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First record of *Phasmarhabditis* sp. from eggs of *Eobania vermiculata* (Müller) snails in Egypt and their response to host size

Karima Mahmoud Azzam and Nema El-Abd

Abstract

Background: Gastropods are very prevalent animals. In Egypt, terrestrial snails represent important economic pests, infesting and causing severe damages to ornamental plants, orchard trees, vegetables, and field crops. The overuse of molluscicides against these destructive pests leads to more environmental pollution. Therefore, searching for biological control agents became necessary to avoid the hazard of chemical molluscicides. *Phasmarhabditis hermaphrodita*, isolated from slugs was used as a bio-control agent against snails and slugs. Few investigations available on gastropod egg parasites. Therefore, the present study interested in the isolation of parasitic nematodes from the snail eggs to study their possible role as biological control agents for gastropod pests.

Results: This is the first record to isolate the snail parasitic nematode, *Phasmarhabditis* sp., from eggs of the Egyptian terrestrial snail, *Eobania vermiculata* (Müller). Infectivity of this nematode was investigated for eggs, juvenile and mature snails, *E. vermiculata* and *Limax flavus* L. slugs, and also eggs of the non-local species, *Achatina fulica* Bowdich and one adult of it was only available. The investigation revealed a capability of the isolated nematode to infect and kill *E. vermiculata* snail, *L. flavus* slugs, and their eggs. Also, it could infect and kill the eggs of non-local snail species, *A. fulica*, and its only individual adult available which was obtained from an agricultural quarantine sample. The results indicated also that released individuals of the nematodes, recovered from snails, were significantly larger in size than those recovered from eggs and vice versa.

Conclusion: It could be concluded that the isolated parasitic nematode may be able to play a role in controlling different stages of the gastropods including eggs. This make the control more effective in protecting host plants before the pest causing damage. The nematode was more effective on local pest species than non-local species. Moreover, the size of the parasite was proportional with the size of the host pest.

Keywords: *Phasmarhabditis*, *Eobania vermiculata* (Müller), *Limax flavus* L., Eggs, Size, Biological control

Background

Gastropods are prevalent animals that belong to Phylum Mollusca, which represent the second largest Phylum after Arthropods (Lydeard et al. 2004). In Egypt, terrestrial snails represent important economic pests that were prevalent in many governorates, infesting and causing severe damages to ornamental plants, orchard trees,

vegetables, and field crops (Desoky 2018). Overuse of molluscicides against these destructive pests has contributed to more environmental pollution (Godan 1983). Therefore, searching for effective biological control agents became a necessity.

The snail parasitic nematode, *Phasmarhabditis tawfiki* Azzam, was first recorded and described by Azzam (2003). This nematode was isolated from *E. vermiculata* snail. *Phasmarhabditis hermaphrodita* (Schneider, 1859) is a parasitic nematode isolated from slugs and capable of killing several species of slug pests (Wilson et al. 1993).

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Coupland (1995) studied the susceptibility of helcid snails infected with *P. hermaphrodita* and found that *Monacha cantiana* (Müller), *Helix aspersa* Müller, *Theba pisana* (Müller), *Ceratomya virgata* (DaCosta), and *Cochlicella acuta* (Müller) could be killed by *P. hermaphrodita* in laboratory bioassays. *Phasmarhabditis hermaphrodita* was used as a bio control agent against snails and slugs (Rae et al. 2007). *Daubaylia* species are nearly always parasitic for freshwater snails of Planorbidae, (Holovachov et al. 2015) and *D. bonaerensis* recorded in *Helix aspersa* Müller (Camino and González 2011). Azzam (2006) recorded 16 snail species and 3 slugs infested about 64 species of different plants in Egypt. She recorded also 5 species of snail parasitic nematodes including, *P. tawfiki*, *P. hermaphrodita*, *Rhabditis* sp., *Diploscapter* sp., and *Cephalobus* sp. Azzam and Abd El-Hady (2018) recorded 7 snail species and 3 slug species infesting about 47 species of plants in 7 governorates in Egypt. All terrestrial snails cause damages to their host plants by feeding on leaves, blooms, flowers, fruits, trunks, limbs, and even on barks.

Since few studies are available on the gastropod egg parasites, the present study aimed to isolate parasitic nematodes from snail eggs to evaluate their possible role as biological control agents against gastropod pests and their eggs.

Methods

Samples collection

Samples of terrestrial snails and slugs were collected from infested plants by hand picking from Cairo, Egypt, during 2016-2019. Snails and slugs were identified according to Godan (1983); Cowie (2002); Auffenberg and Stange (2009); Baker Skinner Park (2009); and Azzam and Tawfik (2011).

Rearing of gastropods

After collecting and identifying the gastropod samples, they were washed thoroughly using a fine metallic net under strong stream of tap water and then washed again by distilled water. Some individuals were placed in a Petri-dish with distilled water and examined under a research microscope, to check for the presence of the microorganisms. This process was repeated several times until the complete removal of all external associated organisms.

Rearing of *Eobania vermiculata* (Müller) snail and *Limax flavus* L. slug were carried out by placing the examined individuals in plastic cages, sterilized with ethanol alcohol, substrated with sterilized clay, and irrigated by distilled water. After egg laying, the eggs were washed gently by distilled water and transferred to sterilized Petri dishes contained sterilized clay.

The Petri dishes were then placed in a dark place until hatching. The newly hatched gastropods were transferred

gently to new sterilized cages as mentioned before and supplied with some lettuce leaves after washing them thoroughly by a strong stream of tap water to remove associated organisms then washed again by distilled water and introduced to the newly hatching gastropods.

Isolation of nematodes from eggs

The collected snail eggs were gently washed by tape water then distilled water and examined under a microscope to remove any external associated nematodes or any microorganisms. If microscopic examination showed any organisms association, the egg washing should be repeated again and again until eggs are looked totally free from these organisms. After the complete removal of external associated microorganisms, the eggs were screened for parasitic nematodes by placing them individually in small Petri-dishes, 6 cm in diameter, with little distilled water (1 ml). The dishes were examined daily by a microscope for releasing nematodes and the distilled water was recompleted. Every 3 days, two eggs were dissected to check the presence of the parasitic nematodes inside. The isolated nematodes were identified according to Andrassy (1976), Azzam (2003), Sudhaus (2018), Tandingan De Ley et al. (2014, 2016), and Singh et al. (2019).



Fig. 1 Adult of *E. vermiculata*



Fig. 2 Adult of *L. flavus*

Count of nematodes

Counting of the released nematodes of recovering individuals, to be used in the infection of tested species and stages, was carried out by taking a 5 μ l of the nematode suspension (by means of a micropipette) after shaking it thoroughly, it was placed on micrometer slide, examined microscopically, and counted the number of nematodes. This procedure was repeated 4 times. The mean number was calculated and multiplied by 200 to estimate the number of nematodes in 1 ml. To prepare adjusting concentration, some of this solution may be diluted to the concentration needed.



Fig. 3 Adult of *A. fulica*

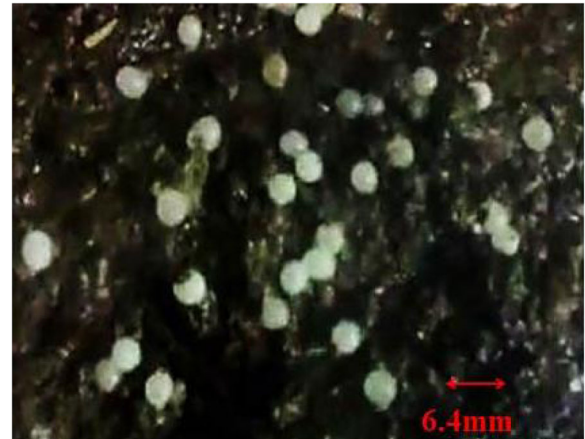


Fig. 4 Healthy eggs of *E. vermiculata*

Infection of snails and eggs

The isolated nematodes infectivity was investigated toward lab bred of *E. vermiculata* snails (Fig. 1), *L. flavus* slug (Fig. 2), and non-local giant terrestrial snails, *Achatina fulica* Bowdich (Fig. 3) which have not been found in Egypt (one snail obtained from the Agricultural Quarantine Office). This sample deposited 125 eggs in the laboratory during examination and identification. These eggs were treated as mentioned above and exposed to infection by the isolated nematodes.

The nematodes were also tested for the infection of eggs of the abovementioned three species (Figs. 4, 5, and 6) respectively at 22 ± 2 °C, using the same technic previously described by Azzam (1998).

The relative infectivity of the isolated nematodes to the abovementioned gastropods was determined by placing the 20 lab bred individuals of each species and stages individually in a Petri dish (9.5 cm in diameter for snails, slugs, and eggs). A little amount of distilled water contained isolated nematodes (1.5 ml contained 500 I.J. S. for snail and slugs and 0.5 ml contained 100 I.J. S. for



Fig. 5 Healthy eggs of *L. flavus*



Fig. 6 Healthy eggs of *A. fulica*

eggs) were placed in their relevant Petri dishes. Another 10 individuals from each species were treated similarly to abovementioned groups but using distilled water only without adding nematodes as control groups.

For the infection of the individual adult of the non-local giant terrestrial snail, *A. fulica* (Fig. 3), 2500 I.J.S. in 3 ml was added into a Petri dish (14 cm in diameter). The concentration increased than those used for adults of *E. vermiculata* and *L. flavus*, to be adequate for its large size. The size of the Petri dish and the volume of nematode suspension were also increased for the same reason. For the comparison of the suitability of this parasitic nematode to infect different hosts, the following capacity index was calculated for each host guided by Azzam (1998).

$$(C.I = M \times R/T)$$

where *M* is the mortality rate of pests at the end of 6 weeks, *R* is the percentage recovery of the infected stage of nematodes in infected individuals, and *T* is the time

needed for the nematodes to reach the infective stage after initial infection.

Statistical analysis

The time needed for the nematodes to reach infective stage after initial infection, body length of nematode in different stages, ratios of mortality, and individuals recovered nematodes were analyzed by ANOVA and *T* test value using PSPP program.

Results

As shown in Table 1, the parasitic nematode could infect all species and stages exposed, killing 100% from *E. vermiculata* eggs, and all individuals of the other species and stages. The table showed also that all infected species and stages recovered the parasitic nematode in different ratios. About 50% of *A. fulica* eggs and 100% from other species and stages were released infective stage of nematode. The lower ratio of *Achatina* eggs may be attributing to the hardness of the calcified egg capsule which was soft for the other species. The results indicated that all species were suitable to serve as hosts for the parasitic nematode, consequently the suitability of isolated nematode to control these pests at their different stages. Statistically, there was a highly significant difference between ratios and individuals recovered nematodes of *A. fulica* eggs and each of the other infected species and stages.

Concerning the time from initial infection to recovered infective stage, the only individual available of *A. fulica* snail recorded the longest period, followed by its eggs then adult of *L. flavus*, while eggs of *E. vermiculata* recorded the shortest period. Statistically, non-significant differences between period for *L. flavus* and juvenile of *E. vermiculata* and between periods for adult *E.*

Table 1 Capability of *Phasmarhabditis* sp. to infect different species and stages of terrestrial gastropods under laboratory conditions of 22±2 °C and 60±5R.H. %

Species and stage	Mortality %	Time from infection to recovered nematodes	Ability of dead individuals to release nematodes	Rate of individuals released nematodes %	Ability of released nematodes to infect healthy pests	Capacity index	Rank
Eggs of <i>E. vermiculata</i>	100%	7.32±0.95 c (6-9)	+	100%	+	1366.12	1
Eggs of <i>L. flavus</i>	100%	8.4±1.05 a (7-10)	+	100%	+	1190.48	3
Eggs of <i>A. fulica</i>	100%	19.2±1.74 (15-21)	+	50	+	260.41	7
Juvenile snail of <i>E. vermiculata</i>	100%	7.95 ±1 a (6-10)	+	100	+	1257.86	2
Juvenile slug of <i>L. flavus</i>	100%	10.8±1.15 b (9-13)	+	100	+	925.93	5
Mature snail of <i>E. vermiculata</i>	100%	10.5±1 b (9-12)	+	100	+	952.38	4
Mature slug of <i>L. flavus</i>	100%	14.4±1.43 d (12-17)	+	100	+	694.44	6
Mature <i>A. fulica</i> *	100%*	40 * e	+	100 *	+	250*	8

Means had the same letter were in significant differences

*Non-local species, only single individual were available from agricultural quarantine sample

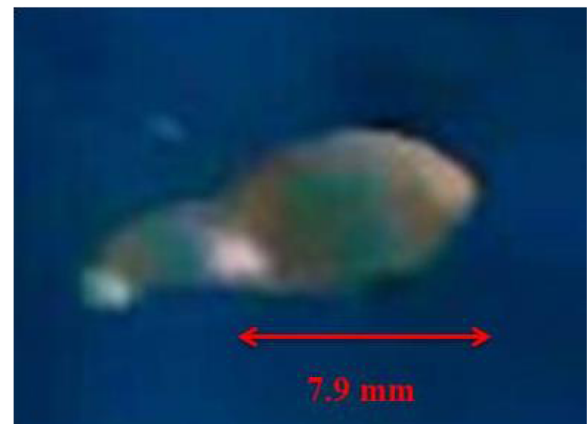
Table 2 Size in micrometer of nematodes recovering from eggs and adult of some gastropods

Gastropod stage	Body length of recovered nematodes		
	Female	Male	Infective stage
Eggs	1180.5 ± 164.06	1016 ± 193.11	767 ± 91.6
Adults	1916 ± 319.03	1313 ± 178.64	941 ± 126.79
T value	7.34	3.32	4.93
Significant	0.001	0.01	0.001

vermiculata and juvenile of *L. flavus*, while highly significant differences among other data were found.

Concerning capacity index, lowest C.I. (250) was reported for the only individual available of *A. fulica*, followed by its eggs (260.41). The highest capacity index (1366.12) was recorded for eggs of *E. vermiculata*, the natural host of the parasitic nematode, followed by juvenile of the same species and then eggs of the slug *L. flavus*. The juvenile and mature slug ranked after mature snails *E. vermiculata*. These results indicated the more suitability of eggs and juvenile of *E. vermiculata* and *L. flavus* and mature snails as hosts for the isolated parasitic nematode than mature slugs, which found more suitable than snail and eggs of the non-local species, *A. fulica*. The results indicated the suitability of isolated parasitic nematode to control gastropods at their different stages including egg stage, thus, make the control programs more effective in protecting host plants before the pest cause damage. The results indicated also that the isolated nematode were more effective on local pest than non-local pests.

Through the investigation, it was noticed that the dead eggs of all species changed in color, appeared yellowish to reddish (Figs. 7, 8, and 9). Also, some of infected *E. vermiculata* snails appeared with swollen tentacle (Fig. 10), but the slugs showed swollen foot (Fig. 11). It was also noticed

**Fig. 8** Dead eggs of *L. flavus*

that the size of nematodes releasing from snails *E. vermiculata* and *L. flavus* slug and *A. fulica* were larger than those released from their eggs (Table 2), but when these large nematodes infected eggs, the eggs released small size nematodes again and vice versa; when the small size nematodes released from eggs infected snails and slugs, they released large size nematodes again. These phenomenon may indicate that the size of host affect the size of parasite. Statistically, high significant differences existed among the body lengths of each of females, males, and infective stages recovered from adult stage of gastropods and those recovered from their eggs.

Discussion

This is the first record of isolating the snail parasitic nematode, *Phasmarhabditis* sp., from eggs of the Egyptian terrestrial snails, *Eobania vermiculata* (Müller). Infectivity of this nematode was investigated for eggs, juvenile and mature stages of different gastropod species

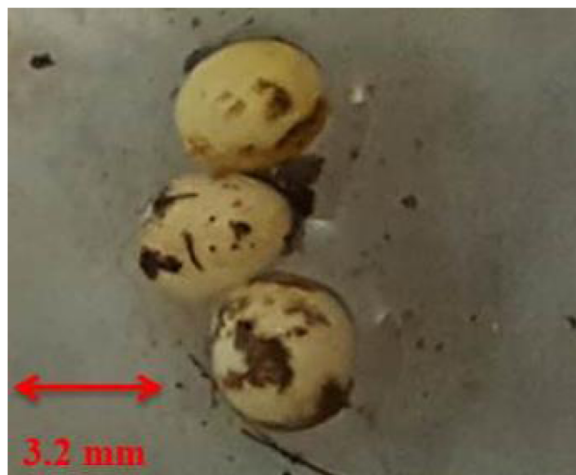
**Fig. 7** Dead eggs of *E. vermiculata***Fig. 9** Dead eggs of *A. fulica*



Fig. 10 Infected adult *E. vermiculata* infected with nematode showing a swollen tentacle

and results indicated the capability of this nematode to infect and kill all species and stages exposed to infection. Azzam (1998) reported the suitability of the parasitic nematode, *Rhabditis* sp., to infect and kill some terrestrial gastropods, their eggs, and insect larvae and pupae. She found that slugs recorded lower C.I. than snails when infected with the snail parasitic nematode *Rhabditis* sp., while the insect larvae reported lowest C.I. Coupland (1995) and Rae et al. (2007) found that *P. hermaphrodita* could kill several snail species and could be used as a bio-control agent against pest snails and slugs. Wilson et al. (2012) found that the New Zealand strain of *P. hermaphrodita* infected and killed field-collected *Deroceras reticulatum* slugs. The associations between nematodes and terrestrial gastropods ranged from accidental phoresis to parasitic or pathogenic relationships (Grewal et al, 2003 and Nermut and Půža 2017).



Fig. 11 Dead infected adult *L. flavus* showed swollen foot

Conclusion

It could be concluded that *Phasmarhabditis* sp. can play a role for controlling gastropods in their different stages including egg stage. These make the control more effective in protecting host plants before the pests reaching the economic damage stage. The results indicated that the isolated nematodes found to be more effective on local pests than on non-local pests, and that the size of the host stage is proportional to the size of the parasite.

Abbreviations

E. vermiculata: *Eobania vermiculata* (Müller); *L. flavus*: *Limax flavus* L.; *A. fulica*: *Achatina fulica* Bowdich.; *P. tawfiki*: *Phasmarhabditis tawfiki* Azzam (2003); *P. hermaphrodita*: *Phasmarhabditis hermaphrodita* (Schneider, 1859); *D. bonaerensis*: *Daubaylia bonaerensis*; C.I.: Capacity Index; M: Mortality rate of pests at the end of 6 weeks; R: Percentage recovery of the infective stage of nematodes in infected individuals; T: The time needed for the nematodes to reach infective stage after initial infection

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Authors' contributions

KA: Identified the gastropods, contribute rearing gastropods, contribute in the examination of the samples, isolate and propagate the nematode, infect different gastropods stage with nematode, record and analyzed the result data, wrote the manuscript. NE: Collect gastropods and their eggs sample from field, prepare the sample for examination, contribute in rearing gastropods, and contribute in recording result data. All authors read and approved the manuscript for submission.

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