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3 Susceptibility of the Giant African Snail (*Achatina*
4 *fulica*) exposed to the gastropod parasitic
5 nematode *Phasmarhabditis hermaphrodita*

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14

15 **Abstract**

16 The Giant African snail (*Achatina fulica*) is a major pest in tropical countries. Current control
17 methods involve the use of slug pellets (metaldehyde) but they are ineffective, therefore new
18 methods of control are needed. We investigated whether *A. fulica* is susceptible to the
19 gastropod parasitic nematode *Phasmarhabditis hermaphrodita*, which has been developed as
20 a biological control agent for slugs and snails in northern Europe. We exposed *A. fulica* to *P.*
21 *hermaphrodita* applied at 30 and 150 nematodes per cm² for 70 days and also assessed
22 feeding inhibition and changes in snail weight. We show that unlike the susceptible slug
23 species *Deroceras panormitanum*, which is killed less than 30 days of exposure to *P.*
24 *hermaphrodita*, *A. fulica* is remarkably resistant to the nematode at both doses. Also *P.*
25 *hermaphrodita* does not reduce feeding in *A. fulica* nor did it have any effect on weight gain
26 over 70 days. Upon dissection of infected *A. fulica* we found that hundreds of *P.*
27 *hermaphrodita* had been encapsulated, trapped and killed in the snail's shell. We found that *A.*
28 *fulica* is able to begin encapsulating *P. hermaphrodita* after just 3 days of exposure and the
29 numbers of nematodes encapsulated increased over time. Taken together, we have shown that
30 *A. fulica* is highly resistant to *P. hermaphrodita*, which could be due to an immune response
31 dependent on the snail shell to encapsulate and kill invading parasitic nematodes.

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33 **Keywords**

34 Nematodes, Giant African snail, shell, parasites, *Phasmarhabditis hermaphrodita*, *Achatina*
35 *fulica*

36

37 **Introduction**

38 Slugs and snails are serious pests in agriculture and cause damage to arable, vegetable
39 and horticultural crops by reducing leaf area, eating stems and leaves as well as
40 contaminating crops with their slime, bodies and faeces (Glen and Moens, 2002; Port and
41 Ester, 2002; Port and Port, 1986; South, 1992). One particularly problematic species is the
42 Giant African snail (*Achatina fulica*), which is a devastating pest of farms and gardens in
43 tropical and subtropical regions. It is an opportunistic, omnivorous and voracious eater which
44 can consume 10% of its own weight daily (Schreurs, 1963). Also *A. fulica* can transmit
45 metastrongylid causative agents of eosinophilic meningoencephalitis e.g. *Angiostrongylus*
46 *cantonensis* (Raut and Barker, 2002). Current control methods have relied on chemical bait
47 pellets containing metaldehyde and methiocarb but have provided limited control (Raut and
48 Barker, 2002). Also chemical bait pellets are both toxic to vertebrates (Homeida and Cooke,
49 1983; Fletcher et al., 1991; 1994) and methiocarb is toxic to beneficial invertebrates such as
50 earthworms and carabid beetles (Purves and Bannon, 1992). Therefore, new more effective
51 means of controlling *A. fulica* are needed.

52 A possible solution for controlling *A. fulica* is the gastropod parasitic nematode
53 *Phasmarhabditis hermaphrodita*. *P. hermaphrodita* is a lethal parasite to numerous slugs and
54 snail species such as *Deroceras reticulatum*, *Arion ater*, and *Helix aspersa* (Wilson et al.,
55 1993) and has been formulated into a biocontrol agent (Nemaslug®) by Becker Underwood-
56 BASF available for farmers and gardeners (Wilson et al., 1993). Nematodes are mixed with
57 water and applied using spraying equipment to soil where they go and search for potential
58 gastropod hosts. They are attracted to slug mucus and faeces (Rae et al., 2006,2009) and upon
59 discovery they penetrate through the slugs mantle and kill it between 4 and 21 days (Wilson
60 et al., 1993; Tan and Grewal, 2001a). Initially it was thought that these nematodes acted as
61 vectors that introduced the bacterium *Moraxella osloensis* into the haemocoel of the slug

62 which caused septicaemia and subsequent death (Tan and Grewal, 2001b) but it has recently
63 been shown that this bacterium is not important for pathogenicity (Rae et al., 2010). *P.*
64 *hermaphrodita* has been used successfully to protect against slug damage in oilseed rape
65 (Wilson et al., 1995), winter wheat (Wilson et al., 1994), strawberries (Glen et al., 2000a),
66 asparagus (Ester et al., 2003a), Brussels sprouts (Ester et al., 2003b), orchids (Ester et al.,
67 2003c) and hostas (Grewal et al., 2001).

68 The host range of *P. hermaphrodita* is not completely understood and some snail
69 species are resistant e.g. *Cepaea nemoralis*, *Oxychilus helveticus*, *Discus rotundatus* and
70 *Clausilia bidentata* (Wilson et al., 2000; Coupland, 1995). However, this resistance can
71 depend on the size and age of snail. For example *H. aspersa* juveniles are susceptible to *P.*
72 *hermaphrodita* but adults are resistant (Glen et al., 1996). We sought to understand whether
73 young stages of *A. fulica* would be susceptible to *P. hermaphrodita* applied at two different
74 doses, as well as investigating whether it would reduce feeding and cause defects in weight
75 gain, a commonly observed symptom of infection in slugs (e.g. Glen et al., 2000b). We also
76 exposed the slug *Deroceras panoramitanum* to assess the virulence of *P. hermaphrodita* to
77 compare to *A. fulica*. Finally, we also sought to understand how many *P. hermaphrodita* had
78 penetrated into *A. fulica* over time and whether they could be encapsulated in the snails shell
79 over 70 days of exposure to nematodes.

80

81 **Materials and Methods**

82

83 **Source and maintenance of invertebrates**

84 Slugs (*Deroceras panormitanum*) (mean weight 0.06 g \pm 0.03, n =90) were collected from
85 LJMU greenhouses. *A. fulica* (mean weight 0.96 g \pm 0.02 g, n =90) were grown from eggs
86 and maintained at room temperature and fed butternut squash and calcium tablets every 7
87 days for 12 weeks. The eggs were F1 progeny from a cross between two *A. fulica* (Zena and
88 Brian), which are personal pets of Dr. Sally Williamson at LJMU. *P. hermaphrodita* was a
89 gift from BASF, UK. Slugs were maintained at 15°C in non-airtight plastic boxes and fed
90 with cabbage *ad libitum*. Nematodes were stored at 15°C until use.

91

92 **Susceptibility assays exposing slugs and snails to *P. hermaphrodita***

93 Nine non-airtight plastic boxes (10 x 9 x 6 cm) were filled with 35 grams of pre-moistened
94 peat soil. Copper tape was placed around the sides of each box to ensure the slugs and snails
95 did not escape nematode infection. To three boxes the recommended rate of *P.*
96 *hermaphrodita* was applied (30 nematodes per cm²) and to another three boxes five times the
97 recommended rate of *P. hermaphrodita* was applied (150 nematodes per cm²). Three boxes
98 received water and no nematodes and acted as the no nematode control. Ten 12 week old *A.*
99 *fulica* were added to each box and were stored at 18°C. The individual weight of each snail
100 was recorded pre addition to soil bioassay. This same experimental set up was repeated with
101 *D. panormitanum* to assess the pathogenicity of the batch of *P. hermaphrodita* used. Survival
102 of slugs and snails was monitored daily for 30 and 70 days, respectively. We also
103 investigated when *A. fulica* began encasing *P. hermaphrodita* by carrying out a time course
104 experiment. By using the above described experimental set up *A. fulica* were exposed to 30
105 and 150 *P. hermaphrodita* per cm² and after 1, 3, 6, 13 and 23 days exposure 10 *A. fulica*
106 were dissected and the numbers of *P. hermaphrodita* found encased in the shell as well as in
107 the snail were determined.

108 **Monitoring feeding inhibition of *A. fulica* exposed to *P. hermaphrodita***

109 At the start of the experiment five discs of cucumber (diameter 3 cm) were added to each box
110 before the addition of snails. Initially every day for seven days the discs were removed and
111 traced onto 1 x 1 mm graph paper and the amount the snail had eaten was quantified for each
112 treatment. After 7 days feeding inhibition was then monitored every 2-3 days. Once
113 cucumber was removed and quantified fresh discs were added.

114

115 **Statistical analysis**

116 Survival of *A. fulica* exposed to *P. hermaphrodita* was analysed using the Log rank test and
117 Kaplan Meier curves in OASIS (Yang et al., 2011). The amount of cucumber eaten by *A.*
118 *fulica* was compared using two way ANOVA. Weight of slugs and snails at the start and end
119 of the experiment and the numbers of *P. hermaphrodita* found in snail shells was compared
120 using student t test. All statistical tests were carried out in SPSS version 20.

121 **Results**

122 *Susceptibility of A. fulica and D. panormitanum to P. hermaphrodita*

123 We exposed both *D. panormitanum* and *A. fulica* to the recommended rate (30 nematodes per
124 cm²) and five times the recommended rate (150 nematodes per cm²) of *P. hermaphrodita* to
125 investigate their susceptibility. We found that *P. hermaphrodita* caused significant mortality
126 to *D. panormitanum* at the recommended rate (P<0.001) and five times the recommended rate
127 of nematodes (P<0.001) (Fig 1a) after just 15 days. In contrast *P. hermaphrodita* had little
128 effect on the survival of *A. fulica* (Fig 1b). Specifically, after 70 days exposure there was no
129 significant difference between the survival of *A. fulica* exposed to *P. hermaphrodita* at the
130 recommended rate (P>0.05) or five times the recommended rate (P>0.05). Therefore even

131 young stages of *A. fulica* are resistant to *P. hermaphrodita* when exposed to high doses of
132 nematodes.

133

134 *Effects of feeding inhibition and weight on A. fulica when exposed to P. hermaphrodita*

135 We monitored feeding of *A. fulica* over 32 days and found that *P. hermaphrodita* was unable
136 to affect the amount of cucumber consumed when exposed to 30 and 150 nematodes per cm²
137 ($P < 0.05$) (Fig 2). As expected, there was a significant difference between the weight (g) of *A.*
138 *fulica* at the start of the experiment (day 0) compared to the end (day 70) in untreated and
139 nematode treated snails (both exposed to 30 and 150 nematodes per cm² ($P < 0.05$) (Fig. 3).
140 But *P. hermaphrodita* had no effect on weight gain in *A. fulica* as there was no significant
141 difference between the weight of *A. fulica* on day 70 exposed to *P. hermaphrodita* (30 or 150
142 nematodes per cm²) and the untreated snails ($P > 0.05$) (Fig 3). Therefore similarly to
143 survival *P. hermaphrodita* has little effect on *A. fulica* feeding or weight gain.

144 *Numbers of P. hermaphrodita infecting A. fulica over time*

145 Interestingly, upon dissection of *A. fulica* we found the majority of *P. hermaphrodita* were
146 not in the body of the snail but were encapsulated in the shell (Fig 4a,b). Numbers of *P.*
147 *hermaphrodita* encased in the snail shell differed significantly (Fig 4c) with 12.28 ± 2.87 *P.*
148 *hermaphrodita* found in *A. fulica* exposed to 30 *P. hermaphrodita* per cm² (n =29) and 164.17
149 ± 17 *P. hermaphrodita* found in *A. fulica* exposed to 150 *P. hermaphrodita* per cm² (n =29)
150 ($P < 0.001$) after 70 days. In a further experiment we could show that *P. hermaphrodita* began
151 encapsulation in the shell of *A. fulica* after just 3 days of exposure and the numbers of
152 nematodes encapsulated increased over the further 6, 13 and 23 days (Fig 5a). *P.*
153 *hermaphrodita* was also found in the body of *A. fulica* to varying degrees and did not show

154 any difference between dose of nematode or over time (Fig 5b). Thus, it seems one of the
155 reasons *A. fulica* is resistant to *P. hermaphrodita* is by encapsulating, trapping and killing of
156 these parasites in the snail's shell, a finding that has never been previously been observed in
157 snails.

158

159 **Discussion**

160 We concentrated on understanding whether young stages of *A. fulica* are susceptible
161 to *P. hermaphrodita* because differences in size susceptibility to slugs and snails have been
162 reported in the literature (Speiser et al., 2001). For example, younger stages of *Arion ater*,
163 *Helix aspersa* and *A. lusitanicus* have been shown to be more susceptible to *P.*
164 *hermaphrodita* than older stages (Glen et al., 1996; Grimm, 2002). We showed that *A. fulica*
165 is incredibly resistant to *P. hermaphrodita* and even after 70 days of exposure there was no
166 reduction in mortality in nematode treated *A. fulica*. This is in stark contrast to susceptible
167 species such as *D. panormitanum* as when it is exposed to *P. hermaphrodita* it is killed
168 rapidly in less than 15 days. Many snail species are susceptible to *P. hermaphrodita* including
169 young stages of *H. aspersa*, *Monacha cantiana*, *Cepaea hortensis*, *Theba pisana*, *Cochlicella*
170 *acuta* and *Cerņuella virgata* but some species are resistant including *C. nemoralis*, *O.*
171 *helveticus*, *D. rotundatus* and *C. bidentata* (Wilson et al., 2000; Coupland, 1995; Rae et al.,
172 2007). The reasons for this difference in susceptibility are unknown but it could potentially
173 be due to the ability to encase and trap invading nematodes. It is unknown where and how *P.*
174 *hermaphrodita* penetrates into snails. In slugs they infect through the pore at the back of the
175 mantle and enter the shell cavity within 8-16 hours of exposure (Wilson et al., 1993; Tan and
176 Grewal, 2001b), but there has not been an equivalent experiment carried out in snails so far.
177 We found that the nematodes were encapsulated and killed in the shell of *A. fulica*. Most of

178 these nematodes were not found in a particular area, some were found on the lip of the shell
179 but not in great amounts and after 70 days exposure most were equally dispersed over the
180 entire shell. We could show that *A. fulica* was able to encase and kill invading *P.*
181 *hermaphrodita* after 3 days exposure to nematodes and this process continued over time. The
182 trapped and killed nematodes were still present in the dauer stage and had not begun self-
183 reproduction. This response to nematodes in snails has not been documented before. In slugs
184 resistant slug species such as *Limax pseudoflavus* have been shown to encapsulate *P.*
185 *hermaphrodita* in the shell beneath the mantle (Rae et al., 2008), but it is unknown how
186 common this is in other slug species. Also there is limited research into using the shell as a
187 defense mechanism but a similar response has been shown in other molluscs. For example
188 mussels encapsulate trematodes such as *Aspidogaster conchicola* (Huehner and Etges, 1981).
189 Our results may therefore demonstrate an evolutionary conserved immune response that is
190 used to trap and kill invading *P. hermaphrodita* in terrestrial gastropods. Yet it remains to be
191 seen whether or not other parasites of slugs and snails are also trapped via the shell including
192 bacteria, microsporidia, mites or flies (Barker, 2004).

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197 **Figures and table legends**

198 Fig 1 a: Survival of *D. panormitanum* exposed to 30 (grey line) and 150 *P. hermaphrodita*
199 per cm² (black dashed line) and untreated control (no *P. hermaphrodita*) (black line). Bars \pm
200 represent one standard error.

201 Fig 1 b: Survival of *A. fulica* exposed to 30 (grey triangles) and 150 *P. hermaphrodita* per
202 cm² (white squares) and untreated control (no *P. hermaphrodita*) (black line). Bars ±
203 represent one standard error.

204 Fig 2: Percentage feeding inhibition of *A. fulica* fed on cucumber exposed to 30 (grey line)
205 and 150 *P. hermaphrodita* per cm² (black dashed line) and untreated control (no *P.*
206 *hermaphrodita*) (black line). Bars ± represent one standard error.

207 Fig 3: Weight of *A. fulica* at start of experiment (white bars) and after 70 days (black bars) of
208 exposure to 30 and 150 *P. hermaphrodita* per cm² and untreated control (no *P.*
209 *hermaphrodita*). Bars ± represent one standard error.

210 Fig 4a,b: *P. hermaphrodita* encased and killed in the shell of *A. fulica* after 70 days exposure.

211 Fig 4c: Numbers of *P. hermaphrodita* found encapsulated in shell of *A. fulica* after 70 days of
212 exposure to 30 *P. hermaphrodita* per cm² (n=29) and 150 *P. hermaphrodita* per cm² (n=29)
213 and untreated control (no *P. hermaphrodita*) (n=29). Bars ± represent one standard error.

214 Fig 5a: Numbers of *P. hermaphrodita* found encapsulated in shell of *A. fulica* after 1, 3, 6, 13
215 and 23 days of exposure to 30 and 150 *P. hermaphrodita* per cm²) (n=10, per time point).
216 Bars ± represent one standard error.

217 Fig 5b: Numbers of *P. hermaphrodita* found inside *A. fulica* after 1, 3, 6, 13 and 23 days of
218 exposure to 30 and 150 *P. hermaphrodita* per cm² (n=10, per time point). Bars ± represent
219 one standard error.

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