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

Williams, A.J. and Rae, R*

Liverpool John Moores University, School of Natural Sciences and Psychology, Byrom Street, Liverpool, L33AF, UK.

*Corresponding author: r.g.rae@ljmu.ac.uk,

Tel: +44(0)1512312486

Abstract

The Giant African snail (*Achatina fulica*) is a major pest in tropical countries. Current control methods involve the use of slug pellets (metaldehyde) but they are ineffective, therefore new methods of control are needed. We investigated whether *A. fulica* is susceptible to the gastropod parasitic nematode *Phasmarhabditis hermaphrodita*, which has been developed as a biological control agent for slugs and snails in northern Europe. We exposed *A. fulica* to *P. hermaphrodita* applied at 30 and 150 nematodes per cm^2 for 70 days and also assessed feeding inhibition and changes in snail weight. We show that unlike the susceptible slug species *Deroceras panormitanum*, which is killed less than 30 days of exposure to *P. hermaphrodita*, *A. fulica* is remarkably resistant to the nematode at both doses. Also *P. hermaphrodita* does not reduce feeding in *A. fulica* nor did it have any effect on weight gain over 70 days. Upon dissection of infected *A. fulica* we found that hundreds of *P. hermaphrodita* had been encapsulated, trapped and killed in the snail's shell. We found that *A. fulica* is able to begin encapsulating *P. hermaphrodita* after just 3 days of exposure and the numbers of nematodes encapsulated increased over time. Taken together, we have shown that *A. fulica* is highly resistant to *P. hermaphrodita*, which could be due to an immune response dependent on the snail shell to encapsulate and kill invading parasitic nematodes.

Keywords

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

Introduction

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

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

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

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

Materials and Methods

Source and maintenance of invertebrates

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

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

Nine non-airtight plastic boxes (10 x 9 x 6 cm) were filled with 35 grams of pre-moistened peat soil. Copper tape was placed around the sides of each box to ensure the slugs and snails did not escape nematode infection. To three boxes the recommended rate of *P. hermaphrodita* was applied (30 nematodes per cm^2) and to another three boxes five times the recommended rate of *P. hermaphrodita* was applied (150 nematodes per cm^2). Three boxes received water and no nematodes and acted as the no nematode control. Ten 12 week old *A. fulica* were added to each box and were stored at 18°C. The individual weight of each snail was recorded pre addition to soil bioassay. This same experimental set up was repeated with *D. panormitanum* to assess the pathogenicity of the batch of *P. hermaphrodita* used. Survival of slugs and snails was monitored daily for 30 and 70 days, respectively. We also investigated when *A. fulica* began encasing *P. hermaphrodita* by carrying out a time course experiment. By using the above described experimental set up *A. fulica* were exposed to 30 and 150 *P. hermaphrodita* per cm^2 and after 1, 3, 6, 13 and 23 days exposure 10 *A. fulica* were dissected and the numbers of *P. hermaphrodita* found encased in the shell as well as in the snail were determined.

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

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

Statistical analysis

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

Results

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

We exposed both *D. panormitanum* and *A. fulica* to the recommended rate (30 nematodes per cm^2) and five times the recommended rate (150 nematodes per cm^2) of *P. hermaphrodita* to investigate their susceptibility. We found that *P. hermaphrodita* caused significant mortality to *D. panormitanum* at the recommended rate ($P<0.001$) and five times the recommended rate of nematodes ($P<0.001$) (Fig 1a) after just 15 days. In contrast *P. hermaphrodita* had little effect on the survival of *A. fulica* (Fig 1b). Specifically, after 70 days exposure there was no significant difference between the survival of *A. fulica* exposed to *P. hermaphrodita* at the recommended rate ($P>0.05$) or five times the recommended rate ($P>0.05$). Therefore even

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

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

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

Numbers of P. hermaphrodita infecting A. fulica over time

Interestingly, upon dissection of *A. fulica* we found the majority of *P. hermaphrodita* were not in the body of the snail but were encapsulated in the shell (Fig 4a,b). Numbers of *P. hermaphrodita* encased in the snail shell differed significantly (Fig 4c) with 12.28 ± 2.87 *P. hermaphrodita* found in *A. fulica* exposed to 30 *P. hermaphrodita* per cm^2 (n =29) and 164.17 ± 17 *P. hermaphrodita* found in *A. fulica* exposed to 150 *P. hermaphrodita* per cm^2 (n =29) ($P<0.001$) after 70 days. In a further experiment we could show that *P. hermaphrodita* began encapsulation in the shell of *A. fulica* after just 3 days of exposure and the numbers of nematodes encapsulated increased over the further 6, 13 and 23 days (Fig 5a). *P. hermaphrodita* was also found in the body of *A. fulica* to varying degrees and did not show

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

Discussion

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

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

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Figures and table legends

Fig 1 a: Survival of *D. panormitanum* exposed to 30 (grey line) and 150 *P. hermaphrodita* per cm^2 (black dashed line) and untreated control (no *P. hermaphrodita*) (black line). Bars ± represent one standard error.

Fig 1 b: Survival of *A. fulica* exposed to 30 (grey triangles) and 150 *P. hermaphrodita* per cm^2 (white squares) and untreated control (no *P. hermaphrodita*) (black line). Bars ± represent one standard error.

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

Fig 3: Weight of *A. fulica* at start of experiment (white bars) and after 70 days (black bars) of exposure to 30 and 150 *P. hermaphrodita* per cm^2 and untreated control (no *P. hermaphrodita*). Bars ± represent one standard error.

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

Fig 4c: Numbers of *P. hermaphrodita* found encapsulated in shell of *A. fulica* after 70 days of exposure to 30 *P. hermaphrodita* per cm^2 (n=29) and 150 *P. hermaphrodita* per cm^2 (n=29) and untreated control (no *P. hermaphrodita*) (n=29). Bars ± represent one standard error.

Fig 5a: Numbers of *P. hermaphrodita* found encapsulated in shell of *A. fulica* after 1, 3, 6, 13 and 23 days of exposure to 30 and 150 *P. hermaphrodita* per cm^2) (n=10, per time point). Bars ± represent one standard error.

Fig 5b: Numbers of *P. hermaphrodita* found inside *A. fulica* after 1, 3, 6, 13 and 23 days of exposure to 30 and 150 *P. hermaphrodita* per cm^2 (n=10, per time point). Bars ± represent one standard error.

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