

## Mating clusters in the mosquito parasitic nematode, *Strelkovimermis spiculatus*



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### ABSTRACT

Mating aggregations in the mosquito parasitic nematode, *Strelkovimermis spiculatus*, were investigated in the laboratory. Female postparasites, through their attraction of males and, remarkably, other females, drive the formation of mating clusters. Clusters may grow in size by merging with other individual or clusters. Female molting to the adult stage and reproductive success are enhanced in larger clusters. Male mating behavior is initiated when the female begins to molt to the adult stage by shedding dual juvenile cuticles posteriorly. Males coil their tail around the adult cuticle, migrating progressively along the female in intimate synchrony with the molting cuticle until the vulva is exposed and mating can occur. The first arriving male is assured of access to a virgin female, as his intermediate location between the vulva and subsequently arriving males blocks these competitors. Males deposit an adhesive gelatinous copulatory plug into and over the vulva before departing the female. Fecundity was greater in larger mating clusters, but this was a function of a greater rate of molting which is a prerequisite for mating. Males compete for virgin females by emerging and molting to the adult stage earlier than females. Mating aggregations have previously only been examined in snakes, but these studies have tended to be observational as snakes offer a challenging system for study. The relatively easy to culture and manipulate mermithid system may offer a model for experimental studies of male–male competition, protandry, copulatory plugs and female choice in mating clusters.

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### 1. Introduction

The Mermithidae (Order Mermithida) is a family of parasitic nematodes mostly known from insects although some species parasitize spiders, leeches, crustaceans, and other invertebrates (Platzer, 2007). Mermithids are roundworms which are free-living as adults and parasitic in their developing stages. Parasitism is invariably lethal to the host upon parasite emergence. This lethality coupled with a host range that includes disease-transmitting insects have been the primary drivers behind the study of mermithid nematodes. A number of mermithid species attack mosquitoes and these species have been of special interest to biological control workers (Platzer, 1981; Gaugler et al., 1984; Santamarina et al., 1999; Kobylinski et al., 2012).

The life cycle of mosquito mermithids is simple. Eggs hatch as second-stage juveniles and these short lived preparasitic nematodes search for early instar hosts and pierce the larval cuticle to enter the hemocoel where they overcome (Shamseldean et al., 2006, 2007) or escape (Gaugler et al., 1984) the host immune response. After 5–9 days of parasitic development, the nematodes emerge from the nutrient exhausted host as third-stage juvenile postparasites. Emergence produces an exit hole through the cuticle which soon kills the host. The emergent postparasites drift to the bottom of the mosquito pool, enter the soil, molt to become adults, mate and lay eggs (Petersen et al., 1978; Platzer, 2007).

Postparasitic mosquito mermithids aggregate to form tightly writhing mating clusters within the host larval habitat. Cluster formation has been observed in the midge mermithid *Gastromermis anisotis* (Poinar and Poinar, 2003), the fire ant mermithid *Allomeris solenopsi* (Poinar et al., 2007), and in the mosquito parasites *Romanomermis iyengari* and *Strelkovimermis spiculatus* (Sanad et al., 2013). Thus mating in clusters appears to be a general feature among mermithids. This behavior closely resembles the mating

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ball recorded for some snake species (Capizzi et al., 1996; Luiselli, 1996; Dubey et al., 2009). Mermithid mating aggregations are unique in that clusters are formed before nematodes have molted to the adult stage and by containing multiple females (Poinar et al., 2007).

The present study attempted to understand the evolutionary benefits derived by mosquito mermithids forming clusters. Our hypothesis was that cluster formation enhances reproductive success. Our test subject was *S. spiculatus*; a mermithid first isolated from *Aedes albifasciatus* larvae in a mosquito pool in Argentina (Poinar and Camino, 1986). This nematode species is adapted to temporary ponds where they oviposit either normal desiccation tolerant eggs to survive seasonal drought or diapause eggs that permit synchrony with the mosquito life cycle (García and Camino, 1990; Wang et al., 2012). The nematode shows particular promise for biological control (Micieli et al., 2012) because of its broad host spectrum of mosquito species, ease of *in vivo* mass culture using laboratory-reared *Culex pipiens*, and tolerance of desiccation and low oxygen (Platzer, 2007).

## 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 °C, 75% RH with a 16L:8D photoperiod. Adults were held in 80 × 80 × 80 cm aluminum screen cages and were supplied with 10% sucrose solution on cotton wicks. Restrained adult quail were used to blood-feed female mosquitoes (animal care and maintenance were in accordance with approved Rutgers University Animal Use Protocol #86-129). Egg rafts were collected and hatched as needed at 26 °C. Larvae were held in enamel trays with 1 L of dechlorinated water and 0.15 g of Brewer's yeast. Food was added daily and the water was replaced with fresh dechlorinated water on alternate days. Second instar mosquito larvae were used exclusively for nematode infections.

### 2.2. Parasite culture

*Strelkovimermis spiculatus* was maintained on larvae of *C. p. pipiens* using procedures previously described for *Romanomeris culicivora*x (Petersen and Willis, 1972). Hosts were infected overnight at a host:parasite ratio of 1:3 in 450 ml containers holding 300 ml of water. Infected mosquito larvae were transferred into enamel trays and reared as described above until nematode emergence was initiated. Infected mosquitoes were then transferred to 17 × 17 × 7 cm trays with 1.3 L water for subsequent emergence. Emergence trays had a 155-mesh screen secured 3 cm above the container bottom which allowed the emerging postparasitic stage nematodes to pass to the container bottom while retaining the moribund mosquito larvae. Postparasites were collected daily by pipette until emergence was completed. Approximately 40–60 postparasites at a 1:1 sex ratio were transferred into 6 × 5 × 4 cm containers filled with water and 50 g of sterilized coarse sand (1.4–2.0 mm diam) for molting, mating and oviposition. Gender separation was easily accomplished in postparasites because, although sexually immature, the male spicules and female vulva are evident.

### 2.3. Cluster formation

Nematode distribution subsequent to host emergence was studied in 28 × 16 × 5 cm enamel trays. A 1.5 cm layer of sterilized

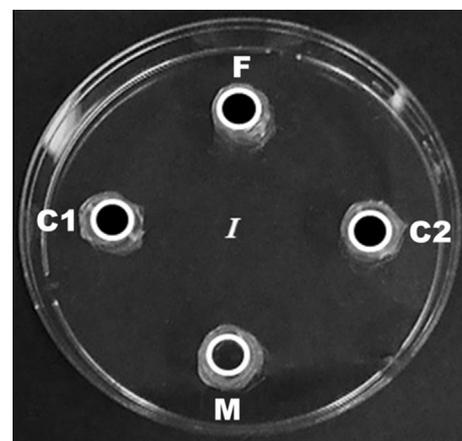
sand (1.4–2.0 mm diam) was added and the tray was half-filled with water. Three hundred infected *C. p. pipiens* were released into each tray 24 h before *S. spiculatus* emergence commenced. Trays were held at 25–27 °C for 1–3 week before extracting postparasites. Distribution was determined by partitioning the tray into 48 sand columns using a 3.5 × 2.6 × 1.5 cm grid pattern. Each 13.65 cm<sup>3</sup> column was removed and the nematodes and mating clusters within each grid column were recorded. A cluster was defined as two or more individuals forming an aggregation which does not separate upon probing. The process of cluster formation was video-recorded under light microscopy for analysis and a selected recording posted at <http://www.youtube.com/watch?v=WljbBaBhd6w>.

### 2.4. Aggregation and attraction

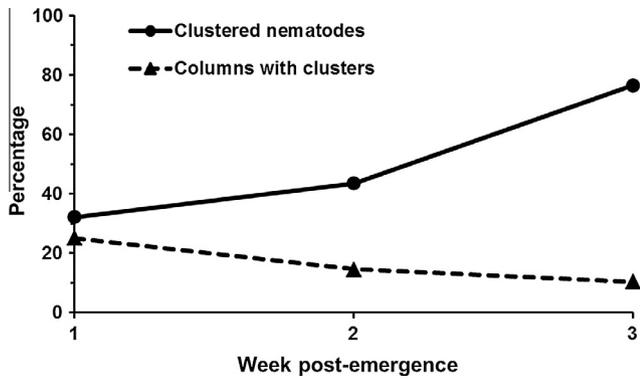
To test whether attraction cues facilitate mermithid cluster formation or the alternative hypothesis that aggregations arise simply via randomly encounters, we designed an attraction assay by modifying a 90 mm petri dish (Fig. 1). Source wells were created by drilling four 7.5 mm diam holes in the dish bottom and gluing a tip cut from a centrifuge tube bottom (11.5 × 7.5 mm diam, 0.8 ml capacity) beneath each hole. The wells were equidistant and 10 mm from the plate edge. Wells were designated as 'F', 'M', 'C1' and 'C2' representing female, male, Control 1 and Control 2, respectively. The dish center was the inoculation site 'I' where nematodes were introduced. Five g of sand (1.4–2.0 mm diam) was distributed over the dish bottom and 40 ml of water was added. An individual male or female postparasite 2–4 days post-emergence was transferred into wells marked 'M' or 'F', respectively, whereas the two control wells were empty. After 30 min for acclimation and for any attractant gradients to form, a test subject male or female postparasite was placed at the inoculation site and the dish top replaced. Sufficient search time (12 h) was allowed for before quantifying nematode distribution. Unlike the preparasite stage, postparasites are poor swimmers and cannot escape from a source well once entered. Response was scored by recording the nematodes captured in each well. The 'C1' and 'C2' means were combined and treated as a single control. Ten replicates for each gender were tested and the experiment was repeated three times at 26 ± 1 °C.

### 2.5. Cluster size effects on molting and fecundity

The effect of cluster size on female molting and fecundity was examined by creating clusters of 2, 3, 5, 9 and 20 postparasites at



**Fig. 1.** Sexual attraction assay plate, top view, showing the inoculation point (I) where test nematodes are introduced into the experiment and the four source wells for the male (M), female (F) and the two controls (C1 and C2).



**Fig. 2.** Mean percentage of individual *Strelkovimermis spiculatus* postparasites released into a gridded sand tray that joined mating clusters from weeks one to three after host emergence (solid line), and the mean percentage of sand columns (grids) containing clusters (dashed line).

male:female ratios of 1:1, 2:1, 4:1, 8:1 and 10:10, respectively. All postparasites were tested at two days post-emergence. The test arena was a  $4.5 \times 2.7 \times 4$  cm cup holding 30 ml of water. Cups for each cluster were examined alternate days for 2 week (oviposition was concluded) when clusters were gently teased apart to permit a determination of molting to the adult stage and oviposition. The mermithids were then transferred into a fresh cup, where mating clusters soon reformed, for further observation. Eggs were counted and microscopically examined for larval development after incubating for 5 days at  $26 \pm 1$  °C. Female mating success was identified by examination for depleted ovaries. Each male:female ratio was replicated ten times and the experiment was repeated three times.

### 2.6. Emergence, molting and mating

Gender differences in the order of postparasite emergence and molting were studied by randomly collecting 30 infected hosts from the infection pool (Section 2.2) five days post-infection. Infected larvae were held in a cup with 400 ml of water for nematode emergence. Postparasites were counted and sexed daily until emergence was completed. Each experiment had twenty replicates (cups) and was repeated twice. Molting and mating behaviors were recorded.

### 2.7. Data analysis

For molting rate and fecundity, one way ANOVA was used to measure variation among the means of treatments using Fisher's Least Significant differences ( $p < 0.05$ ). Nematode molting, mating, and fecundity were correlated with cluster size to test for nematode cluster size-dependent benefit using Pearson's correlation coefficient ( $r$ ). Data are presented as mean  $\pm$  SE.

## 3. Results

### 3.1. Cluster formation

One week after postparasite emergence, 32.1% of the nematodes were found in mating clusters in 25.0% of the sand columns (Fig. 2). The mean number of nematodes per cluster was  $19.92 \pm 2.04$ . After two weeks, 43.6% of the nematodes were found in clusters in 14.6% of the columns, and mean cluster size had increased to  $27.1 \pm 4.4$  nematodes. After three weeks, most nematodes (76.5%) were found in clusters, 10.4% of the sand columns had clusters, and nematodes per cluster had increased to  $67.0 \pm 50.5$  with the smallest cluster holding ten and the largest cluster holding 269 nematodes of

mixed gender. The trend over time, therefore, was for an increasing proportion of nematodes to join clusters and for the number of clusters to decline (negative correlation between clustered nematodes and number of cluster,  $r = -0.87$ ), demonstrating that small clusters merged to form large ones. Fig. 3 illustrates two small mating clusters in the process of merging into a single larger cluster. Females were observed to be responsible for propelling cluster mergers.

### 3.2. Aggregation and attraction

Female postparasites were highly attractive to other females (Fig. 4). A far greater proportion of females ( $68.33 \pm 3.33\%$ ) were attracted to a female than responded to a male held in the well ( $20 \pm 5.77$ ) or the control ( $11.66 \pm 3.33\%$ ) ( $P = 0.00067$ ). Males showed the same pattern where far more males responded to females ( $61.67 \pm 7.26\%$ ) than to either other males ( $23.33 \pm 8.82\%$ ) or the controls ( $15.0 \pm 2.89\%$ ) ( $P = 0.0053$ ). Neither gender was attracted to males.

### 3.3. Cluster size effects on molting and fecundity

As cluster size increased from 2 to 5 and then to 20 nematodes, molting of postparasites to the adult stage increased sharply from  $40.4 \pm 6$  to  $91.3 \pm 1.8$  to  $99.1 \pm 0.4\%$ , respectively ( $F = 50.17$ ,  $Df = 8$ ,  $P = 0.0002$ ) (Fig. 5A). Gender differences in molting were also noted as cluster size increased: at a cluster of two nematodes only  $3.4 \pm 3.3\%$  of females had molted 8 days after host emergence compared to  $33.0 \pm 4.2\%$  of males ( $P = 0.006$ ) (Fig. 5B). When the experiment was ended after 21 days,  $43.3 \pm 6.7\%$  of females in the smallest cluster size had molted compared to  $73.2 \pm 3.3\%$  of males ( $P = 0.023$ ), whereas in 20 nematode clusters virtually all males and females had molting.

Cluster size also impacted fecundity as the average number of eggs per female in clusters increased 360% from  $1145.2 \pm 512.3$  to  $4115.6 \pm 433.6$  as cluster size increased from 2 to 20 nematodes ( $F = 101.54$ ,  $Df = 4$ ,  $P = 0.001$ ) (Fig. 6A). Female mating success showed the same trend with the proportion of mating increasing more than fourfold from  $23.3 \pm 3.3\%$  to  $99.3 \pm 1.4\%$  as cluster size increased from 2 to 20 ( $F = 36.05$ ,  $Df = 4$ ,  $P = 0.0002$ ) (Fig. 6B). Correlation coefficients between cluster size and molting, mating, and fecundity were 0.80, 0.83 and 0.77, respectively. Thus, the greater fecundity per female characteristic of larger mating clusters was a function of more females mating which was due in turn to a greater molting rate. Knots of shed postparasite cuticles (Fig. 7) showed



**Fig. 3.** Two *Strelkovimermis spiculatus* mating clusters beginning merger into a single larger cluster.

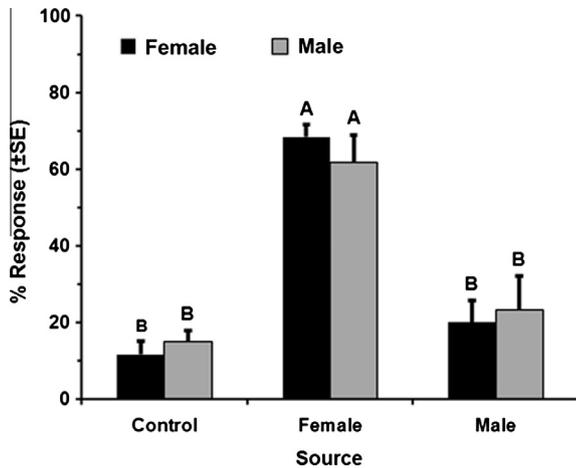


Fig. 4. Sex attraction among *Strelkovimermis spiculatus* male and female postparasites. Bars with the same letter are not significantly different ( $p < 0.01$ ).

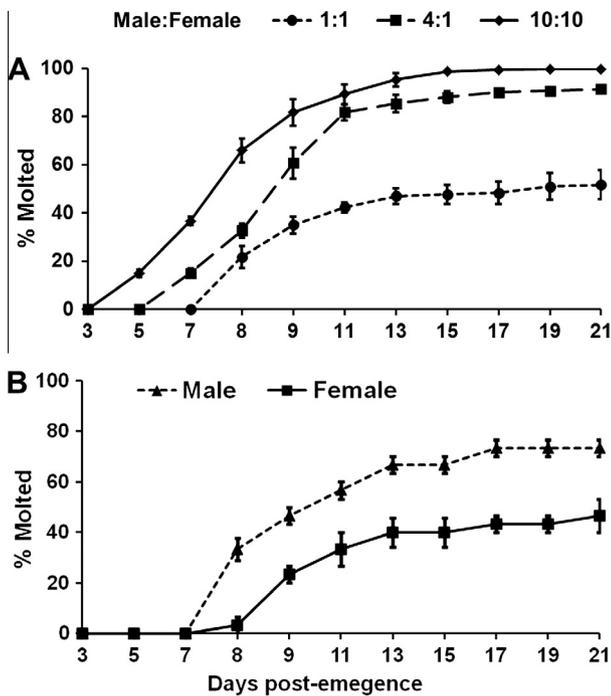


Fig. 5. Molting rate from the postparasite to the adult stage in *Strelkovimermis spiculatus*. (A) Relationship between size of the mating cluster and molting. (B) Molting rate of male versus females postparasites from the 1:1 male:female mating cluster in A.

that postparasite nematodes are tightly aggregated during molting and that molting is closely associated with mating clusters.

### 3.4. Emergence, molting and mating

Male *S. spiculatus* demonstrated a strong tendency to complete parasitic development and emerge from their mosquito hosts earlier than females (Fig. 8A). More than 60% of males exited from their hosts during the first day emergence was initiated (i.e., 7 days post-infection), whereas an additional 2.5 days were required for the same proportion of females to emerge.

Male postparasites also molted to the adult stage faster than females following emergence (Fig. 8B). Three days after emergence,  $28.0 \pm 2.31\%$  of male mermithids had molted compared to

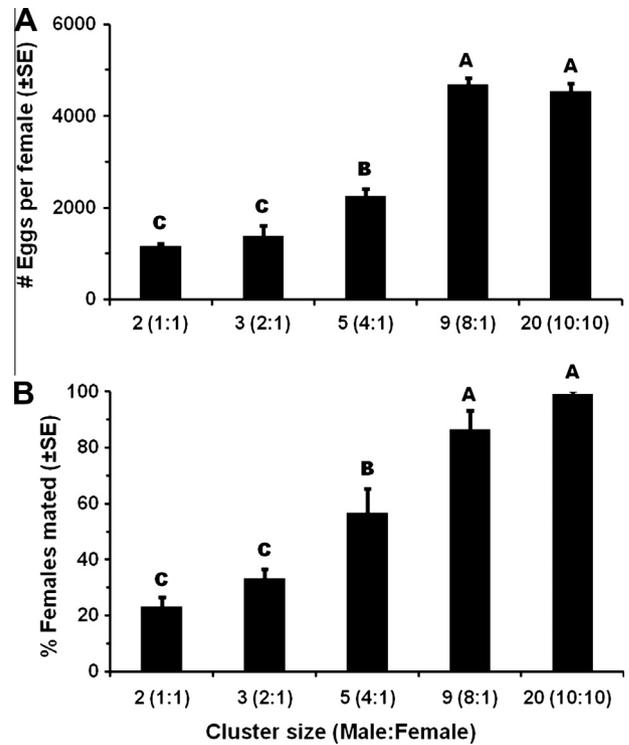


Fig. 6. Effect of mating cluster size (number of nematodes per cluster) on *Strelkovimermis spiculatus* female (A) fecundity and (B) mating success. Bars with the same letter are not significantly different ( $p < 0.05$ ).

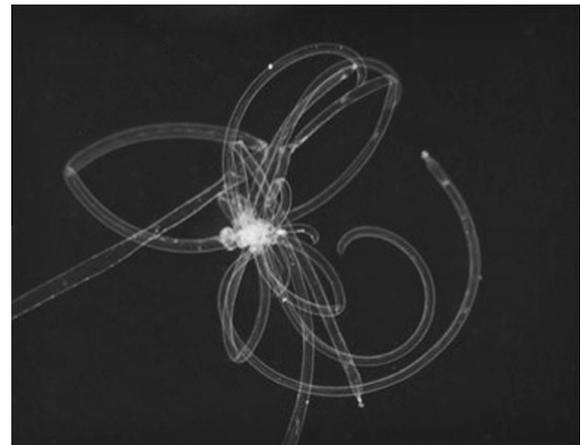
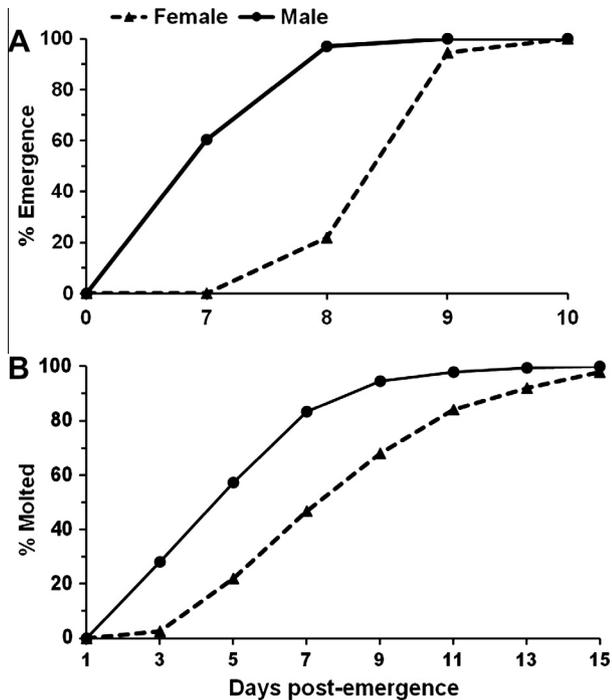


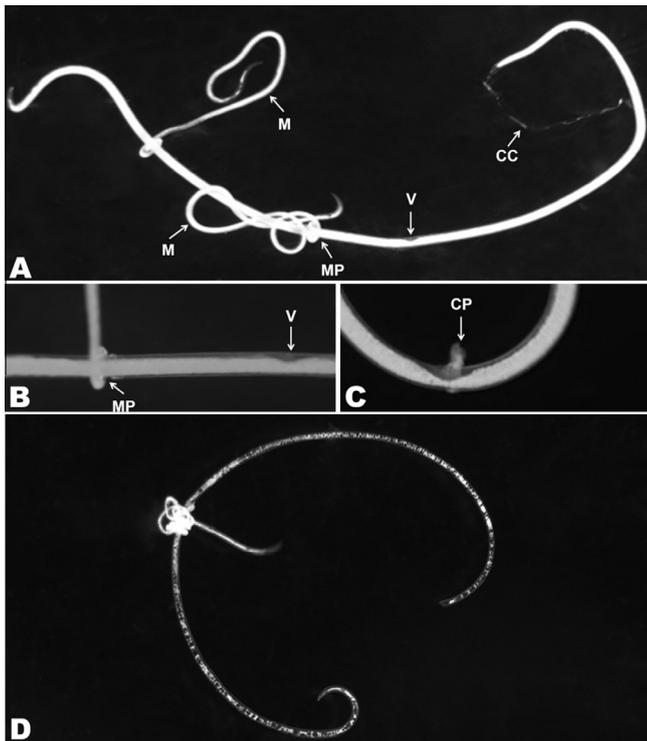
Fig. 7. A tangled knot of molted cuticles from *Strelkovimermis spiculatus*.

$2.7 \pm 1.76\%$  of females ( $P = 0.001$ ); after 5 days,  $57 \pm 3.53\%$  of males had molted and  $22.0 \pm 6.43\%$  of females ( $P = 0.009$ ). The time required for half of the test postparasites to molt ( $MT_{50}$ ) was significantly reduced for males ( $5.1 \pm 0.07$ ) than for females ( $7.2 \pm 0.63$ ) ( $P = 0.025$ ).

Molting occurred when the old cuticle ruptures anteriorly, resulting in a 'cap' detaching together with the accompanying esophageal lining. The cuticles were then slowly sloughed off posteriorly. Mating behavior by males was initiated in concurrence with the cap being shed by females. Males initiated mating by coiling their tail around the freshly exposed anterior cuticle in one to three loops (Fig. 9A). Males moved in a posterior direction by spiraling around the female body to migrate toward the vulva, which is located mid-body in *S. spiculatus*. This spiraling behavior was closely coordinated with the female molt. That is, as additional new



**Fig. 8.** Male and female *Strelkovimermis spiculatus* (A) host emergence and (B) molting patterns.



**Fig. 9.** Molting and mating behaviors of *Strelkovimermis spiculatus*. (A) Virgin female molting. The molting point (MP), a continually migrating site where the double juvenile cuticles are being shed and the new adult cuticle is revealed, is indicated. The cast cuticles (CC) are sloughed to the posterior. Two males (M) have grasped the female and are spiraling toward the vulva (V). Note the vulva's mid-body location. (B) Female molting showing a male attached at the molting point (MP) immediately behind the shedding cuticle. The male constantly makes adjustments to remain in contact with the molting cuticle until the vulva is exposed and copulation can be initiated. Note the pre-copulatory appearance of the vulva. (C) Gelatinous copulatory plug (CP) covering the vulva post-copulation. (D) Male mating with an egg-depleted female.

cuticle was revealed, the males moved in synchrony and proximal to the old cuticles until the vulva was exposed (Fig. 9B) and mating could occur. Multiple males contending for a single female were frequently observed (Fig. 9A). Mating pairs remained in copula for 1–2 days. Males deposited an adhesive mucous copulatory plug into and over the vulva before departing (Fig. 9C). Males were observed mating with egg depleted females (Fig. 9D).

#### 4. Discussion

Aggregation is a social behavior reported from many nematode species (Gaugler and Bilgrami, 2004) in response to food or environmental stimuli (Wharton, 2004). The formation of mermithid mating aggregations has been observed previously but not examined experimentally, although the behavior also occurs in some vertebrates, including fish (Seghers, 1974) and snakes (Luiselli, 1996), a few insects (Tierno and Figueroa, 2003) and is typical of the Nematomorpha (Hanelt et al., 2005). Male *S. spiculatus* attraction to conspecific females was unsurprising as this is one of the most common features of biology, and there are examples of female attraction to males as well even among the Nematoda (Duggal, 1978), but the strong mutual attraction of females to other females in our study was remarkable. By contrast, mating cluster or ball formation in other organisms is due to large numbers of males – as many as 100 males will simultaneously court a single female red-sided garter snake (*Thamnophis sirtalis parietalis*) (Joy and Crews, 1985) – attracted to individual females with no evidence for female–female attraction. Clearly *S. spiculatus* females through their attraction of males and other females are responsible for clusters formation. Furthermore, clusters enlarge not only due to the accumulation of individuals but also by frequent merger with other clusters to form larger clusters. Female postparasites are responsible for mergers as the large (19 mm length; Poinar and Camino, 1986) and powerful females partially extend themselves from a cluster to drag a cluster forward.

Asynchrony among sexes in emergence timing and realizing maturity is common and typically related to reproduction (Bulmer, 1983). The competitive advantage inherent in a *S. spiculatus* male grasping the female as soon she begins her molt to become an adult creates strong selection pressure for emergence and molting before females, that is, for protandry. Protandry is an adaptive reproductive strategy for early male emergence that provides an advantage in scramble competition for females. In contrast with our results, concurrent gender emergence was reported for *S. spiculatus* by Sanad et al. (2013), although the same study found the closely related mermithid *R. iyengari* exhibited protandry. This difference in conclusions between the earlier and present study might be attributed to experimental design. Postparasite emergence was observed individually in petri dishes in the Sanad et al. (2013) experiment, which required selecting parasitized hosts for observation, creating an opportunity for bias if hosts infected with the more evident female parasites were selected. Further, continuous observation under a microscope could add stress to the host and parasite and therefore an opportunity to skew the outcome. We observed emergence in a group of 30 host larvae selected randomly from a large pool of infected mosquitoes, and did not observe continuously or microscopically. In a species that places heavy emphasis on being the first to mate, it would be expected for protandry to occur in *S. spiculatus*.

Dual postparasite cuticles enclose the *S. spiculatus* female post-parasite, so that mating is prevented until molting is 50% completed and the vulva is exposed. Thus the first arriving male is assured of access to a virgin female, and his intermediate location between the vulva and subsequently arriving males blocks these competitors. The significance of this for male reproductive success

is obvious in single mating species, but is less clear in multiple mating species such as *S. spiculatus*. The implicit assumption is that there is a genetic advantage of mating with virgin females despite the threat of multiple paternities. We propose that the first male achieves a greater fitness advantage in offspring than succeeding males due to first-male sperm precedence (Jones and Parker, 2008). By grasping the female at the molting point between the old and new cuticles, and tightly maintaining this precise position until the vulva is revealed, the male is assured of first-male sperm precedence and enhanced fitness.

Copulatory plugs are regarded as a passive form of mate guarding employed by males to enhance their paternity in polyandrous mating systems (Devine, 1977; Barker, 1994). Female *S. spiculatus*, however, mate multiple times irrespective of a copulatory plug. Thus males may deploy plugs for reasons other than to prevent females from additional matings. For example, in *Caenorhabditis remanei* plugged females mated as frequently as unplugged females; however, plugging improved fecundity suggesting that plugs benefited the female partner rather than being a paternity assurance mechanism for males (Timmermeyer et al., 2010). The benefit of the copulatory plug remains unclear for *S. spiculatus* females, but may include reduced male harassment, increased fecundity, a seal to hold sperm within the female, or even a nutritive 'gift'.

The benefits for *S. spiculatus* males to participate in mating clusters are obvious: an opportunity to maximize fitness by having reproductive access to multiple females. Benefits for females are less apparent, but clearly participation in clusters was a prerequisite for efficient mating in our study. Female mating success in single pairs was low whereas virtually all females in larger clusters mated. This was attributed to greater molting in clusters: female molting to the adult stage was low when clusters were small, and females unable to molt are unable to mate, thus explaining the low average egg production per female characteristic of small clusters. In short, clusters enhance female molting. The constant writhing characteristic of large mating clusters generates resistance and torsion that assists females in shedding their cuticles. Improved molting appears to be a key motive for females forming clusters.

The benefits for females to mate with multiple males in species such as *S. spiculatus* where males offer no evident resources other than sperm are often uncertain (Jennions and Petrie, 2000). Increased fecundity can be a direct benefit of polyandrous mating shown in bark beetles (Lewis and Austad, 1994). Although we found *S. spiculatus* fecundity to increase in relation to cluster size, we attribute this to enhanced molting and therefore mating. Mated females had the same fecundity regardless of the number of mating partners. Mating clusters, however, do provide opportunities for intense male-male competition, serving the female's evolutionary need for high quality descendants (Luiselli, 1996; Møller et al., 2009; Luiselli and Rugiero, 2005). Certainly *S. spiculatus* males engage in competition for mates. Also mermithid sex ratio is female-biased at low parasite burdens and male-biased at high parasite burdens (Petersen, 1973; Miceli et al., 2012; Sanad et al., 2013), so males are rare when parasite densities are low. Mating clusters that bring multiple females together may offer advantages in this scenario since a single male can inseminate multiple partners. Other potential benefits that require evaluation include enhanced fertility assurance and increased offspring fitness through increased genetic variation.

Organisms adapted to ephemeral aquatic habitats such as *S. spiculatus* must evolve desiccation tolerance mechanisms. The principal means of avoiding fatal water loss in *S. spiculatus* is desiccation tolerant eggs (Wang et al., 2010), but mating clusters may provide an additional means. Single postparasites of *S. spiculatus* are much more susceptible to desiccation than those in a cluster

(unpublished data). Mating clusters may benefit both sexes by extending the period available to complete molting, mating and oviposition of desiccation tolerant eggs.

We have demonstrated that mermithid nematodes form mating aggregations to increase their fitness by enhanced molting and reproductive success. Mermithids may provide a model for the study of mating clusters as they relate to protandry, male-male competition, copulatory plugs and female choice systems.

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