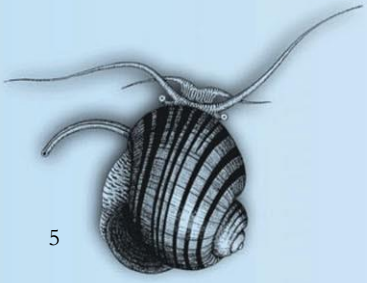

Journal of
Molluscan Studies

The Malacological Society of London

*Journal of Molluscan Studies* (2017): 1–4. doi:10.1093/mollus/eyx045

# Shell encapsulation of parasitic nematodes by *Arianta arbustorum* (Linnaeus, 1758) in the laboratory and in field collections

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(Received 28 July 2017; editorial decision 8 November 2017)

## ABSTRACT

There are 108 species of nematode that are known to have co-evolved with gastropods and use them as definitive, intermediate or paratenic hosts. One nematode (*Phasmarhabditis hermaphrodita*) is lethal to eight species of snail, but nine species are resistant for unknown reasons. This study investigated whether a previously untested snail species, *Arianta arbustorum*, was susceptible to *P. hermaphrodita*. Snails were exposed to 0, 30 and 90 *P. hermaphrodita* per $cm^2$ applied to the soil surface for 40 days. Survival, feeding inhibition and differences in weight were monitored. It was found that *A. arbustorum* was resistant to *P. hermaphrodita*. The nematode did not cause mortality, induce feeding inhibition or affect weight. At the end of the experiment, surviving *A. arbustorum* had encapsulated and killed the invading nematodes in their shells. Inspection of shells of *A. arbustorum* collected on sand dunes in the north of Scotland, and in those collected by others in 1966 and 1908, revealed the presence of encapsulated nematodes. *Arianta arbustorum* can encapsulate and kill invading nematodes under laboratory conditions and in the wild, and the evidence of encapsulation remains for a long time.

## INTRODUCTION

Nematodes are common parasites of terrestrial slugs and snails, and 108 species have been recorded using them as intermediate, definitive or paratenic hosts (Grewal *et al.*, 2003). One of these, *Phasmarhabditis hermaphrodita* is a lethal parasite of several species of slugs and snails (Wilson, Glen & George, 1993) and has been formulated into a biological control agent (Nemaslug®, available from BASF-Becker Underwood Agricultural Specialities; Rae *et al.*, 2007). The dauer larvae of the nematodes are mixed with water, then applied to soil where they hunt out and kill slugs in 4–21 days (Wilson *et al.*, 1993; Tan & Grewal, 2001). The treatment provides protection against slug damage in a range of important agricultural crops (Rae *et al.*, 2007).

While *P. hermaphrodita* can kill eight species of snail, nine are resistant for unknown reasons (Coupland, 1995; Glen *et al.*, 1996; Rae, Robertson & Wilson, 2009). Terrestrial molluscs are known to have a variety of defences against parasites and infections (Barker, 2004; Loker, 2010). It has been shown that some terrestrial snails trap, encase and kill the invading nematodes (Williams & Rae, 2015, 2016). The nematodes are targeted by cells that adhere to the nematode cuticle and fuse it to the inner surface of the shell (Rae, 2017). This process is similar to the immune response in mussels that encase trematodes like *Aspidogaster conchicola* in their shells (Huehner & Etges, 1981).

The aim of this research was to examine whether the previously untested snail *Arianta arbustorum* (Linnaeus, 1758) was susceptible to infection by *P. hermaphrodita*. *Arianta arbustorum* is common in the British Isles and northern and central Europe, living in moist woods, hedges and along river banks (Janus, 1965). It has been used as a model organism in ecology, ethology, morphology and evolutionary biology for over 20 years (Gittenberger, Piel & Groenenberg, 2004). Here, I monitored the survival of *A. arbustorum* when exposed to *P. hermaphrodita* in the laboratory; I examined whether the parasite could also induce feeding inhibition or affect weight (Glen *et al.*, 1996; Williams & Rae, 2015) and whether *A. arbustorum* could encase and kill nematodes in its shell. I extended this study by looking for evidence of encapsulation in shells of *A. arbustorum* collected from sand dunes from the north of Scotland. Finally, in order to investigate how long evidence of encapsulation persists in empty shells, I examined collections of *A. arbustorum* housed in Liverpool Museum.

## MATERIAL AND METHODS

### *Source of study animals*

To assess the susceptibility of *Arianta arbustorum* to *Phasmarhabditis hermaphrodita*, snails were collected from Dunnett Beach (GPS latitude: 58.62, longitude: −3.34) near Thurso, Scotland, and placed in non-airtight plastic boxes with premoistened paper and fed on lettuce *ad libitum*. *P. hermaphrodita* (Nemaslug®) was purchased from BASF and stored at 15 °C.

### *Infection of* A. arbustorum *by* P. hermaphrodita *under laboratory conditions*

Nine non-airtight plastic boxes (10 × 10 $cm^2$) were filled with *c.* 50 g of premoistened compost soil. The top 2 cm of the boxes

were lined with copper tape, which snails will not cross (Moens *et al.*, 1967), thus ensuring constant exposure to *P. hermaphrodita*. As a control, three boxes received water applied over the soil surface, while a further three boxes had nematodes applied at each of two doses (30 and 90 *P. hermaphrodita* per $cm^2$) over the soil surface (based on the field application rate of *P. hermaphrodita*; Rae *et al.*, 2007). Ten *A. arbustorum* (mean weight 1.84 ± 0.05 g; $n = 180$) were added to each box along with five discs of lettuce (3 cm diameter) and maintained at 20 °C. The weight of the individual snails was recorded at the start and the end of the experiment, which ran for 40 d. Survival of the snails was monitored every 4 d and any dead snails were removed. The amount of lettuce eaten by the snails in each box was quantified by tracing the remaining amounts on 1 × 1 $mm^2$ graph paper and lettuce was replaced every 4 d (Rae *et al.*, 2009; Williams & Rae, 2015). At the end of the experiment the inner aperture of all living snails was examined for nematodes fixed in the shell. This experiment was repeated twice.

### *Encasement of parasitic nematodes in field-collected* A. arbustorum *shells*

I also investigated whether wild *A. arbustorum* encapsulated and killed nematodes using their shell, by collecting 205 dead *A. arbustorum* from locations near those used by Cain, Cameron & Parkin (1969), including sand dunes at Strathy beach ($n = 139$) (GPS latitude: 58.56, longitude: −3.99), Durness Beach ($n = 16$) (GPS latitude: 58.58, longitude: −4.75) and Dunnett Beach ($n = 50$) (same location as previously mentioned). Using a dissecting microscope, shells were examined for any nematodes trapped in the inner surface of the aperture and last whorl.

I examined into how long encapsulated nematodes remained visible in shells by examining 73 *A. arbustorum* shells housed in Liverpool Museum. These shells were collected from four sites across England: Maidwell ($n = 20$; 1964); Ganton ($n = 13$; no record of year); Castleton ($n = 30$; 1966) and Wendover ($n = 10$; 1908).

### *Statistical analysis*

Survival of *A. arbustorum* after 40 d exposure to *P. hermaphrodita* at three concentrations was compared using a one-way ANOVA. The amount of lettuce eaten over 40 d was compared using a two-way ANOVA. The mean weights of *A. arbustorum* on day 0 and day 40 were compared using an unpaired Student's *t*-test. The numbers of nematodes found encased in shells on day 40 were compared using a one-way ANOVA.

## RESULTS

### *Survival of* Arianta arbustorum *exposed to different doses of* Phasmarhabditis hermaphrodita

At the end of the experiment, there was no significant effect on survival for *A. arbustorum* exposed to 0, 30 or 90 *P. hermaphrodita* per $cm^2$ ($P > 0.05$) (Fig. 1). Similarly, there was no significant difference between the amount of lettuce eaten by *A. arbustorum* during the experiment, with snails eating 100% of the lettuce every 4 d in all treatments ($P > 0.05$). Treatment with *P. hermaphrodita* did not affect the weight of the snails at either dosage of nematodes ($P > 0.05$) (Fig. 2).

When the shells of *A. arbustorum* were examined for the presence of nematodes after 40 d exposure at 30 and 90 *P. hermaphrodita* per $cm^2$, nematodes were found fused to the inner shell layer within the aperture and lip (Fig. 3; Table 1). No nematodes were found in the shells of the control snails that were not exposed to nematodes, apart from a single shell that had two small nematodes

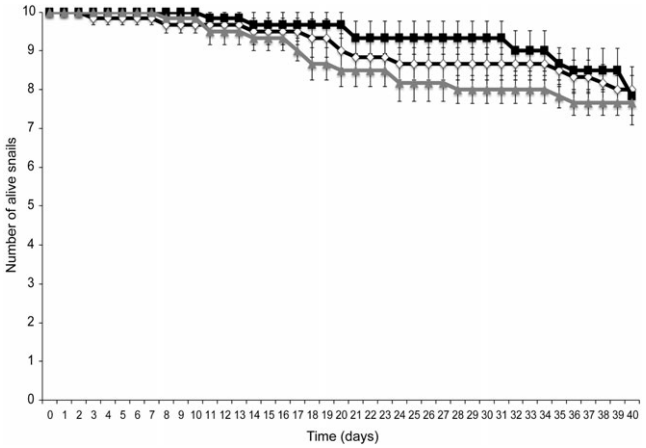


Figure 1. Survival of *Arianta arbustorum* exposed to 0 (white), 30 (black) and 90 (grey) *Phasmarhabditis hermaphrodita* per $cm^2$ for 40 days. Bars represent ± one standard error.

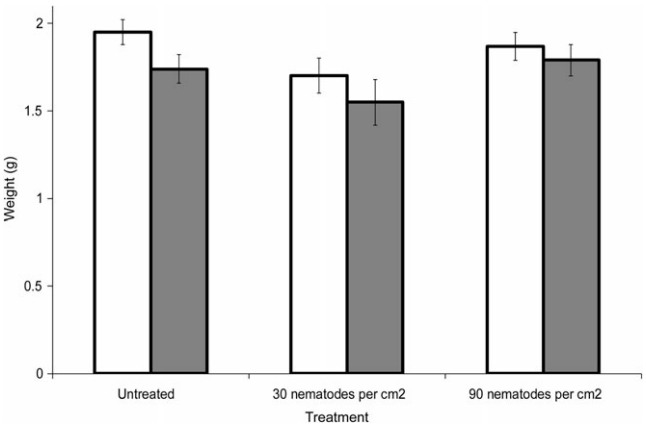


Figure 2. Mean weight of *Arianta arbustorum* at day 0 (white) ($n = 60$) and after 40 days (grey) exposure to 0 ($n = 48$), 30 ($n = 49$) and 90 ($n = 46$) *Phasmarhabditis hermaphrodita* per $cm^2$. Bars represent ± one standard error.

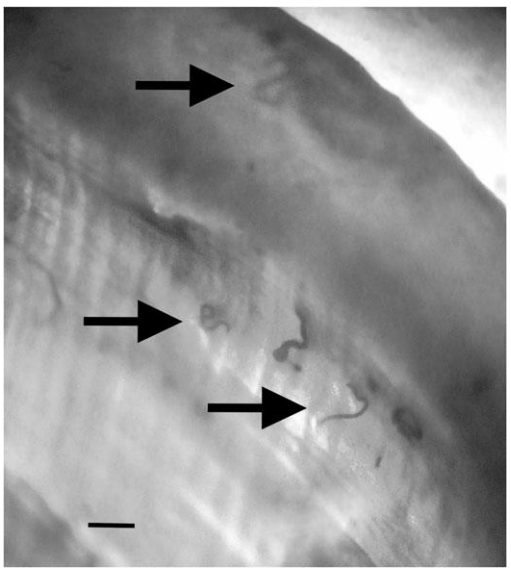

Figure 3. *Arianta arbustorum* exposed to *Phasmarhabditis hermaphrodita* can encapsulate and kill nematodes in the aperture of the shell near the lip. Scale bar = 500 μm.

Table 1. Mean, range and percentage of *Arianta arbustorum* with nematodes encapsulated in the shell when infected experimentally with *Phasmarhabditis hermaphrodita* and in field-collected shells.

| | Dose or location (year) | *n* | Number infected (%) | Mean number of nematodes per shell ± SE | Range of nematodes per shell |
|---|---|---|---|---|---|
| Treatment of *A. arbustorum* with *P. hermaphrodita* | 0 nematodes per cm$^2$ | 48 | 1 (2.1%) | 2 | 2 |
| | 30 nematodes per cm$^2$ | 49 | 30 (61.2%) | 1.43 ± 0.18 | 1–4 |
| | 90 nematodes per cm$^2$ | 46 | 33 (71.7%) | 2.69 ± 0.42 | 1–12 |
| Newly collected *A. arbustorum* | Strathy Beach | 139 | 31 (22.3%) | 2.10 ± 0.35 | 1–8 |
| | Durness Beach | 16 | 1 (6.3%) | 2 | 2 |
| | Dunnett Beach | 50 | 8 (16%) | 2.43 ± 0.69 | 1–5 |
| Historical collections of *A. arbustorum* | Maidwell (1964) | 20 | 0 | 0 | 0 |
| | Ganton (no year) | 13 | 0 | 0 | 0 |
| | Castleton (1966) | 30 | 1 (3.3%) | 1 | 1 |
| | Wendover (1908) | 10 | 1 (10%) | 1 | 1 |

fused in the shell, which I considered to have been present due to natural infection in the wild. Significantly more nematodes were encapsulated in shells of *A. arbustorum* exposed to 90 nematodes per cm$^2$ compared with 30 nematodes per cm$^2$ ($P < 0.05$) (Table 1).

### *Nematodes naturally fixed in shells of* A. arbustorum *from sand dunes*

Among 205 *A. arbustorum* shells examined, 40 had nematodes fixed in their shells (19.5% infection rate). Thirty-one shells from Strathy beach, one from Durness Beach and eight from Dunnett Beach had nematodes present. The numbers of nematodes, of unknown species, ranged from 1 to 11 per shell (Table 1). Therefore, it seems that *A. arbustorum* routinely encases parasitic nematodes in their shell in the wild.

Of the historical shells examined, those collected from Maidwell and from Ganton were free of encapsulated nematodes. Among the shells from Castleton and from Wendover (collected in 1908) there was a single shell in each sample with one encapsulated nematode.

## DISCUSSION

The mechanisms of parasite resistance in terrestrial gastropods are poorly known (Barker, 2004; Loker, 2010). The majority of knowledge comes from research on the immune response of medically important snails such as *Biomphalaria* species and their interactions with schistosome trematodes, where downstream effectors such as lectins and antimicrobial peptides controlled by the MAPK/ERK pathway are used to combat parasites (Zelck, Gege & Schmid, 2007). However, it has been shown recently that nematodes can be found encased in the shell of several snails such as *Lissachatina fulica* (Williams & Rae, 2015), *Cepaea nemoralis* (Williams & Rae, 2016) and slugs such as *Limax pseudoflavus* and *Deroceras panormitanum* (Rae, Robertson & Wilson, 2008; Rae, 2017). Some of these previous studies, which have used a means of infection with *P. hermaphrodita* similar to that used here, have reported hundreds of nematodes being encapsulated in shells, which is in sharp contrast to the results reported here for *A. arbustorum*. For example, *L. fulica* had an average of 15 nematodes per shell after exposure to 30 nematodes per cm$^2$ and at 90 nematodes per cm$^2$ there were over 160 per shell (Williams & Rae, 2015). For *A. arbustorum* in the present study the maximum number of *P. hermaphrodita* found encased in a single shell was 12. While the size of the snail may be a factor, it may also be the case that species differ in the extent to which they are susceptible to infestation (Rae *et al.*, 2009). Although nematodes were encapsulated in low numbers in *A. arbustorum*, the ability to do so is important, as infection by just one *P. hermaphrodita* is known to cause mortality in the slug *Deroceras reticulatum* (Tan & Grewal, 2001).

So far, *P. hermaphrodita* has been shown to be capable of killing eight terrestrial snail species, whereas it is apparently unable to do so in nine other terrestrial and freshwater species (Coupland, 1995; Glen *et al.*, 1996; Wilson *et al.*, 2000; Rae *et al.*, 2009; Whitaker & Rae, 2015; Williams & Rae, 2015, 2016). While only a few species have been tested to see if they can encapsulate *P. hermaphrodita*, unknown nematodes have been found encapsulated in many snail species across the Stylommatophora (Rae, 2017). The results of this study demonstrate that *A. arbustorum* is not only capable of resisting parasitic attack, but to do so without any evident stress in terms of feeding or reduction in weight. Nematode infestation does occur in *A. arbustorum*, but is quickly disposed of. If DNA could be extracted from encapsulated nematodes in samples of field-collected shells, it might be possible to identify the species involved and to track changes over time and among snail populations. Clearly, encapsulated remains remain visible in dead shells for long periods.

## ACKNOWLEDGEMENTS

I am very grateful to Tony Hunter of Liverpool Museum who provided information and access to the conchology collection.

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