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Behavioural avoidance by slugs and snails of the parasitic nematode *Phasmarhadditis hermaphrodita*

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3 **Behavioural avoidance by slugs and snails of the parasitic**
4 **nematode *Phasmarhabditis hermaphrodita***

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19 **Abstract**

20 The nematode *Phasmarhabditis hermaphrodita* has been developed as a biological control
21 agent for slugs and snails. Slugs avoid areas where *P. hermaphrodita* is present. We
22 investigated whether behavioural avoidance of *P. hermaphrodita* is a common feature of
23 slugs and snails by exposing eight species to *P. hermaphrodita*. We showed that slugs
24 generally avoided *P. hermaphrodita* whereas snails did not. We also showed that slugs
25 specifically avoided the commercial strain and a natural isolate of *P. hermaphrodita* and were
26 not deterred by other nematodes such as *Steinernema kraussei* or *Turbatrix aceti*. We also
27 showed that slugs avoided the dauer stage of *P. hermaphrodita* and not mixed stage cultures.
28 Furthermore, slugs do not avoid dead *P. hermaphrodita* or exudates from live nematodes.
29 Taken together, we have unravelled further factors that are essential for slugs to avoid *P.*
30 *hermaphrodita* in soil, which could have important implications for the biological control of
31 slugs and snails.

32

33 **Keywords**

34 *Phasmarhabditis hermaphrodita*, nematodes, slugs, snails, behaviour, avoidance

35 1. Introduction

36 Slugs and snails cause damage to arable, vegetable and horticultural crops by
37 reducing leaf area by eating stems and leaves (Glen and Moens, 2002; Port and Ester, 2002;
38 Port and Port, 1986; South, 1992). They are usually controlled by applications of chemical
39 bait pellets containing metaldehyde, methiocarb and iron phosphate (Purves and Bannon,
40 1992; Speiser and Kistler, 2002). Other methods of slug control have been developed
41 including the parasitic nematode *Phasmarhabditis hermaphrodita* DMG0001 which is a
42 lethal parasite of numerous slug and snail species such as *Deroceras reticulatum*, *Arion ater*,
43 and *Helix aspersa* (Wilson et al., 1993; Glen et al., 1996). Formulations of *P. hermaphrodita*
44 DMG0001 are sold as Nemaslug[®] by Becker Underwood-BASF Agricultural Specialties and
45 are routinely used by farmers and gardeners (Rae et al., 2007). Nematodes are mixed with
46 water and applied using spraying equipment to soil where they search for hosts. They are
47 attracted to mucus and faeces from slugs (Rae et al., 2006, 2009) and upon discovery they
48 enter the shell cavity beneath the dorsal surface of the mantle and kill the slug between 4 and
49 21 days later (Wilson et al., 1993; Tan and Grewal, 2001a). *P. hermaphrodita* DMG0001 has
50 been used successfully to protect against slug damage in oilseed rape (Wilson et al., 1995),
51 winter wheat (Wilson et al., 1994a), strawberries (Glen et al., 2000a), asparagus (Ester et al.,
52 2003a), orchids (Ester et al., 2003b) and hostas (Grewal et al., 2001).

53 Slugs such as *Deroceras reticulatum* and *Arion ater* avoid *P. hermaphrodita*
54 DMG0001 in soil and spend less time feeding and resting where these nematodes have been
55 applied (Wilson et al., 1999). Although this avoidance behaviour is an interesting phenomenon
56 it is uncharacterised and there remain many questions to be answered. For example, what
57 other slug and snail species avoid *P. hermaphrodita* DMG0001? Are susceptible species
58 more likely to avoid *P. hermaphrodita* DMG0001 than resistant gastropods? Do other

59 nematodes provoke such an extreme behavioural avoidance? Also does *P. hermaphrodita*
60 DMG0001 secrete a chemical or exudate that the slugs detect?

61 **2. Materials and methods**

62 **2.1 Source and maintenance of invertebrates**

63 Slugs species used in this study were: *Arion subfuscus*, *A. ater*, *Deroceras reticulatum*,
64 *D. panormitanum*, *Limax flavus* and *Lehmannia valentiana*, which were collected from
65 Festival gardens in Liverpool and in greenhouses at Liverpool John Moores University
66 (LJMU). Snails used in the study consisted of *Cepaea nemoralis* and *H. aspersa*. Slugs and
67 snails were kept in non-airtight containers with moist tissue paper and were fed lettuce and
68 cabbage *ad libitum*. Nematodes (*Steinernema kraussei* and *P. hermaphrodita* DMG0001 were
69 supplied by Becker Underwood BASF Agricultural Specialities. *Turbatrix aceti* was supplied
70 by Sciento, U.K. All nematodes were kept at 15°C until use. A natural strain of *P.*
71 *hermaphrodita* was isolated from a separate study looking at the distribution of
72 *Phasmarhabditis* species from around the U.K. This strain, designated “AB38”, was
73 dissected from a collected *L. flavus* from Aberdeen and was identified using DNA sequencing
74 of the 18SrRNA gene. It was grown on rotting *L. flavus* in White traps for 28 days at 20°C.

75 **2.2 Behavioural avoidance assay exposing slugs and snails to *P. hermaphrodita***

76 DMG0001

77 We used a similar assay to Wilson et al. (1999). Briefly, three non-airtight plastic
78 boxes (9 x 24 x 6 cm) were half filled with moist peat soil (approx. 50 g) and fitted with
79 copper tape to stop slugs escaping *P. hermaphrodita* DMG0001. Nematodes were evenly
80 applied to one side (4.5 x 12 cm) at a rate of 120 nematodes per cm² (Wilson et al., 1999). To
81 the other side an equal volume of water was added. Five slugs or snails were added to the

82 middle of each of the three boxes and stored at 18°C. Three discs of fresh cabbage (diameter
83 3.5 cm) were added to each side of each box and replaced every two days. The number of
84 slugs or snails resting on each side was monitored every 12 hours for 4 days. Once recorded,
85 slugs and snails were placed back to the midline of each assay box. Each experiment was
86 repeated twice. The same soil bioassay was used to investigate whether slugs would avoid
87 other nematodes such as *S. kraussei*, *P. hermaphrodita* AB38 and *T. aceti*.

88

89 **2.3 Assessing the effect of heat killed nematodes, different life stages and nematode** 90 **suspension on slug behaviour**

91 For further experiments we concentrated on using just one species of slug (*A.*
92 *subfuscus*) as it was readily available around LJMU gardens. In order to investigate whether
93 nematodes have to be alive to induce behavioural avoidance in *A. subfuscus*, *P.*
94 *hermaphrodita* DMG0001 were heat killed by placing nematodes at 80°C for 20 mins and the
95 same soil bioassay was used as above. This temperature and time was chosen as in
96 preliminary studies all nematodes were deceased after 20 mins even with high numbers of *P.*
97 *hermaphrodita* DMG0001 present. To test whether *P. hermaphrodita* DMG0001 released a
98 compound into the environment which slugs and snails avoided approximately 15,000 alive *P.*
99 *hermaphrodita* DMG0001 were placed in PBS buffer, mixed and stored in non-airtight
100 plastic boxes (9 x 24 x 6 cm) at 18°C for 7 days. We incubated the nematodes for 7 days as
101 this should be sufficient time to release any potential exudates that slugs may avoid. We then
102 harvested the supernatant of the suspension by centrifugation at 5,000 rpm for 10 mins and
103 applied at the treated side of the soil assay compared with PBS, which was applied to the
104 other side. In order to discover whether slugs were deterred by other life stages of *P.*
105 *hermaphrodita* DMG0001 we grew nematodes on rotting *L. flavus* which had previously

106 been killed by placing it at -80°C for 20 mins. We grew the nematodes for 5 days after which
107 cultures consisted of mixed life stages of J1-J4 and adult stages. The nematodes were washed
108 several times in PBS, quantified and applied to the soil bioassay at the same rate as used in
109 previous experiments. In each of these experiments, three replicate boxes were used and the
110 experiment was repeated twice.

111

112 **2.4 Quantification of movement of *P. hermaphrodita* DMG0001 in soil bioassay**

113 We also determined how far *P. hermaphrodita* DMG0001 could move throughout the
114 soil during the 4-day experiment as this may affect the avoidance behaviour of the slugs as
115 the nematodes possibly were not confined to one location. *P. hermaphrodita* DMG0001 were
116 evenly applied to one side of the soil bioassay (4.5 x 12 cm) at a rate of 30 nematodes per
117 cm². Over 1, 2, 3 and 4 days, 3 soil samples (approx. 1-2 g) were removed from 3 different
118 boxes from 0-2, 4-6 and 10-12 cm from the midline of the box and the numbers of *P.*
119 *hermaphrodita* DMG0001 were extracted via sugar solution centrifugation and quantified.
120 The experiment was repeated 3 times. Overall, this revealed how far *P. hermaphrodita*
121 DMG0001 moved throughout the soil in the bioassay.

122

123 **2.5 Data analysis**

124 Numbers of slugs or snails on each side of the assay box were recorded every 12
125 hours for 4 days. The mean number of slugs on each side over 4 days was compared using a
126 Two Way Repeated Measures ANOVA. Movement of *P. hermaphrodita* DMG0001 was
127 analysed using a One-way Analysis of Variance (ANOVA).

128

129 3. Results

130 3.1 Slugs but not snails avoided *P. hermaphrodita* DMG0001

131 *D. reticulatum* and *D. panormitanum* are rapidly killed by *P. hermaphrodita*
132 DMG0001 (Wilson et al., 1993) and avoided the nematode as significantly more slugs were
133 recorded in the untreated than nematode-treated half of the box ($P < 0.001$, Fig 1). The slug
134 species *A. ater* and *A. subfuscus* are only killed by *P. hermaphrodita* DMG0001 when
135 juveniles but adult slugs (which were used in our assays) are resistant but still avoided the
136 nematode ($P < 0.001$, Fig 1). Similarly, *L. valentiana* is not killed by *P. hermaphrodita*
137 DMG0001 (Dankowska, 2006; Ester et al., 2003b) but also is deterred by it ($P < 0.001$). In
138 contrast to these species, *L. flavus* is resistant to *P. hermaphrodita* DMG0001 (Rae et al.,
139 2008) but does not avoid the nematode (Fig 1) as similar numbers of slugs were recorded in
140 the untreated and nematode-treated halves of the boxes ($P > 0.05$). The snail species used in
141 this study (*C. nemoralis* and *H. aspersa*) are resistant to *P. hermaphrodita* DMG0001 and
142 remain alive even when exposed to high doses of this nematode (Rae et al., 2009, Wilson et
143 al., 2000). Equal numbers of *H. aspersa* and *C. nemoralis* were recorded in the untreated and
144 nematode-treated halves of the boxes ($P > 0.05$, Fig 1). In general, slugs avoided *P.*
145 *hermaphrodita* DMG0001 but snails were not deterred by the nematode.

146 3.2 *A. subfuscus* avoided *P. hermaphrodita* DMG0001 but not other nematodes

147 *A. subfuscus* also avoided the recently isolated natural strain of *P. hermaphrodita*
148 AB38 as significantly more slugs were recorded in the untreated and nematode-treated halves
149 of the boxes (Fig 2) ($P < 0.001$). In contrast, *A. subfuscus* did not avoid either *T. aceti* or *S.*
150 *kraussei* and an equal number of slugs were recorded in the untreated and nematode-treated
151 halves of the boxes ($P > 0.05$) (Fig 2). Therefore, *A. subfuscus* can differentiate between

152 nematode species and is deterred specifically by *P. hermaphrodita* (strains AB38 and
153 DMG0001).

154 **3.3 Factors affecting behavioural avoidance of *A. subfuscus* exposed to treated *P.*** 155 ***hermaphrodita* DMG0001**

156 *A. subfuscus* did not avoid heat killed *P. hermaphrodita* DMG0001 ($P>0.05$) (Fig 3)
157 or the supernatant of a suspension of *P. hermaphrodita* DMG0001 ($P>0.05$) (Fig 3).
158 Therefore, in order to avoid these nematodes they must be alive and in contact with *A.*
159 *subfuscus*. Also when *P. hermaphrodita* DMG0001 was applied as a mixture of J1-J4 and
160 adult stages *A. subfuscus* did not exhibit avoidance behaviour and equal numbers of slugs
161 were recorded in the untreated and nematode-treated halves of the boxes $P>0.05$) (Fig 3).
162

163 **3.4. Movement of *P. hermaphrodita* DMG0001 in the soil bioassay**

164 Over the four days of the assay the numbers of *P. hermaphrodita* DMG0001 that
165 moved from the nematode side to the water treated side was negligible (Table 1). There was
166 no significant difference between the numbers of *P. hermaphrodita* DMG0001 moving from
167 the nematode treated side to 0-2, 4-6 and 10-12 cm on the untreated side of the boxes over 4
168 days ($P>0.05$). Only 0.78 ± 0.46 nematodes per gram of soil had moved onto the water
169 treated side after 4 days. These results are similar results to Wilson et al. (1999), who found *P.*
170 *hermaphrodita* DMG0001 remained within 2 cm of the point of application. Therefore,
171 natural nematode movement did not affect the results of the bioassay.

172

173 **4. Discussion**

174 Avoidance behaviour is the first line of defence used by free-living animals in their
175 struggle to maintain fitness in the face of parasite threat, and is likely the most cost-effective
176 strategy as compared to resistance and tolerance (Curtis, 2014). Avoidance behaviour can be
177 defined as the actions taken by an animal (or group of animals) to reduce its (or their)
178 chances of becoming infected with pathogens or parasites (Curtis, 2014) and is widespread in
179 the animal kingdom. For example, pine weevils (*Hylobius abietus*) avoid areas where
180 *Steinernema carpocapsae* are present (Ennis et al., 2010). The model organism
181 *Caenorhabditis elegans* avoids pathogenic bacteria such as *Bacillus thuringiensis*
182 (Schulenburg and Muller, 2004). Bumblebees (*Bombus terrestris*) avoid flowers
183 contaminated with *Escherichia coli* (Fouks and Lattorff, 2011). Rainbow trout
184 (*Oncorhynchus mykiss*) avoid flukes (*Diplostomum spathaceum*) that cause eye infections
185 (Karvonen et al., 2004). Here we have shown that slugs avoided *P. hermaphrodita* (both
186 strains DMG0001 and AB38) but snails did not. We were interested to discover if gastropods
187 that are susceptible to *P. hermaphrodita* DMG0001 were more likely to avoid the nematode.
188 Slugs such as *D. reticulatum* and *D. panormitanum* and juveniles of *A. ater* and *A. subfuscus*
189 are susceptible to *P. hermaphrodita* DMG0001 (Wilson et al., 1993; Tan and Grewal, 2001a;
190 Rae et al., 2009) and avoided the nematode. In comparison to susceptible slug species the
191 behavioural response of resistant slug species differed. For example, *L. valentiana* is resistant
192 to *P. hermaphrodita* DMG0001 (Dankowska, 2006; Ester et al., 2003b;) but is deterred by the
193 nematode. Also *L. flavus* is resistant to *P. hermaphrodita* DMG0001 (Rae et al., 2008) and
194 did not show avoidance behaviour. The snails tested (*H. aspersa* and *C. nemoralis*) did not
195 avoid *P. hermaphrodita* DMG0001 but as both snails used in this study are resistant to *P.*
196 *hermaphrodita* DMG0001 it is perhaps not surprising that these species would not avoid it
197 (Rae et al., 2009; Wilson et al., 2000).

198 *A. subfuscus* only avoided dauer stage *P. hermaphrodita* DMG0001 and was not
199 deterred by mixed stage nematodes. As the dauer stage is the life cycle stage responsible for
200 killing slugs and juvenile or adult stages cannot infect slugs (Tan and Grewal, 2001a) it
201 seems reasonable to presume slugs would avoid this stage exclusively. We also showed that
202 slugs were not repelled by the supernatant of a liquid suspension of *P. hermaphrodita*
203 DMG0001. This suggests that nematodes were probing the slug's body and trying to
204 penetrate inside and the slugs were avoiding the mechanical stimulus rather than a chemical
205 cue.

206 We could also show that *A. subfuscus* specifically avoided both the commercial and
207 recently isolated strain of *P. hermaphrodita* (strains DMG0001 and AB38) and did not avoid
208 other nematodes such as *S. kraussei* or *T. aceti*. Entomopathogenic nematodes (EPNs) such as
209 *S. kraussei*, harbour symbiotic bacteria in their intestines that are transported into insect hosts
210 and released which kills the insect in 24-48 hours (Forst et al., 1997). EPNs cannot kill slugs
211 (Wilson et al., 1994b) therefore there is no need for them to avoid these parasites in soil.
212 Similarly, there are no reports of *T. aceti* parasitizing slugs or snails; hence there is no need
213 for gastropods to avoid them either.

214 Other factors have to be taken into account when trying to understand why slugs
215 avoid *P. hermaphrodita* DMG0001. For example, the commercial strain of *P. hermaphrodita*
216 DMG0001 is grown on the bacterium *Moraxella osloensis* and is thought to be responsible
217 for slug death (Tan and Grewal, 2001b). The slugs could possibly be avoiding *M. osloensis*
218 present in the nematodes and future research will investigate this possibility. Also another
219 caveat is that slugs follow slime trails of similar species to find mates, aggregate and avoid
220 desiccation (Ng et al., 2013). As we only looked at groups of slugs together and did not carry
221 out experiments with individual slugs perhaps when slugs began moving towards one side
222 others would follow.

223 Our results may have important implications for biocontrol and the use of *P.*
224 *hermaphrodita* DMG0001 in controlling slugs in the field. For example, two outdoor plot
225 trials have shown that a reduced application of *P. hermaphrodita* DMG0001 has the potential
226 to reduce slug damage in Chinese cabbage and winter wheat due to slugs being deterred by
227 areas applied with nematodes (Hass et al., 1999a,b). These studies concentrated on just one
228 slug species (*D. reticulatum*) but our data shows that other pestiferous slug species such as *A.*
229 *subfuscus*, *D. panormitanum* and *L. valentiana* (as well as *A. ater* and *D. reticulatum*) would
230 also be deterred by *P. hermaphrodita* DMG0001.

231

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235

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333 **Figure legends**

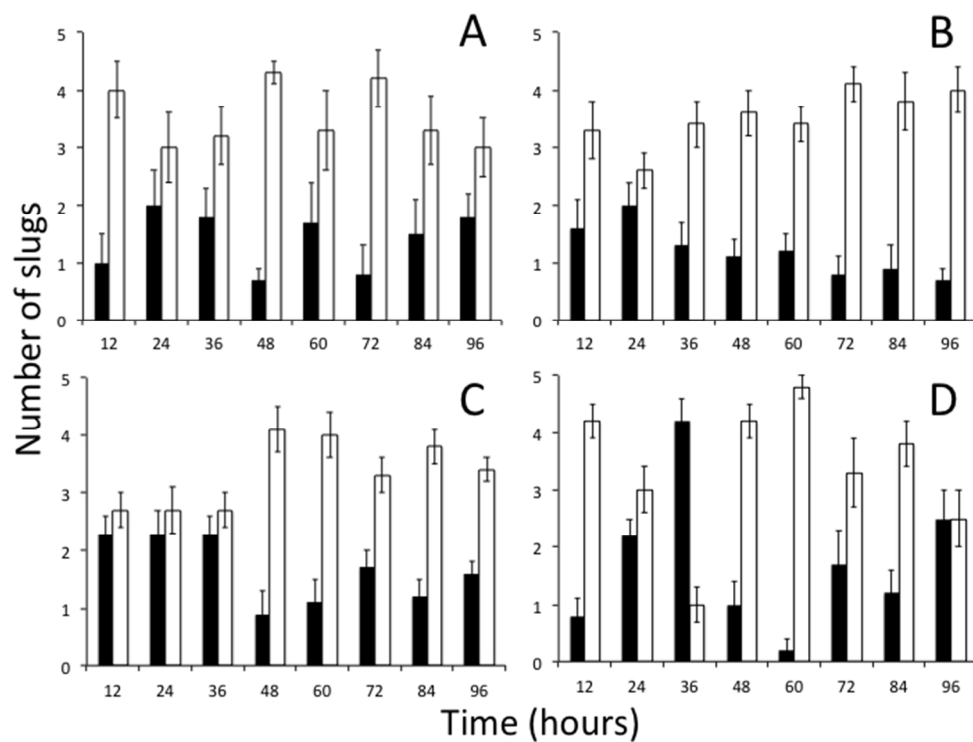
334 Fig 1. Numbers of slugs or snails recorded on each side of soil bioassay treated with either *P.*
335 *hermaphrodita* DMG0001 (black) or no nematodes (white) every 12 hours for 4 days. Slug
336 and snail species tested include *D. reticulatum* (A), *D. panormitanum* (B), *A. ater* (C), *A.*
337 *subfuscus* (D), *L. valentiana* (E), *L. flavus* (F), *C. nemoralis* (G) and *H. aspersa* (H). Bars
338 represent \pm one standard error.

339 Fig 2. Numbers of *A. subfuscus* recorded on each side of soil bioassay treated with either
340 nematodes (black) or on the side with no nematodes (white) every 12 hours for 4 days.
341 Different nematode species tested were: a natural isolate of *P. hermaphrodita* AB38 (A), *T.*
342 *aceti* (B) and *S. kraussei* (C). Bars represent \pm one standard error.

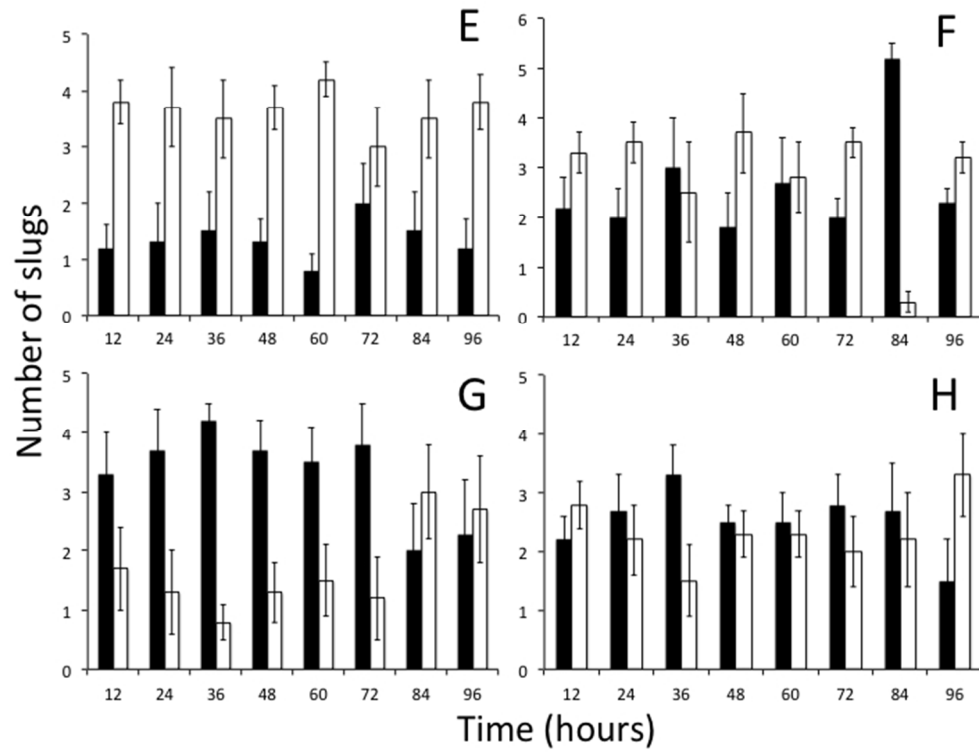
343 Fig 3. Numbers of *A. subfuscus* recorded on each side of soil bioassay treated with either
344 nematodes (black) or on the side with no nematodes (white) every 12 hours for 4 days. *A.*
345 *subfuscus* were exposed to heat killed *P. hermaphrodita* DMG0001 (A), the supernatant of a
346 suspension of *P. hermaphrodita* DMG0001 (B) and mixed stage *P. hermaphrodita*
347 DMG0001 (C). Bars represent \pm one standard error.

348 **Table legend**

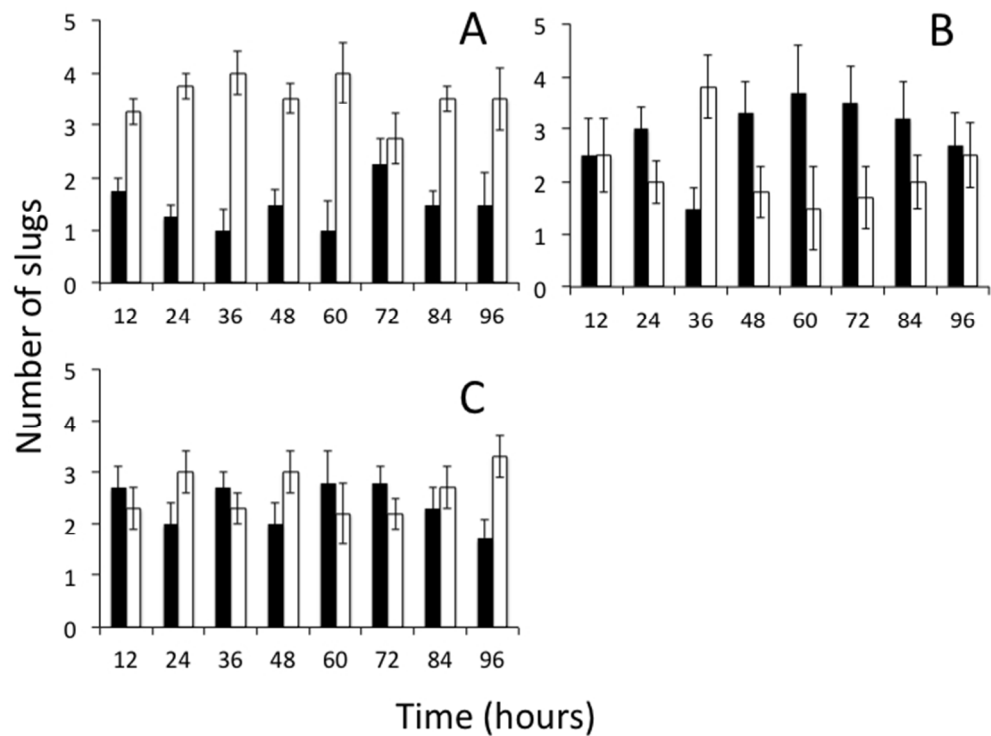
349 Table 1. Mean numbers of *P. hermaphrodita* DMG0001 per 1-2 g of soil in behavioural
350 bioassay monitored daily for 1, 2, 3 and 4 days. Nematodes were extracted from 0-2, 4-6 and
351 10-12 cm from midline in water treated and nematode treated sides.



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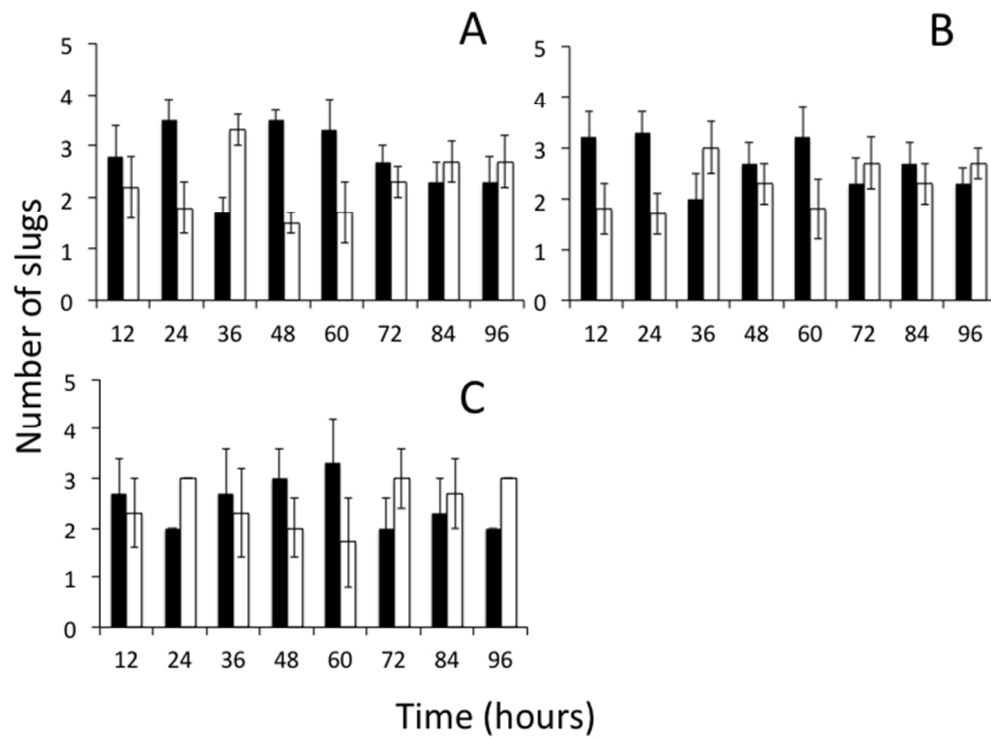


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Treatment	Day	Distance* from midline	Numbers of <i>P. hermaphrodita</i> (\pm st. err)
Water treated side	1	0-2	0 \pm 0
		4-6	0 \pm 0
		10-12	0 \pm 0
	2	0-2	0.11 \pm 0.11
		4-6	0 \pm 0
		10-12	0 \pm 0
	3	0-2	0.33 \pm 0.24
		4-6	0 \pm 0
		10-12	0 \pm 0
	4	0-2	0.8 \pm 0.46
		4-6	0 \pm 0
		10-12	0 \pm 0
Nematode treated side	1	0-2	12.1 \pm 3.34
		4-6	6.6 \pm 1.73
		10-12	2.4 \pm 0.73
	2	0-2	17.3 \pm 2.36
		4-6	7.7 \pm 1.12
		10-12	3.7 \pm 0.62
	3	0-2	24.2 \pm 2.85
		4-6	11.2 \pm 1.26
		10-12	9.8 \pm 3.57
	4	0-2	5.4 \pm 1.12
		4-6	5.9 \pm 0.98
		10-12	3 \pm 0.76

*Distance in centimeters

254x190mm (96 x 96 DPI)

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