Host cues induce egg hatching and pre-parasitic foraging behaviour in the mosquito parasitic nematode, *Strelkovimermis spiculatus*

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**Abstract**

The responses of eggs in diapause and the infective stage of the nematode, *Strelkovimermis spiculatus*, to larvae of its host, *Culex pipiens pipiens*, were investigated in the laboratory. The results indicated that the presence of the host induced the egg hatching. The hatching rate increased when larger numbers of host larvae were present. Second instar mosquito larvae induced significantly higher hatching rates than any other stages. These findings explain how *S. spiculatus* synchronizes its life cycle with its host life cycle and population dynamics to increase its fitness when the natural habitat is constantly covered by water. Direct exposure of the nematode eggs to host larvae resulted in consumption of as many as 20 eggs per host. The eggs consumed caused 0–70% host mortality depending on the number consumed, which indicated an infection path other than cuticle penetration although it may represent a rare situation in nature. The result of host cue assays showed that the combination of chemical cues and physical vibration induced the highest egg hatching, which may increase the chance of host availability after hatching. However, once hatched, the nematodes ignored vibrations and used only chemical cues for host location. These findings suggest that eggs hatch synchronously with the most susceptible mosquito stage and with peak mosquito larval density.

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**1. Introduction**

Mosquito parasitic nematodes of the family Mermithidae have attracted the interest of mosquito control workers due to their potential for biological control (Petersen et al., 1978; Platzer, 2007; Achinelly and Micieli, 2009, 2011). The parasite life cycle is simple. The infective stage or L2s hatch from eggs deposited at the bottom of pools. The larvae use their stylet to penetrate into the hemocoel of the mosquito larva where they avoid the host immune system (Shamseldean and Platzer, 1989; Shamseldean et al., 2006, 2007). Developing within the mosquito as L3s, this parasitic stage feeds transcuticularly to deplete host nutrients and emerges after 5–9 days as a post-parasitic stage. Emergence generates a hole which kills the host. The emerging larvae moult twice to become adults, form mating clusters and lay eggs to complete the life cycle (Platzer, 2007).

*Strelkovimermis spiculatus* shows particular promise for biological control due to its broad host range, ability to be reared in the laboratory using *Culex pipiens pipiens*, and its tolerance of desiccation (Platzer, 2007). Originally isolated from larvae of *Aedes albifasciatus* in temporary ponds in Argentina (Poinar and Camino, 1986), *S. spiculatus* is adapted for both temporary and permanent water mosquito habitats (García and Camino, 1990; García et al., 1994). As temporary pools diminish, the nematode eggs dehydrate, permitting survival. Attempts to use various species of mermithids to control mosquitoes have focused on releasing infective stage nematodes, obtained by the rehydration of desiccated eggs, into mosquito larval habitats (Peng et al., 1998; Petersen et al., 1978; Mijares et al., 1999; Achinelly and Micieli, 2009).

*Strelkovimermis spiculatus* form fully developed L2s 8 days after egg laying at 20 °C (Camino and Reboredo, 1994), yet larvae remain unhatched within the egg shell. Even after flooding, some nematode eggs remain unhatched for a long period (Walker et al., 1985; Achinelly and Micieli, 2011). Similarly, we have observed that more than 70% of *S. spiculatus* eggs do not hatch even 2 months after inundation at 24 °C, with the pre-parasitic larvae becoming completely immobile within the egg after the first moult (unpublished observations). It was concluded that these dormant eggs are in diapause, a state of arrested activity used to survive unfavourable conditions, usually extremes in the biotic environment (e.g., temperature) but also reduced food availability (Chapman, 1998). Although most often described from arthropods, diapause in eggs has been previously reported in the mosquito mermithid, *Romanomermis iyengari* by Pailly and Balaranan (1994) as well as in plant pathogenic nematodes such as the yellow potato cyst nematode, *Globodera rostochiensis*, and the soybean cyst nematode, *Heterodera glycines* (Tefft and Bone, 1985; Devine et al., 1996). Once induced, specific stimuli or
conditions are required for the termination of diapause (Clarke and Perry, 1977; Perry, 1989).

Diapausing nematode eggs survive in a desiccated or flooded state for several months (Walker et al., 1985; Achinelly and Miceli, 2011), but once diapause is broken and the pre-parasitic larvae emerge from the egg, the vigorously swimming larvae quickly exhaust their resources. Death ensues within 24–48 h unless a suitable host is found and parasitized (Camino and Reboredo, 1994; Platzer, 2007). This extremely brief window of opportunity for infection suggests that parasite egg hatching must be precisely synchronized with host availability. Our hypothesis is that natural selection favours eggs able to accurately detect host presence. That is, mosquito larvae provide specific stimuli that terminate diapause and cause nematode larval emergence (i.e., egg hatching), thereby synchronizing host and parasite life cycles.

2. Material and methods

2.1. Host culture

Larvae of Culex pipiens pipiens were used as the host insect and were obtained from a colony established from eggs collected in New Jersey, USA. The colony was maintained at 26 ± 1 °C, 75% r.h. and 16:8 light:dark photoperiod. Adults were held in 80 × 80 × 80 cm aluminium screen cages and were supplied with 10% sucrose solution on cotton wicks. Restrained adult quail was used to feed female mosquitoes (animal care and maintenance were in accordance with Animal Use Protocol #86-129 approved by Rutgers University, USA) and eggs were collected from a black plastic water container (400 ml) and hatched at 26 ± 1 °C. The larvae were held in enamel trays with 1 L of dechlorinated water and 0.15 g of Brewer’s yeast:lactalbumin (50:50). The water was replaced with fresh dechlorinated water every other day and the food was added daily. Second instar mosquito larvae used for nematode infection were of uniform size.

2.2. Nematode culture

Streptkovermis spiculatus were maintained on larvae of C. p. pipiens with procedures described for Romanomermis culicivorax (Petersen and Willis, 1972) under standard laboratory conditions as mentioned above (2.1). Second instar larval stages of C. p. pipiens were infected overnight at a host:parasite ratio of 1:3 in plastic containers (450 ml) containing 300 ml of dechlorinated water. Infected mosquito larvae were transferred into enamel trays and reared as described in Section 2.1 until the first nematode larvae emerged from the hosts. The larvae were then transferred to a container (17 × 17 × 7 cm) filled with dechlorinated water using a strainer (155 mesh). The distance between the mesh and the container bottom was 3.0 cm and the mesh size allowed larval nematodes to migrate through to the bottom of the container while retaining the mosquito larvae. The emerging nematode larvae were collected from the bottom of the container with a hair brush on a daily basis until no more emerged. Approximately 20–30 pairs of post-parasitic nematode larvae were inoculated into plastic containers (6 × 5 × 4 cm) filled with dechlorinated water and 50 g of autoclaved sand particles (1.4–2.0 mm depth) in which nematodes moulted into adults, mated and laid eggs. To obtain desiccated eggs, the water was removed 2 weeks post-inoculation, the container was sealed with Parafilm and incubated at 26 ± 1 °C.

2.3. Nematode eggs in diapause

To obtain eggs in diapause, the same containers were used (6 × 5 × 4 cm) as described in Section 2.2 without the sand particles. The containers containing adult nematodes were cultured at 24 ± 1 °C and checked every 2 days from day 7 following inoculation. Once eggs were laid, the adult nematodes were transferred into a new container with dechlorinated water for additional egg production. The eggs were collected, labelled and held at room temperature (24 ± 1 °C) for further experiments. Eggs remaining unhatched for 8–10 weeks post oviposition were considered to be in diapause and were used for the hatching assay.

2.4. Indirect exposure of nematode eggs to the hosts

To determine whether the presence of the host could trigger the hatching of nematode eggs, 60 eggs in diapause were introduced into each well of a 12-well cell culture plate (Fisher Scientific, USA) with 4 ml of dechlorinated water. To prevent physical contact between the nematode eggs and the hosts, a mini-strainer constructed by replacing the top of a cap from a 14 ml centrifuge tube with a nylon mesh (250 mesh) was inserted in each well. The mesh was fixed to the cap with sealant. The distance between the mesh and the bottom of the well was kept above 5 mm to ensure that the mosquito larval feeding activity did not change the position of eggs on the bottom of the well. Twenty second instar larvae of C. p. pipiens were introduced into the mini-strainer with a plastic pipette for the first treatment group. For the second treatment group, the water in each well was preconditioned with 20 2nd instar larvae of C. p. pipiens for 24 h before the experiment. Dechlorinated water was used in the control group. Unhatched eggs were counted in each well 24 h after exposure to assess hatching. In each experiment, there were 12 replicates for each treatment and control, and the experiment was repeated three times.

2.5. Direct exposure of nematode eggs to the host

Most nematode eggs were missing less than 30 min after exposure to second instar host larvae in a preliminary experiment. To determine whether the mosquito larvae consumed the nematode eggs and whether the eggs consumed result in host infection, 1, 5, 10 or 20 eggs per host larva were inoculated into individual 100 μl water droplets in a Petri dish. A single second instar larva of C. p. pipiens was subsequently introduced into each water droplet. The droplet was observed continuously under a stereomicroscope until all of the eggs were observed to have been consumed by the larva, which required from 10 to 60 min. If all available eggs were not ingested, the droplet was discarded. No egg hatching was observed during the egg feeding experiment. The larva was then transferred into a container containing 60 ml of dechlorinated water and fed with Brewer’s yeast as described in Section 2.1 until the emergence of either post-parasitic nematodes or an adult mosquito. In each experiment, there were six replicates for each treatment and control, and the experiment was repeated three times.

2.6. Host density and egg hatch

We tested the relationship between host density and nematode egg hatching in the 12-well plates with mesh separation as described in Section 2.4. Sixty S. spiculatus eggs were exposed to 1, 5, 10, or 20 2nd instar larvae of C. p. pipiens for 24 h. Dechlorinated water was used in control wells. Unhatched eggs were counted and the percentage of eggs which hatched was calculated. In each experiment, there were 12 replicates for each treatment and control, and the experiment was repeated three times. Pearson’s correlation and coefficient was calculated with software StatGraphics 5.0.
2.7. Host stage and egg hatch

To determine the effect of host stage on nematode egg hatching, nematode eggs to 1st, 2nd, 3rd and 4th instar C. p. pipiens larvae were exposed in assay plates as described in Section 2.4. Sixty nematode eggs were exposed to 20 larvae of each instar. Unhatched eggs were counted to determine the hatching rate 24 h post-exposure. Dechlorinated water was used in control plates. In each experiment, there were 12 replicates for each treatment and control, and the experiment was repeated three times.

2.8. Host cues and egg hatch

To determine whether the cues inducing nematode egg hatching were chemical cues released by the host larva or mechanical cues generated by larval movement, artificial metal larvae (1 x 3 mm) were created by cutting iron wire and coating the wire with hot glue. Metal larval vibration was generated by placing the assay plate on a Thermo Scientific Ceramic 18.4 x 18.4 cm Stirring Plate at 300 rpm. The exposure assay was conducted as described in Section 2.4 with larval conditioned water (i.e., chemical cues), one metal larva in dechlorinated water (i.e., chemical and mechanical cues), or larval conditioned water plus one metal larva (i.e., chemical and mechanical cues). Dechlorinated water without a metal larva was used for the control group. Unhatched eggs were counted after 24 h exposure. In each experiment, there were 12 replicates for each treatment and control, and the experiment was repeated three times. To reduce any temperature variation generated by the stirring plate, the experiments for each treatment and control were conducted on the same stirring plate. The water temperature in each well was monitored with a Fisher Scientific digital thermometer by placing the probe at the Centre of each individual well.

2.9. Host cues and host finding

To determine whether the hatched parasites used host cues to locate mosquito larvae, we designed an attraction assay plate that linked three 35 x 10 mm Petri dish bottoms (Fisher Scientific) with silicon glue. The interconnection uniting each dish wall was a 15 mm long cut in the side. The resulting clover leaf-shaped unit was then glued onto the bottom of a larger Petri dish (90 x 15 mm) to create a tri-circle plate with a central triangular indentation (15 x 15 mm) (Fig. 1). Each of the three smaller dishes were labelled either “P”, representing positive, “N1” and “N2”, representing negative 1 and 2, respectively. The central indentation was labelled “I” to represent the inoculation point. The plate was prefilled with 12 ml of dechlorinated water and the unit was placed onto an 18.4 x 18.4 cm stirring plate running at 300 rpm. Larval-conditioned water was prepared by placing 400 2nd instar mosquito larvae in 80 ml of dechlorinated water for 24 h. In the positive plate “P”, we used a metal larva plus 0.1 ml of water for treatment 1, 0.1 ml of larval-conditioned water in treatment 2, and a metal larva plus 0.1 ml of larval-conditioned water in treatment 3. Dechlorinated water (0.1 ml) was used in the control. Approximately 40 infective-stage nematodes were inoculated with 0.1 ml of water to the inoculation point, and 20 min were allowed for the nematodes to migrate. The nematodes in each of the three small plates were counted after each assay. The percentages of nematodes migrating into each plate were used to calculate the net positive response (NPR). Residual nematodes at the inoculation point were also counted to establish the total number inoculated. The NPR, percentage attraction, was calculated by the formula: NPR = (P - (N1 + N2)/2)/T x 100, where P is the number of nematodes migrating into the positive dish, N1 and N2 represent the number of nematodes migrating into the negative dishes numbered 1 and 2, respectively, and T represents the total number of nematodes inoculated. All experiments were conducted at room temperature (24 ± 1 °C). Water temperature in positive and negative dishes was monitored using a digital thermometer probe placed in the dish centres. In each experiment, there were nine replicates for each treatment and control, and the experiment was repeated three times.

2.10. Data analysis

Egg hatching and nematode migration to potential host cues were analysed by one-way ANOVA using Fisher’s least significant difference (LSD) in multiple range tests among the means (P ≤ 0.05). Data in text and figures are presented as mean ± S.E.

3. Results

3.1. Host induced egg hatching

The presence of host larvae induced 73.3 ± 3.9% of S. spiculatus eggs to hatch within 24 h, which was significantly higher than that triggered by larval-conditioned water (14.1 ± 1.9%) and controls (5.9 ± 1.8%) (see Fig. 2).

3.2. Direct exposure

Direct exposure of the nematode eggs to the host larvae resulted in ingestion of the eggs. As many as 20 eggs were consumed...
by each mosquito larva within 1 h. Host mortality resulting from the consumption of nematode eggs was recorded, ranging from 33.3% at five eggs per host to 66.7% at 20 eggs per host (Fig. 3). From 3.3–5.0% of eggs completed their development as parasitic stage nematodes within the mosquito hemocoel and ultimately emerged from the host.

3.3. Host density and egg hatching

Egg hatching was host density dependent (Fig. 4). The linear correlation coefficient \( r = 0.915 \) indicated that increasing the number of 2nd instar larvae of *C. p. pipiens* from five to 10 to 20 induced a corresponding increase in egg hatching of \( 21.9 \pm 3.9, \) \( 46.4 \pm 4.6 \) and \( 63.9 \pm 4.5\% \), respectively.

3.4. Host stage and egg hatching

Second instar host larvae induced the highest rate of nematode egg hatching (Fig. 5). When 20 different instars of *C. p. pipiens* were tested in this assay, 2nd instar larvae induced \( 69.1 \pm 6.0\% \) egg hatching, which was significantly higher than the other three instars: \( 41.2 \pm 4.6\% \) for first, \( 34.2 \pm 3.4\% \) for third, and \( 45.1 \pm 4.6\% \) for fourth instar. No significant difference was detected among first, third and fourth instar larvae.

3.5. Host cues and egg hatching

This experiment was designed to identify whether the host cues responsible for egg hatching were chemical, mechanical vibration or a combination of both cue types (Fig. 6). We found that host conditioned water induced \( 33.1 \pm 6.5\% \) of eggs to hatch, which was significantly higher than the controls \( (12.2 \pm 4.2\%) \). Vibration alone did not induce significant nematode egg hatching \( (16.7 \pm 4.1\%) \) compared with controls. However, the combination of the host conditioned water and vibration induced greater egg hatching \( (56.9 \pm 5.7\%) \) than any other treatment or control. Although a slightly higher temperature \( (0.91 \pm 0.03\ ^\circ C) \) was detected on the stirring plate than the bench top, no significant temperature difference was observed between the treatment and control wells \( (24.82 \pm 0.02 \) and \( 24.77 \pm 0.03\ ^\circ C, P = 0.24) \).

3.6. Host cues and host finding

Once hatched, infective stage or ‘preparasitic’ nematodes responded to host cues differently. The results indicated that host conditioned water and host conditioned water plus vibration induced similar nematode attraction \( (24.58 \pm 5.44\% \) and \( 25.55 \pm 2.64\%, \) respectively) (Fig. 7). Vibration alone resulted in no significant nematode attraction \( (3.3 \pm 4.6\%) \) compared with the control group \( (0.13 \pm 3.78\%) \). This result, in contrast to the egg hatching result, showed that host seeking nematodes use only chemical cues released by the host for host location. No temperature difference was detected between the positive \( (24.5 \pm 0.05\ ^\circ C) \) and the two negative plates \( (24.44 \pm 0.06\ ^\circ C, P = 0.18 \) and \( 24.46 \pm 0.05\ ^\circ C, P = 0.21) \).

4. Discussion

Our data indicate that host presence induces *S. spiculatus* eggs to break their diapause and hatch. Egg hatching was host density
host induced the hatching of the eggs in the trematode Microcotyle salpae but he also reported egg hatching followed water agitation. It is not known if the chemical and mechanical cues function in combination. The aquatic environment seems well suited for using mechanical disturbance to stimulate hatching.

The chemical(s) released by the host which induced *S. spiculatus* eggs to hatch was not identified in our study. High concentrations of carbon dioxide (CO₂) however, have been shown to enhance egg hatching for the closely related mermithid *R. rygeri* (Paily, 1990). We have also noted that water pH changes from basic to acidic after 24 h of mosquito larval conditioning (unpublished observations), an increase consistent with CO₂ conversion to carbonic acid. CO₂ may play a role in inducing hatching, but other chemicals such as urea released by mosquito larvae need further study. Whether a vibration signal assists the chemical cues in binding to the egg-shell, aids in eggshell permeability, or is perceived by nematode mechanical receptors is unknown. Infective entomopathogenic nematodes, including *Heterorhabditis megidis*, *Steinernema carpocapsae* and *Steinernema feltiae*, are attracted to vibration signals in soil substrates (Torr et al., 2004), which indicates that infective larval nematodes in terrestrial environments possess the ability to use vibration signals in host finding.

Parasitic nematodes using host cues to synchronize egg hatching with host life cycles have been well documented in sedentary soil nematodes that are plant parasitic. For example, infective juveniles of the potato cyst nematode, *Globodera rostochiensis*, hatch when chemical cues from potato root diffusates are provided (Clarke and Hennessy, 1984). Our results are unusual in involving an insect-parasitic nematode, an aquatic environment and most notably an additional layer of complexity by the use of mechanical as well as chemical cues.

Egg hatching in mermithid parasites of mosquitoes is not random. Timing of hatching is strongly influenced by a range of host cues including host chemicals, mechanical vibration, density and stage of development. These hatching strategies are adaptations that increase parasite success by facilitating host-parasite life-cycle synchrony. Once hatched, *S. spiculatus* no longer require the combination of chemical and vibration cues generated by the host. The infective juveniles ignore the vibration cue we offered from our artificial host and needed only chemical cues for host finding. The reason for this is not clear. However, it should be more energy efficient to respond to a relatively stationary chemical cue released by one or a group of mosquito larvae than to trace a moving target that has high mobility.

Mermithid eggs hatching inside the host gut could cause infection, although the low success rate shows that this is unlikely to represent an important route of entry. Consumption of multiple eggs resulted in considerable host mortality, which was attributed to microbial contamination introduced inadvertently by nematode penetration of the mosquito midgut wall. The mechanism of shedding surface contaminants as described by Shamseldean and Platzer (1989) during normal integument penetration may not occur in the midgut.

The mosquito parasitic nematode *S. spiculatus* has adapted to both temporary and permanent standing water habitats (*Garcia and Camino, 1990; Garcia et al., 1994*). In temporary habitats, eggs survive partially desiccated in soil (Platzer, 2007) and hatch when the habitat is flooded, thereby synchronizing with the available mosquito population which also respond to flooding by hatching. However, in permanent standing water habitats such as in ponds, our results suggest that most eggs hatch in response to chemical and mechanical cues generated by hosts, which assure suitable host availability. Once the infective stage of the nematode hatches, host chemical cues play a critical role in assisting the nematode to find the host. Mosquito management strategies to deploy *S. spiculatus* in temporary pools would achieve optimal host-parasite syn-
chronization by releasing infective stage nematodes from rehydrated eggs when surveillance reveals appropriate host density and stage. By contrast, in permanent ponds the mermithids perform their own ‘surveillance’ to synchronize their life cycle with the host life cycle, so releasing short-lived infective stage nematodes would be suboptimal. Adults, eggs in diapause, or even infected hosts could be a better release strategy (Petersen and Willis, 1974).

Acknowledgments

We thank Linda McCuiston and Jennifer Sun for assistance with maintenance of mosquito and nematode cultures.

References