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

5

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

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23

1 **Abstract**

2

3 *Phasmarhabditis hermaphrodita* is a terrestrial gastropod parasitic nematode
4 that has been formulated into a biological control agent for farmers and gardeners to
5 kill slugs and snails. In order to locate slugs it is attracted to mucus, faeces and
6 volatile cues however, there is no information about whether these nematodes are
7 attracted to snail cues. It is also unknown how wild isolates of *P. hermaphrodita* or
8 different *Phasmarhabditis* species behave when exposed to gastropod cues.
9 Therefore, we investigated whether *P. hermaphrodita* (commercial and wild isolated
10 strains), *P. neopapillosa* and *P. californica* were attracted to mucus from several
11 common snail species (*Cepaea nemoralis*, *Cepaea hortensis*, *Arianta arbustorum* and
12 *Cornu aspersum*). We also examined whether snails (*C. aspersum*) collected from
13 different locations around the U.K. differed in their attractiveness to wild isolates of
14 *P. hermaphrodita*. Furthermore, we also investigated what properties of snail mucus
15 the nematodes were attracted to including hyaluronic acid and metals (FeSO₄, ZnSO₄,
16 CuSO₄ and MgSO₄). We found that the commercial strain of *P. hermaphrodita*
17 responded poorly to snail mucus compared to wild isolated strains and *C. aspersum*
18 collected from different parts of the U.K. differed in their attractiveness to the
19 nematodes. We found that *Phasmarhabditis* nematodes were weakly attracted to all
20 metals tested but were strongly attracted to hyaluronic acid. In a final experiment we
21 also showed that pharmacological application of cyclic GMP increased
22 chemoattraction to snail mucus, suggesting that the protein kinase EGL-4 may be
23 responsible for *Phasmarhabditis* sp. chemoattraction.

24

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

1 **Introduction**

2

3 Parasitic nematodes detect and respond to specific cues in order to locate and
4 parasitise hosts (Lee, 2002). For example, entomopathogenic nematodes (*Steinernema*
5 and *Heterorhabditis* sp.) respond to odour blends and carbon dioxide emitted by live
6 insect hosts (Dillman et al., 2012). The human parasite *Strongyloides stercoralis* is
7 attracted to skin and sweat odorants (Castelletto et al., 2014). Similarly,
8 *Heligmosomoides polygyrus* (a parasite of rodents) is attracted to sweat odorants,
9 faeces and carbon dioxide (Ruiz et al., 2017). The terrestrial gastropod parasitic
10 nematode *Phasmarhabditis hermaphrodita* is a lethal parasite of several pestiferous
11 slug species (Wilson et al., 1993) and is attracted to slug faeces, mucus and volatiles
12 (Rae et al., 2006; 2009; Hapca et al., 2007a,b; Nermut et al., 2012, Small & Bradford,
13 2008). *P. hermaphrodita* has been formulated into a biological control agent
14 (Nemaslug®) used to kill slugs and snails across northern Europe (Rae et al., 2007).
15 Nematodes are applied to soil where they seek out hosts, penetrate through the mantle
16 and kill slugs within 4 to 21 days (Wilson et al., 1993, Tan and Grewal, 2001). The
17 nematodes then reproduce on the decaying cadaver and go in search for more hosts
18 (Rae et al., 2009). *P. hermaphrodita* has been shown to successfully protect crops
19 such as lettuce and oilseed rape against slug damage (Wilson & Rae, 2015).

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

1 *aspersum* (Glen et al., 1996) and adult *Monacha cantiana* and *Cerneuella virgata*
2 (Wilson et al., 2000; Coupland, 1995) however; species such as *Arianta arbustorum*
3 and *Cepaea nemoralis* are resistant (Wilson et al., 2000; Williams & Rae, 2016; Rae,
4 2018). The reasons for their resistance to *P. hermaphrodita* are unknown but it could
5 be due to the presence of the shell, which has the ability to trap, encase and kill
6 nematodes (Rae, 2017b). We decided to investigate whether *P. hermaphrodita* and
7 other *Phasmarhabditis* species were attracted to snail mucus.

8 All behavioural studies using *P. hermaphrodita* (Rae et al., 2006; 2009; Hapca
9 et al., 2007a,b; Nermut et al., 2012; Small & Bradford, 2008), have concentrated on
10 using one strain (the commercial isolate, designated “DMG0001” by Hooper et al.,
11 1999), which has been in production for over 20 years. There is no information about
12 how wild strains of *P. hermaphrodita* and other *Phasmarhabditis* species respond to
13 gastropod cues such as mucus. Therefore, we utilised a collection of recently isolated
14 wild strains of *P. hermaphrodita* and *Phasmarhabditis* species (including *P.*
15 *californica* and *P. neopapillosa*) (Andrus & Rae, 2018) to examine their
16 chemoattraction behaviour to snail mucus to see if it differed from the commercial
17 isolate.

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

1 including metals (FeSO₄, ZnSO₄, CuSO₄ and MgSO₄), hyaluronic acid and examined
2 whether heat treatment of mucus (which denatures large glycoproteins) would alter
3 the chemoattraction of the nematodes.

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

16

17 **Materials and methods**

18

19 **Source of invertebrates**

20

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

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

9

10 **Chemotaxis assay**

11

12 To assess the behaviour of *Phasmarhabditis* nematodes exposed to snail
13 mucus an agar plate chemotaxis assay was used as in previous studies (Rae et al.,
14 2006, 2009). Briefly, 10 cm Petri dishes were half filled with 1.2% technical agar and
15 left to dry for 48 hours. Using a 1 cm² piece of Whatman number 1 filter paper 0.01 g
16 of snail mucus was gently swabbed from the foot of each snail and placed 0.5 cm
17 from the edge of the plate. On the opposing side of the Petri dish 10 µl of distilled
18 water was added to a 1 cm² piece of Whatman number 1 filter paper and acted as the
19 control. Approximately 50 dauer stage *Phasmarhabditis* nematodes were added to the
20 middle of the plate and each plate was sealed with Parafilm® and stored at 20°C. The
21 following morning the numbers of nematodes that had graduated to each piece of
22 filter paper and the numbers that remained in the middle of the plate were recorded.
23 Wild strains of *Phasmarhabditis* were sub-cultured by growing them in White traps
24 (described in Andrus & Rae, 2018) where approximately 100 nematodes were added
25 to a rotting piece of *Limax flavus* and left for 28 days until they grew to the dauer

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

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

14

15 **Investigating the properties of snail mucus that *Phasmarhabditis* nematodes are** 16 **attracted to**

17

18 We attempted to discover what properties of mucus *Phasmarhabditis* sp. were
19 attracted to. To do this we used the same chemotaxis assay described above with
20 modifications. We added four 1 cm² pieces of filter paper to each plate and added
21 different concentrations (0, 10, 50 and 100 µM) of each metal (FeSO₄, ZnSO₄,
22 MgSO₄ and CuSO₄) to each piece of filter paper. Approximately 50 dauer stage *P.*
23 *hermaphrodita* (DMG0007), *P. neopapillosa* (DMG0014) or *P. californica*
24 (DMG0019) were added to three replicate plates and the whole experiment was
25 repeated three times. It should be noted that it is unknown whether the higher salt

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

8 We also investigated whether any large (unknown) glycoproteins may be
9 involved in the attraction of *Phasmarhabditis* nematodes to snail mucus. Proteins in
10 snail mucus can be denatured using heat treatment. *C. aspersum* mucus was harvested
11 (as previously described), placed into 1.5 ml Eppendorfs and heated at two
12 temperatures (41°C or 82°C) for 45 mins in a heat block. The first treatment (41°C)
13 was used to destroy smaller proteins (>40,000 kDa) present in the mucus (Branden,
14 1999). The second treatment (82°C) was used to target large glycoproteins found in
15 mucus (>120,000 kDa) (Kubota et al., 1985). The heat-treated filter paper with mucus
16 was then placed on the agar plate (as previously described) and a control piece of
17 filter paper with water and treated at the same temperatures was placed opposite.
18 Three replicate plates were used for each heat-treatment and the experiment was
19 repeated three times with *P. hermaphrodita* (DMG0007), *P. neopapillosa*
20 (DMG0014) and *P. californica* (DMG0019).

21

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

24

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

17

18 **Statistical analysis**

19

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

25

1 **Results**

2

3 ***Phasmarhabditis* nematodes are attracted to mucus from several snail species**

4

5 There was a significant difference between the numbers of *P. hermaphrodita*
6 (DMG0001) found in mucus from pink *C. nemoralis* with zero bands ($p = 0.023$),
7 yellow *C. nemoralis* with 5 bands ($p = 0.0007$) and *C. aspersum* ($p = 0.0035$)
8 compared to the water control (Fig 1A). However, there was no significant difference
9 between the numbers of *P. hermaphrodita* (DMG0001) found in mucus of yellow or
10 pink *C. nemoralis* (1 band), *C. hortensis* or *A. arbustorum* and water ($p > 0.05$; Fig.
11 1A). In general, very few nematodes (<5) moved towards the mucus, whereas the
12 majority (23-36) were found still at the point of application. In contrast, the recently
13 isolated strain of *P. hermaphrodita* (DMG0007) was more active and attracted to snail
14 mucus with significantly more nematodes found in mucus from yellow *C. nemoralis* 1
15 band ($p = 0.0052$) and 5 band ($p = 0.0002$); pink *C. nemoralis* (0 bands) ($p = 0.046$),
16 *C. hortensis* ($p = 0.0008$), *A. arbustorum* ($p = 0.0135$) and *C. aspersum* ($p = 0.0002$)
17 compared to water (Fig 1B). There was no significant difference between the numbers
18 of *P. hermaphrodita* (DMG0007) found in mucus from pink *C. nemoralis* (1 band)
19 and water ($p = 0.066$; Fig 1B).

20 The numbers of *P. californica* (DMG0019) found in the mucus from pink *C.*
21 *nemoralis* 0 band ($p = 0.007$) and 1 band ($p = 0.0002$), yellow *C. nemoralis* 1 band (p
22 $= 0.005$) and 5 bands ($p = 0.035$), *C. hortensis* ($p = 0.015$) and *C. aspersum* ($p =$
23 0.0002) was significantly greater than the number of nematodes found in water (Fig
24 1C). However, there was no significant difference between the numbers of *P.*

1 *californica* (DMG0019) found in mucus from *A. arbustorum* compared to water ($p =$
2 0.43; Fig 1C).

3

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

6

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

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

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

4

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

6

7 Significantly more *P. hermaphrodita* (DMG0007) were found in 10, 50 and
8 100 mM FeSO₄ compared to the 0 mM control ($p < 0.05$; Fig 3A). There was no
9 significant difference between the numbers of nematodes found in 10, 50 or 100 mM
10 FeSO₄ ($p > 0.05$). When exposed to a range of concentrations of ZnSO₄ there were
11 significantly more *P. hermaphrodita* (DMG0007) found in 10 and 50 mM of ZnSO₄
12 ($p < 0.05$) but not 100 mM of ZnSO₄ ($p > 0.05$) compared to 0 mM. There was a
13 significant difference between the numbers of *P. hermaphrodita* (DMG0007) that
14 were found in 0 and 50 or 100 mM of MgSO₄ ($p < 0.05$) but not 10 mM ($p < 0.05$).
15 There was no significant difference between the numbers of *P. hermaphrodita*
16 (DMG0007) that were found in 0, 10, 50 or 100 mM of CuSO₄ ($p > 0.05$).

17 There were significantly more *P. neopapillosa* (DMG0014) found in 10, 50 or
18 100 mM of FeSO₄, ZnSO₄ and MgSO₄ compared to the control (0 mM) ($p < 0.05$; Fig
19 3B). CuSO₄ was also attractive to the nematodes with significantly more found
20 nematodes found in 50 or 100 mM ($p < 0.05$) than the 0 mM control but not at 10 mM
21 ($p > 0.05$; Fig 3B).

22 The numbers of *P. californica* (DMG0019) found in 10, 50 or 100 mM of
23 FeSO₄, ZnSO₄ and CuSO₄ compared to the 0 mM control was significantly different
24 ($p < 0.05$; Fig 3C). There was no significant difference between the numbers of *P.*

1 *californica* (DMG0019) found in 0, 10 and 100 mM MgSO₄ ($p > 0.05$) but there was
2 significantly more nematodes found in 50 mM MgSO₄ than 0 mM ($p < 0.05$).

3

4 **Attraction of *Phasmarhabditis* nematodes to mucus is attenuated by heat** 5 **treatment**

6

7 As previously reported *P. hermaphrodita* (DMG0007), *P. neopapillosa*
8 (DMG0014) and *P. californica* (DMG0019) were significantly attracted to *C.*
9 *aspersum* mucus compared to the water control ($p < 0.001$; Fig 4A-C). This was also
10 the case when mucus from *C. aspersum* was treated at 41°C and 82°C with all species
11 ($p < 0.001$; Fig 4A-C). However, the mucus from *C. aspersum* exposed to 41°C and
12 82°C was significantly less attractive than mucus than was untreated ($p < 0.001$; Fig
13 4A-C). This implies that a protein (or proteins) present in the mucus is important in
14 attraction towards mucus for *Phasmarhabditis*.

15

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

17

18 *P. hermaphrodita* (DMG0007), *P. neopapillosa* (DMG0014) and *P.*
19 *californica* (DMG0019) were significantly attracted to sodium hyaluronate at 1%, 5%
20 and 10% compared to the 0% control ($p < 0.001$; Fig 5). There was no significant
21 difference between the numbers of *P. hermaphrodita* (DMG0007), *P. neopapillosa*
22 (DMG0014) or *P. californica* (DMG0019) found at 1% or 5% sodium hyaluronate (p
23 < 0.05) but there were significantly more *P. hermaphrodita* (DMG0007) found in
24 10% sodium hyaluronate than *P. neopapillosa* (DMG0014) or *P. californica*
25 (DMG0019) ($p < 0.0001$).

1

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

4

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

13 When *P. hermaphrodita* (DMG0007) dauers were exposed to *C. aspersum*
14 mucus 10.56 ± 0.97 moved to it (compared to 0.67 ± 0.17 to water control) ($p <$
15 0.001) (data not shown). When *P. hermaphrodita* (DMG0007) dauers were treated
16 with 8-bromo-cGMP 7.78 ± 1.22 nematodes moved to the mucus (compared to $0.78 \pm$
17 0.28 to water control) ($p < 0.001$). There was no significant difference between the
18 numbers of *P. hermaphrodita* (DMG0007) dauers found in the mucus when they were
19 treated with 8-bromo-cGMP or not ($p < 0.05$) (data not shown).

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

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

13

14 **Discussion**

15

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

1 also shown to be attracted to mucus from *C. aspersum* and *C. hortensis*, scoring
2 chemotaxis indices of 0.45 and 0.2, respectively (Rae et al., 2009). As this nematode
3 has been in commercial production for over 20 years this suggests there may be a
4 degree of in lab evolution occurring. This is not uncommon in nematodes commonly
5 used in research. For example, through decades of being propagated under lab
6 conditions using the same monoxenic diet of *Escherichia coli* OP50 and being
7 cultured at the same temperature (20-25°C) *C. elegans* N2 is phenotypically different
8 from wild strains in terms of aggregation behaviour, maturation time, fecundity, body
9 size and many other traits (Sterken et al., 2015). At the genetic level this continued
10 culturing has lead to laboratory derived variation in three genes including *npr-1*, *glb-5*
11 and *nath-10*, which have striking effects on behaviour (Andersen et al. 2014), oxygen
12 sensing (McGrath et al. 2009) and several other life history traits (Duveau & Félix,
13 2012) compared to wild isolated strains. *P. hermaphrodita* (DMG0001) was initially
14 discovered in a moribund slug (*D. reticulatum*) showing signs of infection from Long
15 Ashton Research Station, U.K. in 1988 (Wilson et al. 1993). Since then it has been
16 under commercial production fed the bacterium *Moraxella osloensis* which was
17 chosen as it produces high yields of nematodes that are consistently virulent (Wilson
18 et al., 1995a.b). It is therefore possible that decades of growth under the same
19 laboratory conditions away from natural conditions and gastropod hosts may have
20 affected chemoattraction in *P. hermaphrodita* (DMG0001). Similar results showing
21 that lack of chemotactic ability towards several slug species have been reported
22 (Andrus and Rae, submitted). However, it should be noted that even if a potentially
23 deleterious mutation may have hindered the ability of this nematode to respond to
24 snail mucus it remains highly pathogenic to slugs (Williams & Rae, 2015).

1 We also observed striking intra and interspecies differences in chemotaxis in
2 *Phasmarhabditis* nematodes. When exposed to mucus from *C. aspersum* collected
3 from five different locations from around the U.K. the two recently isolated strains of
4 *P. hermaphrodita* (DMG0007 and DMG0008) were significantly attracted to mucus
5 from snails from all locations (unlike the commercial strain DMG0001). In contrast,
6 *P. californica* (DMG0019) did not find *C. aspersum* mucus attractive apart from those
7 collected from Halifax. Presumably, this strain of *C. aspersum* produces some sort of
8 attractive compound in greater quantity than the others that is detected by *P.*
9 *californica* (DMG0019). *P. californica* was first discovered in California (Tandingan
10 de Ley et al. 2016), and has since been found in Ireland (Carnaghi et al., 2017) and
11 Wales (Andrus & Rae, 2018). Our strain was isolated from a snail (*Oxychilus*
12 *draparnaudi*) collected from Pembrokeshire, Wales (Andrus & Rae, 2018). Research
13 into *P. californica* has concentrated on its recent description (Tandingan de Ley et al.
14 2016) but there is little information about its biology. It seems curious that this
15 species displays such limited attraction to snail mucus from *C. aspersum* yet was
16 found parasitising *O. draparnaudi*.

17 We have gained some insight into the properties that *Phasmarhabditis*
18 nematodes use to detect mucus from snails. Mucus is mainly made of water and a
19 plethora of compounds including glycoproteins, carbohydrates, metals and hyaluronic
20 acid (Smith et al., 2009; Kubota et al., 1985; Kim, et al., 1996; Sallam et al., 2009;
21 Werneke et al., 2007; Burton, 1965). We have shown that *Phasmarhabditis*
22 nematodes are weakly attracted to several metals that are abundant in terrestrial
23 gastropod mucus. Werneke et al. (2007) found zinc concentrations ranging from 70-
24 340 p.p.m and levels of iron, manganese and copper ranging from 2-7 p.p.m in mucus
25 from individual slugs (*Arion hortensis*). We also showed that heat treatment of mucus

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

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

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

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

21

22 **Figure legends**

23

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

1 *C. nemoralis* (0 and 1 bands), yellow *C. nemoralis* (1 and 5 bands), *A. arbustorum*, *C.*
2 *hortensis* and *C. aspersum* or the control (water) or the application point. Significant
3 differences between the numbers of nematodes found in mucus and the control at $p <$
4 0.05 are denoted by * and at $p < 0.001$ denoted by **, n.s. means non-significant ($p >$
5 0.05). Bars represent \pm one standard error.

6

7 Fig 2. The mean numbers of *P. hermaphrodita* (DMG0001) (A), *P. hermaphrodita*
8 (DMG0007) (B), *P. hermaphrodita* (DMG0008) (C) and *P. californica* (DMG0019)
9 (D) that were found in mucus of *C. aspersum* collected from Formby, Liverpool,
10 Thurso, Whitby and Halifax or the control (water) or the application point. Significant
11 differences between the numbers of nematodes found in mucus and the control at $p <$
12 0.05 are denoted by * and at $p < 0.001$ denoted by **, n.s. means non-significant ($p >$
13 0.05). Bars represent \pm one standard error.

14

15 Fig 3. The mean numbers of *P. hermaphrodita* (DMG0007) (A), *P. neopapillosa*
16 (DMG0014) (B) and *P. californica* (DMG0019) (C) that were found in 0, 10, 50 and
17 100 mM of FeSO₄ (black bars), ZnSO₄ (white bars), MgSO₄ (dark grey bars) and
18 CuSO₄ (light grey bars). Significant differences between the numbers of nematodes
19 found in 0 and 10, 50 or 100 mM are denoted by * at $p < 0.05$, n.s. means non-
20 significant ($p > 0.05$). Bars represent \pm one standard error.

21

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

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

3

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

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11 Fig 6. The mean numbers of untreated or treated adult *P. hermaphrodita* (DMG0007)
12 (black bars), *P. neopapillosa* (DMG0014) (white bars) and *P. californica* (DMG0019)
13 (grey bars) found in mucus from *C. aspersum*. Pharmacological treatment consisted of
14 1-hour exposure to 500 μ M 8-bromo-cGMP. Significant differences between the
15 numbers of nematodes found in mucus and the control at $p < 0.05$ are denoted by *
16 and at $p < 0.001$ denoted by **, n.s. means non-significant ($p > 0.05$). Bars represent
17 \pm one standard error.

18

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

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