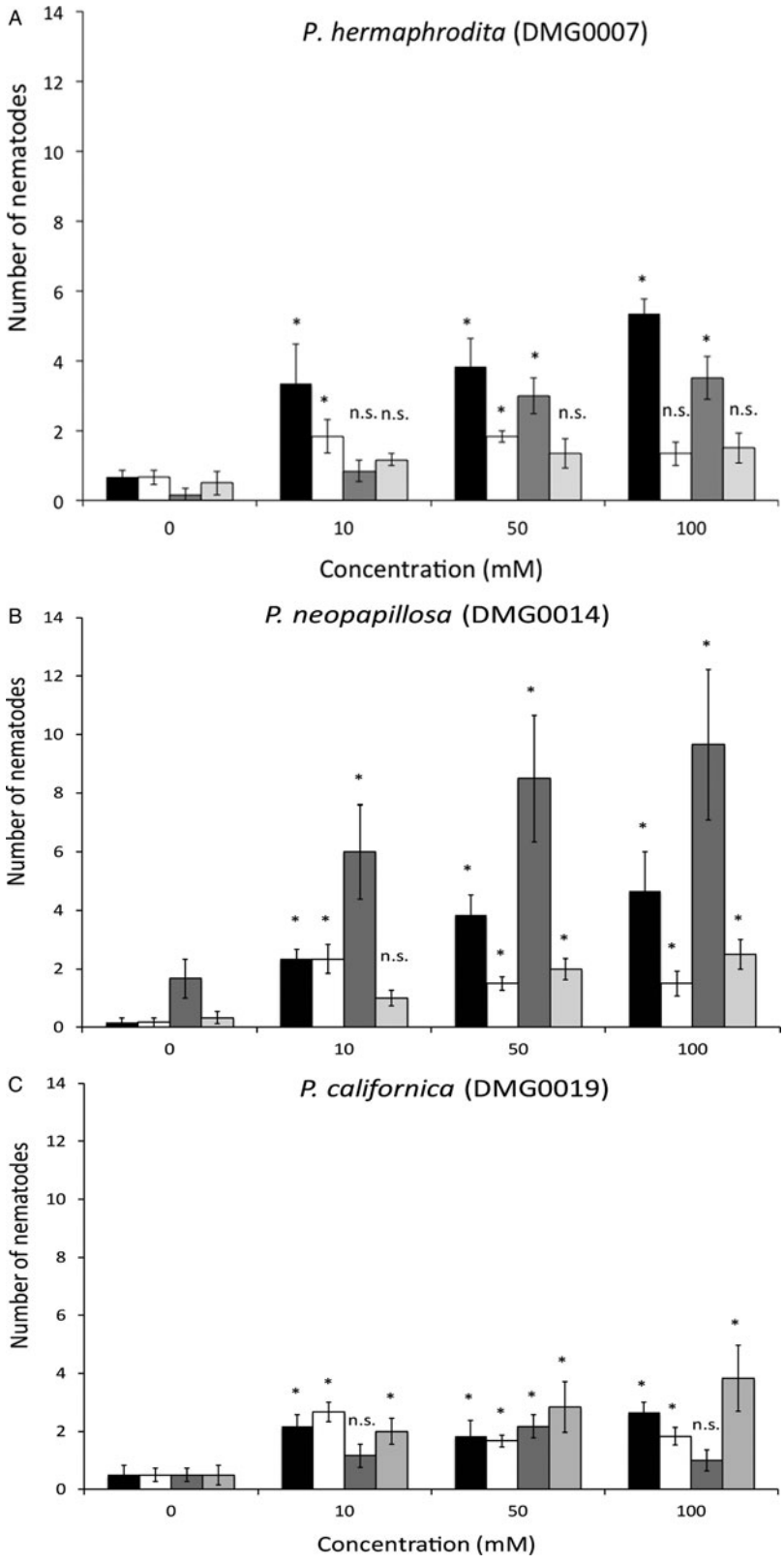


**Fig. 3.** The mean numbers of *P. hermaphrodita* (DMG0007) (A), *P. neopapillosa* (DMG0014) (B) and *P. californica* (DMG0019) (C) found in 0, 10, 50 and 100 mM of $FeSO_4$ (black bars), $ZnSO_4$ (white bars), $MgSO_4$ (dark grey bars) and $CuSO_4$ (light grey bars). Significant differences between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

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 the 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 the water control) ($P < 0.001$). There was no significant difference between the numbers of *P. hermaphrodita* (DMG0007) dauers found in the mucus when

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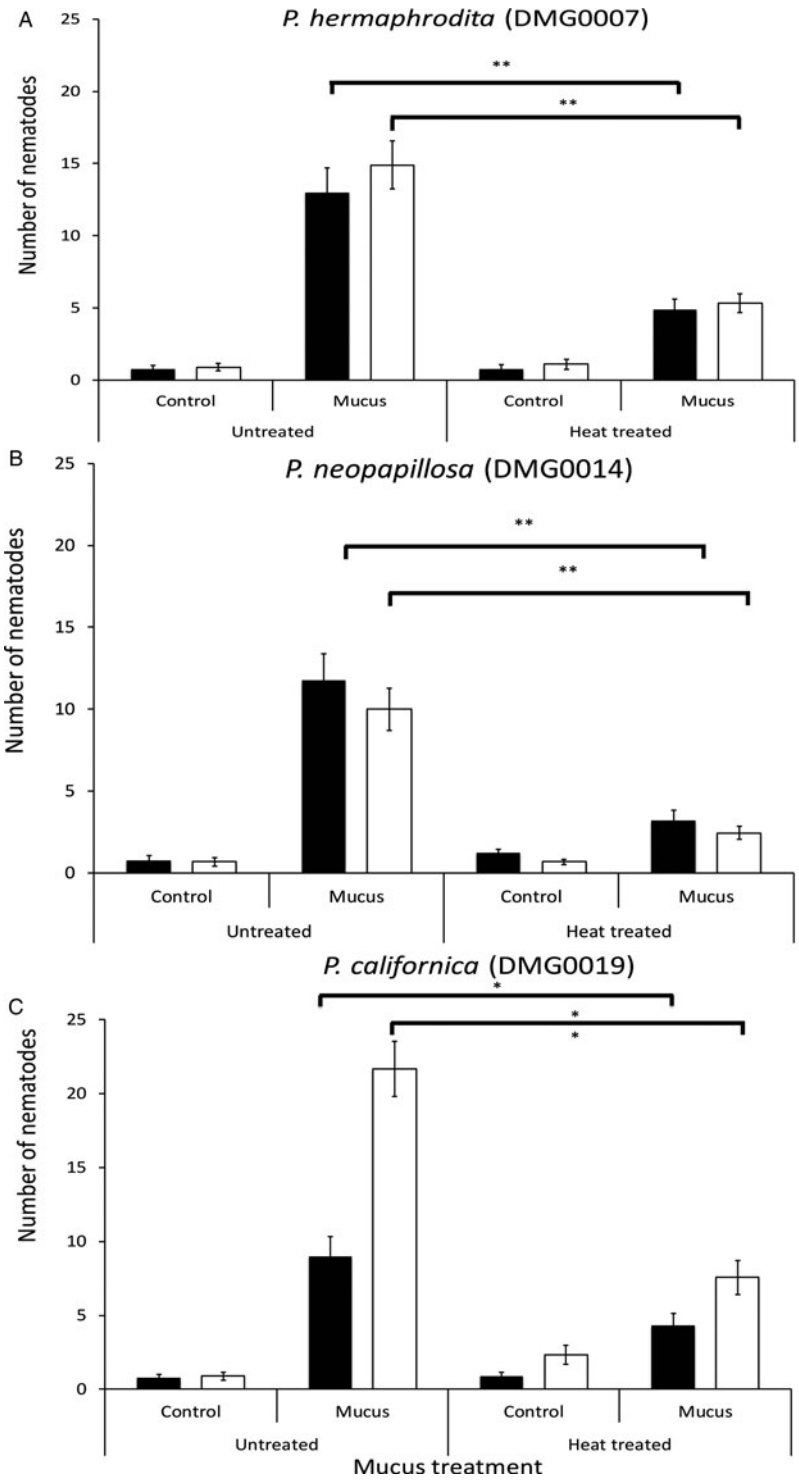


**Fig. 4.** The mean numbers of *P. hermaphrodita* (DMG0007) (A), *P. neopapillosa* (DMG0014) (B) and *P. californica* (DMG0019) (C) found in untreated mucus from *C. aspersum* and mucus exposed to 41°C (black bars) and 82°C (white bars). Significant differences between the numbers of nematodes found in untreated mucus and heat-treated mucus at $P < 0.05$ are denoted by * and at $P < 0.001$ denoted by **; n.s. = non-significant ($P > 0.05$). Bars represent ± one standard error.

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. *Phasmarhabditis 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 tested only two strains of *P. hermaphrodita* (DMG0001 and DMG0007). It may not be the dauer stage that is resistant but just these two isolates, therefore there may be other strains or species of *Phasmarhabditis* 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. *Phasmarhabditis 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 ($P > 0.05$; figs 6 and 7A). However, when adults of each species were exposed to 8-bromo-cGMP this

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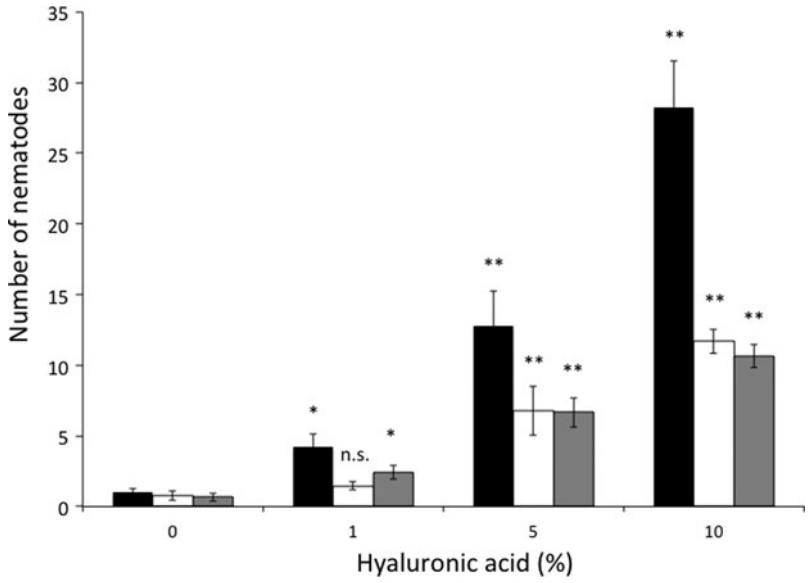


**Fig. 5.** The mean numbers of *P. hermaphrodita* (DMG0007) (black bars), *P. neopapillosa* (DMG0014) (white bars) and *P. californica* (DMG0019) (grey bars) found in 0, 1, 5 and 10% sodium hyaluronate. Significant differences between the numbers of nematodes found at each concentration of sodium hyaluronate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

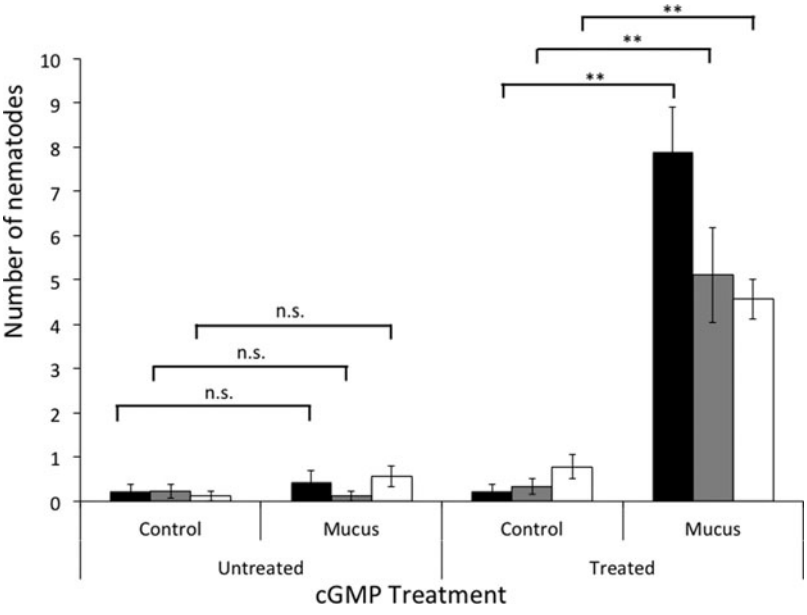


**Fig. 6.** The mean numbers of untreated or treated adult *P. hermaphrodita* (DMG0007) (black bars), *P. neopapillosa* (DMG0014) (grey bars) and *P. californica* (DMG0019) (white bars) found in mucus from *C. aspersum*. Pharmacological treatment consisted of 1-hour exposure to 500 μM 8-bromo-cGMP. Significant 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. = non-significant ($P > 0.05$). Bars represent ± one standard error.

had a highly significant effect and increased their attraction to *C. aspersum* mucus ($P < 0.0001$; figs 6 and 7B). The most extreme effect was found with *P. hermaphrodita* (DMG0007), for which 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. *Phasmarhabditis 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 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 laboratory 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

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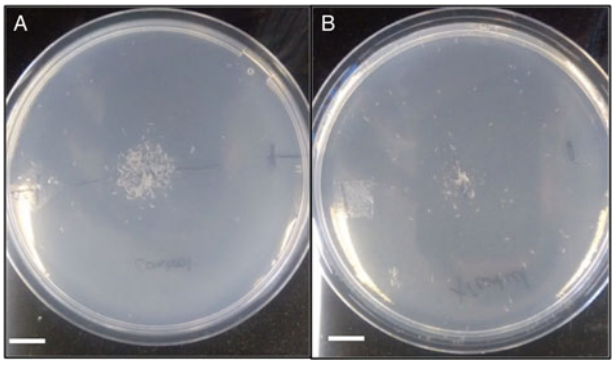


**Fig. 7.** After 12–16 hours of being added to the chemotaxis plate testing the behavioural response of adult *P. hermaphrodita* (DMG0007) to *C. aspersum* mucus the majority remain at the point of application at the centre of the plate (A). However, if treated for 1 hour with 500 μM 8-bromo-cGMP then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

(Sterken *et al.*, 2015). At the genetic level this continued culturing has led to laboratory-derived variation in three genes, *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 and Félix, 2012) compared to wild isolated strains. *Phasmarhabditis hermaphrodita* (DMG0001) was initially discovered in 1988 in a moribund slug (*D. reticulatum*) showing signs of infection, from Long Ashton Research Station, UK (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, 2018b). 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 and 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 around the UK 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, which is detected by *P. californica* (DMG0019). *Phasmarhabditis 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 and Rae, 2018a). Our strain was isolated from a snail (*Oxychilus draparnaudi*) collected from Pembrokeshire, Wales (Andrus and Rae, 2018a). 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 parasitizing *O. draparnaudi*.

We have gained some insight into the properties that *Phasmarhabditis* nematodes use to detect mucus from snails. Mucus is composed mainly of water and a plethora of compounds, including glycoproteins, carbohydrates, metals and hyaluronic acid (Burton, 1965; Kubota *et al.*, 1985; Kim *et al.*, 1996; Werneke *et al.*, 2007; Sallam *et al.*, 2009; Smith *et al.*, 2009). We have shown that *Phasmarhabditis* nematodes are weakly attracted to several metal salts that are abundant in terrestrial gastropod mucus. Werneke *et al.* (2007) found zinc concentrations ranging from 70–340 ppm and levels of iron, manganese and copper ranging from 2–7 ppm 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 point 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 and 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 (Osborne *et al.*, 1997) and honeybees (Ben-Shahar *et al.*, 2002). We did not observe 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 maximize the uptake of 8-bromo-cGMP. We concentrated on using adult stage nematodes. This is not the host-seeking stage in *P. hermaphrodita* (Tan and 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 and Rae, 2018a), 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 emphasize 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

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mucus. We have shown that the commercial strain seems to have a reduced chemotactic response towards snail mucus, perhaps due to artificial selection as a result of mass production, but this has had little effect on its pathogenic potential towards pestiferous slugs (Williams and 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.

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