AN INVESTIGATION INTO NEMATODES ENCAPSULATED IN SHELLS OF WILD, FARMED AND MUSEUM SPECIMENS OF *CORNU ASPERSUM* AND *HELIX POMATIA*

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Abstract *Nematodes are prolific and diverse parasites of gastropods that have evolved to use slugs and snails as definitive and intermediate hosts. Recently, several snail species have been shown to encapsulate and kill nematodes by encasing them in their shell under laboratory conditions. However, it is unknown how common this process is in wild populations. Also there has been little research on how the morphology and physiology of the shell may influence the successful identification of nematodes trapped in snails' shells using molecular biology. We examined shells from several U.K. populations that consisted of wild* Cornu aspersum *from Formby and Littlebourne and farmed* C. aspersum *from Lurgan, as well as* Helix pomatia *from snail farms in France (Brumath and La Rivière Drugeon). We found nematodes present in shells collected from four out of five locations with the number of shells infected with nematodes ranging from 17% to 100%. Using previously described protocols we attempted to amplify nematode DNA from the shells with nematodes. Nematode DNA could be amplified from* C. aspersum *from Lurgan (as previously reported) but not from the other three snail populations. This could potentially be due to low starting numbers of nematodes in the shell and high calcium content prohibiting amplification of DNA. In a final experiment we discovered that museum collections of* C. aspersum *and* H. pomatia *had nematodes present in their shells that were over 100 years old. Taken together, these results show that* C. aspersum *and* H. pomatia *frequently trap and fix nematodes using their shell in wild and farm environments. However, in order for these nematodes to be identified successfully nematode infection load and calcium content should be considered.*

Key words Cornu aspersum, Helix pomatia, C. elegans, *nematodes, shells*

INTRODUCTION

The immune system of terrestrial gastropods is poorly understood (South, 1992) and there is little information about how these animals tackle infection by common parasites such as nematodes, trematodes, viruses, microsporidia, bacteria and flies (Barker, 2004). Most research has concentrated on investigating the relationship between aquatic snails and vectors of medically important pathogens (e.g. *Biomphalaria* spp. and schistosome trematodes) that produce anti-microbial peptides, Reactive Oxygen Species (ROS) and lectins to defend themselves (Loker, 2010). However, recently it has been shown that upon infection by the parasitic nematode *Phasmarhabditis hermaphrodita*, snails (*Lissachatina fulica* and *Cepaea nemoralis*) can trap, encase and kill these nematodes using their shell (Williams & Rae, 2015; 2016). The shell is made from an outer proteinaceous periostracum of conchiolin and sub-layers of crystalline calcium carbonate (Lowenstam & Weiner, 1989) that allows protection from predators and extreme environmental conditions. But upon infection by nematodes cells of the shell adhere to the surface of the nematode and engulf the entire animal and fix it to the aperture of the shell within 3 days (Rae, 2017). Although this process has been shown in several snail species under laboratory conditions (Williams & Rae, 2015; 2016; Rae, 2017) there is little information about how prevalent nematode encapsulation is in wild populations from different locations or ecological niches.

It has been shown recently that nematode DNA can be extracted from shells and used for species identification (Rae, 2017) but there is little information on how to carry out these procedures routinely on different snail species. Furthermore, there is little information on how long these nematodes are encased in shells. Rae (2017) showed that nematodes are found in *C. nemoralis* collected from 1864 and even >500 years old but there is no information for any other snail species. If nematodes are preserved indefinitely in shells and their DNA can be routinely extracted and analysed then this could allow an unparalleled look into nematode evolution over time.

We therefore examined whether snails (*Cornu aspersum* and *Helix pomatia*) collected from the wild and snail farms from several locations in the U.K and France had encased nematodes in their shells. We chose these two species as they

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are very common in the U.K. and northern Europe (Janus, 1965) and as they are routinely bred in large numbers as a delicacy we managed to obtain many shells easily from several locations. Also as one of the most frequent parasites of snails reared in farms are nematodes (Cabaret *et al*., 1988) we thought they could provide an ample source of material for DNA extraction. We also examined conchology collections of *C. aspersum* and *H. pomatia* at the World Museum, Liverpool to discover how long nematodes could be encased in shells.

There is no information on the factors that may affect successful amplification of nematode DNA from snail shells using Polymerase Chain Reaction (PCR). Rae (2017) managed to identify nematodes from *C. nemoralis* shells using molecular biology but only from several shells. The molluscan shell is made of calcium carbonate, conchiolin, proteins, glycoproteins, chitin and acidic polysaccharides (Marin *et al*., 2008). Some of these components (e.g. calcium) can inhibit the efficacy of Polymerase Chain Reaction (PCR). Also the size and thickness of the shell surrounding the encapsulated nematodes may hinder molecular verification. Therefore, we attempted to investigate how several factors may affect the success of PCR including: the numbers of nematodes in the shell, the size of the section of shell where DNA is extracted from, species of snail and the size (length and weight) of shell and calcium carbonate content. Ultimately, this research could pinpoint morphological and physiological factors that may adversely affect the successful amplification of nematode DNA from snail shells.

Materials and Methods

Source of invertebrates

Adult *C. aspersum* shells were purchased from a snail farm in Lurgan, Northern Ireland (n=100); collected from woodland in Littlebourne, southeast England (n=100) and sand dunes in Formby, northwest England (n=100) and were examined for presence and abundance of nematodes. Also adult *H. pomatia* shells were purchased from snail farms in Brumath, northeast France (n=60) and La Rivière Drugeon, east France (n=100) (Fig 1A). For each shell the aperture and inner body whorl of the shell was examined for nematode encapsulation using a dissecting microscope with a light source.

Molecular analysis of nematodes extracted from shells

Once concentrated areas of nematodes were observed in the shells they were carefully removed in sections of varying sizes using a scalpel and placed in separate 1.5 ml Eppendorf tubes. Six to eight shells were used from each location (apart from Brumath as from 60 shells no nematodes were found). We recorded the size, weight and numbers of nematodes found in each piece of shell used for molecular verification. Specifically, pieces of *C. aspersum* shell from Lurgan (n=8) were 47±5mm^2, weighed 0.011±0.002g and contained 44±4 nematodes. Shell pieces of *C. aspersum* from Littlebourne (n=8) were 33±7mm^2, weighed 0.013±0.003g and had 4±1 nematodes encased. Sections of *C. aspersum* shells from Formby (n=6) were 21±6mm^2, weighed 0.0093±0.0034g and had 18±7 nematodes present. Fragments of *H. pomatia* shells from La Rivière Drugeon (n=9) were 92±11mm^2, weighed 0.079±0.012g and had 10±5 nematodes. Shells of *C. aspersum* from Lurgan were used as a positive control, as nematode DNA had been successfully extracted and amplified from these shells in a previous study (Rae, 2017). Each piece of shell was homogenised in 1.5 ml Eppendorf tubes with a mini-plastic pestle to a fine dust. DNA was extracted using a DNA extraction kit from Qiagen. Using PCR the ITS-1 gene (which is commonly used for nematode identification) was then amplified (Tandingan De Ley *et al*., 2014). Primers for the ITS gene were N93 (5′-TTGAACCGGGTAAAAGTCG-3′) and N94 (5-TTAGTTTCTTTTCCTCCGCT-3′). PCR cycling conditions consisted of the following: 3 mins at 95°C followed by 35 cycles of 15 secs at 95°C, 15 secs at 50°C, 2 min at 72°C and a final step of 7 mins at 72°C. The PCR products were checked using gel electrophoresis and any bands were then purified and sequenced in both forward and reverse directions.

Characteristics of the shells and quantification of calcium carbonate

To gain more information about the composition and basic characteristics of the shells that could influence the efficiency of PCR, height (mm), weight (g) and calcium carbonate content were recorded from shells from each location (apart from Brumath). Calcium carbonate content was measured by taking 9 individual shells from each location then homogenizing them for 3 mins

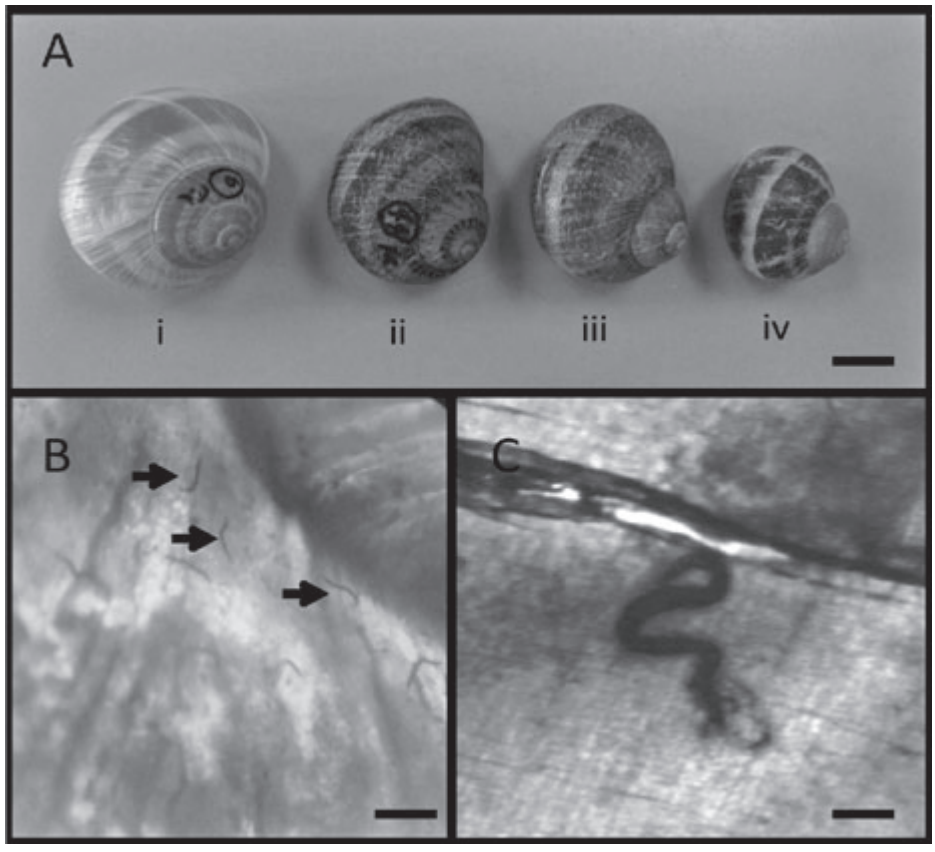

Figure 1 *H. pomatia* shells from La Rivière Drugeon (i) and *C. aspersum* shells from Littlebourne (ii), Lurgan (iii) and Formby (iv) (A). Nematodes were found in the inner whorl of shells (B) and in the inner lip (C). Scale bar represents 1cm (A), 1mm (B) and 100 µm (C)

until ground to a fine dust. After which 100mg was removed and added to 10 ml of deionized water and mixed for several minutes. A Total Water Hardness Test kit (Simplex Health) was used to quantify the amount of calcium carbonate in each shell from 0 to 1000 ppm.

Examination of historic collections of C. aspersum and H. pomatia for nematode encapsulation

In order to understand how long parasitic nematodes were encased in shells of *C. aspersum* and *H. pomatia* collections of both species were examined in the World Museum, Liverpool. These shells were mainly collected in the early 20th century but some shells were collected in the early 19th century from numerous countries including Spain, Switzerland, Tunisia, Greece, U.K. and Ireland. In total the aperture and inner whorl of 54 shells of *C. aspersum* and 42 of *H. pomatia* were examined for presence of nematodes.

Data analysis

The numbers of nematodes that were found in shells of *C. aspersum* from Lurgan, Littlebourne and Formby were compared using a One Way ANOVA with Tukey's post hoc test. The numbers of nematodes that were found in shells of *H. pomatia* shells from Brumath and La Rivière Drugeon were compared using a Student's t test. Differences in weight, length and calcium content of shells of *C. aspersum* from Lurgan, Littlebourne and Formby and *H. pomatia* shells from La Rivière Drugeon were compared using a One Way ANOVA with Tukey's post hoc test.

Results

Nematodes encapsulated in C. aspersum and H. pomatia

There were significantly more nematodes found encased in *C. aspersum* shells from Lurgan (mean 42.1±2.8 nematodes per shell) than *C. aspersum* shells from Littlebourne (mean 2.9±0.3 nematodes per shell) and Formby (mean 4.6±0.3 nematodes per shell) (P<0.0001) (Fig. 1A–C, Table 1). The numbers of nematodes found in shells from Littlebourne and Formby did not differ

Table 1 The mean number of nematodes, length (mm), weight (g) and calcium content (ppm) of *C. aspersum* shells from Littlebourne, Formby and Lurgan and *H. pomatia* shells from Brumath and La Rivière Drugeon.

Species	Location	Habitat	Number of shells examined	Number of shells with nematodes present	Mean number of nematodes per shell (range)	Length of shell (mm)	Weight of shell	Calcium content
C. aspersum	Lurgan	Snail farm	100	100	42 (4–123)	37.64±0.55	1.77±0.08	80 to 120
	Littlebourne	Woodland	100	52	2.9 (1–11)	42.64±0.48	6.21±0.65	120 to 180
	Formby	Sand dunes	100	65	4.6 (1–11)	28.69±0.62	1.83±0.15	80 to 120
H. pomatia	La Rivière Drugeon	Snail farm	100	17	17.6 (2–194)	43.74±0.54	4.39±0.14	120 to 180
	Brumath	Snail farm	60	0	0	N/A	N/A	N/A

significantly ($P>0.05$). There were significantly more nematodes found in *H. pomatia* shells from La Rivière Drugeon (mean 17.6±11.1 nematodes per shell) compared to Brumath which had no evidence of nematode encapsulation from 60 shells ($P<0.0001$) (Table 1).

Characteristics of the shells and quantification of calcium carbonate in shells

C. aspersum shells from Littlebourne (42.64±0.48mm) were significantly larger than *C. aspersum* from Formby (28.69±0.62mm) and Lurgan (37.64±0.55mm) ($P<0.01$) (Table 1). *C. aspersum* collected from Formby were significantly smaller than snails from all locations ($P<0.01$). *C. aspersum* from Littlebourne were significantly heavier (6.21±0.65g) than *C. aspersum* collected from Lurgan (1.77±0.08g) and Formby (1.83±0.15g) ($P<0.0001$) (Table 1). There was no significant difference between the weight of *C. aspersum* collected from Formby and Lurgan ($P>0.05$). *H. pomatia* from La Rivière Drugeon were significantly larger than *C. aspersum* shells from Formby and Lurgan ($P<0.01$) but not Littlebourne ($P>0.05$). These shells were also significantly heavier than *C. aspersum* shells from Formby and Lurgan ($P<0.01$) but were significantly lighter than those from Littlebourne ($P<0.01$).

Overall *C. aspersum* shells collected from Littlebourne were the heaviest and largest and they also had significantly higher calcium carbonate content (ppm) compared to the shells from Lurgan and Formby ($P<0.01$) (Table 1) but not *H. pomatia* from La Rivière Drugeon ($P>0.05$). There was no difference between the amount of calcium carbonate found in shells of Lurgan and Formby ($P>0.05$).

Identification of nematodes present in the shell of C. aspersum and H. pomatia

From the four locations that had nematodes present in the shells of *C. aspersum* or *H. pomatia* (Littlebourne, Formby, La Rivière Drugeon and Lurgan) nematode DNA was only successfully amplified and sequenced from nematodes in *C. aspersum* shells from Lurgan (as reported previously by Rae, 2017 and used as a positive control). Analysis of the ITS-1 gene amplified and sequenced from 5 out of 8 PCR reactions (2 failed and 1 was contaminated) showed that the closest species match using BLASTN searches on NCBI was *Caenorhabditis elegans* (99% similarity). Although there were nematodes present in shells collected from Littlebourne, Formby and La Rivière Drugeon there was no successful PCR amplification of ITS-1 from each nematode DNA extraction (for unknown reasons).

The lack of amplification could be due to the high amounts of calcium found in shells of *C. aspersum* and *H. pomatia* that lead to competitive binding of the DNA polymerase and reduces magnesium levels, which are essential for the activity of the polymerase (Opel *et al*., 2010). This can be reversed by adding more magnesium to the PCR reaction (Bickley *et al*., 1996). Therefore, we added an extra 2 and 4 µl of $MgCl_2$ to counteract the increased calcium per PCR reaction but this had no effect (data not shown).

Examination of historic collections of C. aspersum and H. pomatia for nematode encapsulation

From 54 *C. aspersum* examined for presence of nematodes, 7 shells were found with nematodes encased ranging from 1 to 3 nematodes per shell (Table 2). These shells were collected from Northern Ireland (1904), England (1908), Tunisia (1911), and from an unknown location in 1901. From 42 *H. pomatia* shells observed 6 had nematodes encased and ranged from 1 to 4 nematodes per shell. These shells dated back to 1904, 1911 and 1920 and were collected in England and Italy. Nematodes were also observed in *H. pomatia* from a snail farm in Switzerland but there was no recording of the year they were collected. Therefore, when nematodes are encased in the shells of both *C. aspersum* and *H. pomatia* they can be preserved for over 100 years.

Discussion

Under laboratory conditions *L. fulica* and *C. nemoralis* can encase and kill parasitic nematodes (*P. hermaphrodita*) using their shell (Williams & Rae, 2015; 2016), however there is little information about how common this process is in nature. Here we show that both *C. aspersum* and *H. pomatia* collected from snail farms and from field sites in the U.K. and France commonly trap and encase nematodes in their shell. Therefore, it seems that shell encapsulation of these parasites is a very common process; especially in *C. aspersum* from Lurgan where all snails examined had nematodes present in their shells. Interestingly, from

Table 2 Analysis of collections of *C. aspersum* and *H. pomatia* shells at World Museum, Liverpool.

Species	Location	Year	n	Number of shells with nematodes (% of shells infected)	Number of nematodes in shells (range)
C. aspersum	Tranmere, England	1861	7	0 (0%)	0
	Rome, Italy	1868	6	0 (0%)	0
	Arosa, Spain	1899	3	0 (0%)	0
	Arosa, Spain	1898	4	0 (0%)	0
	Windermere, England	1900	1	0 (0%)	0
	Location unknown	1901	4	3 (75%)	1 to 3
	Raghly, Ireland	1904	1	0 (0%)	0
	Larne, Northern Ireland	1904	6	1 (16.7%)	1
	Land's end, England	1908	5	0 (0%)	0
	Kendal, England	1908	6	0 (0%)	0
	Cambridge, England	1911	6	0 (0%)	0
	Tunisia, Africa	1911	2	1 (50%)	2
	Penzance, England	1908	3	2 (66.7%)	1 to 2
H. pomatia	Surrey, England	1900	3	0 (0%)	0
	Gallows Hill, England	1904	6	1 (16.7%)	4
	Dorking, England	1905	3	0 (0%)	0
	Denbies, England	1908	1	0 (0%)	0
	Athens, Greece	1814	1	0 (0%)	0
	Cheltenham, England	1871	2	0 (0%)	0
	Le Havre, France	1897	2	0 (0%)	0
	Mt. Pilatus, Switzerland	1898	2	0 (0%)	0
	Lezzeno, Italy	1911	2	1 (50%)	1
	Painswick, England	1920	3	1 (33%)	2
	Escargot farm in Switzerland	unknown	17	3 (17.6%)	1

60 shells from a snail farm in Brumath we found no evidence of any nematodes. It is difficult to pinpoint exact reasons for the lack of nematode infection. Perhaps this is due to the method of rearing, which may prohibit prolonged contact with soil and therefore nematode parasites.

We tried to identify nematodes encased in the shells through homogenization, DNA extraction and PCR amplification of the ITS-1 gene (Tandingan De Ley *et al*., 2014). We were only able to successfully identify *C. elegans* from *C. aspersum* shells from Lurgan as reported in similar studies (Rae, 2017), which could be due to the large numbers of nematodes that were found in these shells compared to shells from the other locations. *Caenorhabditis elegans* and *Caenorhabditis* species nematodes have been shown to be associated with slugs and snails (Kiontke & Sudhaus, 2006; Petersen *et al*. 2015; Caswell-Chen *et al*., 2005; Maupas, 1900). There is little understanding of what effect these nematodes are having on the snails but it has been suggested that they use gastropods for travelling purposes (Schulenburg & Fèlix, 2017) or as necromenic hosts (Kiontke & Sudhaus, 2006), where they infect and wait for the host to die to reproduce.

We attempted to unravel the factors that affected the molecular identification of nematodes from shells of *C. aspersum* and *H. pomatia*. One of most variable factors was the numbers of nematodes fixed in the pieces of shell from which DNA was extracted. *C. aspersum* from Lurgan had a mean of 42.1 nematodes per section, but those from Littlebourne had 2.9 nematodes, Formby had 4.6 nematodes and La Rivière Drugeon had 17.6 nematodes. This difference could mean that in order to successfully amplify DNA from nematodes from shells in museum collections a large population of nematodes will be needed. As well as the numbers of nematodes in the shells, components of shell may have interfered with the success of the PCR. Nematodes were not identified from *C. aspersum* shells from Littlebourne or Formby or from *H. pomatia* shells from La Rivière

Drugeon. In general, these shells were significantly larger and heavier than *C. aspersum* from Lurgen. The only exception was from *C. aspersum* from Formby, which were the same size as those from Lurgen. It may however, be the calcium of the shell that was interfering with the success of PCR. Ninety seven percent of the shell weight is made of calcium chloride in pulmonate land snails (Heller & Magaritz, 1983). Calcium (as well as polysaccharides) can inhibit PCR (Bickley *et al*., 1996; Monteiro *et al*., 1997) by competing with the amount of magnesium used in the reaction (Schrader *et al*., 2012). We found that many of the shells used in our analysis were calcium rich so to remedy this we added extra magnesium chloride to the PCR but this had mixed results (data not shown). This means that either the levels of calcium are so high that addition of magnesium has no effect or that there are other unidentified PCR inhibitors present. Interestingly, *C. aspersum* from Formby were low in calcium and did not have a thick shell but the PCR failed repeatedly, which would suggest that other PCR inhibitors were present. Future research will focus on reducing the effect of calcium ions and other potential sources of inhibition from the homogenised shell debris as well as purifying DNA pre-PCR. One method might be to treat the shells with an agent that could degrade the shell and release the nematodes into solution. For example, vinegar breaks down the snail shell so much so that nematodes are released into solution (data not shown) (although it is unknown whether these animals would be suitable for PCR amplification).

Four out of five clades of the Nematoda parasitise molluscs which include at least 108 nematode species (Grewal *et al*., 2003; Blaxter *et al*., 1998) that have evolved to use slugs and snails for transport e.g. *C. elegans* (Petersen *et al*., 2015); definitive hosts e.g. *P. hermaphrodita* (Wilson *et al*., 1993) or as intermediate hosts to facilitate transmission to mammalian hosts e.g. *Angiostrongylus vasorum* (Bolt *et al*., 1994). The co-evolution of nematodes and molluscs has been ongoing for over 500 MY (Grewal *et al*., 2003), and this intimate relationship led to the gastropod exoskeleton evolving a new co-opted role 120 MY ago to encase and kill invading nematodes (Rae, 2017). This ability may facilitate DNA analysis of ancient nematodes preserved in shells of museum collections (provided current PCR problems can be solved). We found nematodes present in shells of both *C. aspersum* and *H. pomatia* collected over 100 years ago. As edible land snails, such as *C. aspersum*, have been reared by humans from the late Pleistocene and Holocene (Lubell, 2004) and are often abundant in archeological deposits there is a possibility of tracking nematode evolution using molecular biology. Nematodes in conchology collections in museums are preserved from agents that break down DNA such as water and extreme temperatures (Shapiro, 2013), as well as microbial contamination, they could therefore be harvested for DNA analysis and compared to modern day parasitic nematodes. However, for successful amplification a large number of starting nematodes would be needed and shells high in calcium may be problematic.

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