The Role of Copulatory Plugs in Mosquito-Parasitic Nematode *Strelkovimermis spiculatus*

By

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ABSTRACT OF THE THESIS

The Role of Copulatory Plugs in Mosquito-Parasitic Nematode *Strelkovimermis spiculatus*

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The mosquito-parasitic nematode, *Strelkovimermis spiculatus* (Mermithidae: Nematoda) emerges from hosts and aggregates to form mating clusters characterized by intense male-male competition for females. Successful males deposit a copulatory plug over the female vulva after mating. In choice experiments, males strongly preferred virgin females, whereas plugged females were ignored. Males were not observed attempting to remove the plug nor endeavoring to mate. Females with a copulatory plug repelled males. The observed chemical repellency was independent of females, since excised plugs alone showed the same negative male response. The plug contributes significantly to female fitness because removal of the plug after mating was found to reduce fecundity by 90%. About average of 805 spermatids were found to leak out from a

female in the first 2 h after plug removal. Our initial hypothesis that the plug provides a nutritional gift was rejected due to the fact that there was no post-mating reduction in plug size that would have indicated absorption.

ACKNOWLEDGEMENTS

It is a pleasure to thank the people who made this thesis possible. I would never have been able to finish it without the guidance of my advisor and assistance of my committee members. We thank Linda McCuiston and Dr. Kshitij Chandel for assistance with maintenance of mosquito cultures.

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1. Introduction

Mermithidae are relatively well known because they can be found in other invertebrates such as spiders and crustaceans, where these nematodes are free-living roundworms as adults, and parasitic during the developmental phase. Five Nematoda orders are insect parasites, but only the Mermithidae have been found in the natural mosquito population (Poinar, 1975). Their lethality, during host exit, is of interest in biological control. Of its natural and laboratory infected hosts, 53% are Aedes sp., 20% are Anopheles sp., and 19% are Culex sp. (Poinar, 1979). Mermithids were successfully used as mosquito biological control agent in large scale in South America (Petersen et al., 1978).

Strelkovimermis spiculatus was first found in *Aedes albifasciatus* larvae in Argentina, and cultured in *Culex pipiens* in the laboratory (Poinar and Camino, 1986; Platzer, 2007). The life cycle of this aquatic mermithids is relatively simple. After being hatched from eggs, preparasites search and enter the body cavity of mosquito larvae through the cuticle in 24 h, and then absorb host nutrients in the hemocoel. After 7-10 days developing into larvae, nematodes then emerge from the mosquito larvae as postparasites. Approximately one week later, these nematodes

gather under sands and molt into adults. They mate and lay eggs during the next two weeks to complete their life cycle (Platzer, 2007).

After parasitic nematodes emerge from mosquito larvae, these postparasites form a mating cluster which varies in size due to gender differences and population size (Dong et al., 2014). In the cluster, each male must compete with others to successfully mate with females (Parker, 1970). In order to prevent re-mating, males may employ a variety of strategies. These include guarding while mating, direct sperm competition, and a copulatory plug.

Male place a plug on female genital opening after mating. Such plugs can be found in many taxa, including drosophila, rats, snakes, and spiders. Previous studies show that copulatory plugs can prevent females from re-mating.

In garter snakes, the males make no attempt to mate with plugged females; there appears to be a chemical cue involved, because no contact is required for this effect (Devin, 1977). In *Drosophila*, the plug prevents sperm leaking from the female body, as it is not permeable. Male *Drosophila* do not have appendages that could pierce or remove this structure, which is also indirect evidence that the plug serves as a physical barrier (Polak et al., 1998). Copulatory plugs prevent sperm

leakage not only in *Drosophila* but also in garter snakes. Apparent sperm leakage was observed when removing copulatory plugs from mated females (Friesen et al., 2013). In guinea pig, plug assures paternity of the first copulating male. Only progeny of first male was found if plug present (Martin et al., 1976).

In *C. elegans*, the effect of the copulatory plug is that of a chemical barrier. Male nematodes lose interaction with plugged females on the first encounter. Rather than contact with plug (Barker, 1994). In *Caenorhabditis remanei*, plugs do not decrease the chance for contact and mating, however, male spend more time on interacting with plugged female to locate where is vulva. In fact, those females with plug show a higher reproductive fitness, although it is not known whether this is due to the plug acting as a physical seal, or providing additional nutrients (Timmermeyer et al., 2010).

However, the function of plug was not reported in parasitic nematode, which is important in pest control. Our question is why does male spend time and energy to form a plug. According to previous plug study in different taxa, we had four hypotheses. First, plug is a chemical signal that repels other males to come in contact with the mated female. Second, it is a physical mating barrier that prevent other males to attempt to mate again. Third, it is a nutritional gift that provides

more nutrition for the female egg production. Last, it is a seal that prevents sperm from leaking out from vulva.

2. Materials and Methods

2.1 Host Culture

Mosquito larvae were obtained from a colony established from eggs collected in New Jersey, U.S.A. The colony was maintained at 26°C, 75% RH. in a 16L:8D photoperiod. Adults were maintained in cages and supplied with 10% sucrose solution on cotton wicks. Guinean pig were used to blood-feed female mosquitoes (animal care and maintenance were in accordance with approved Rutgers University Animal Use Protocol #86-129), and 500 mL cups were used for females to lay eggs. Egg rafts were collected and hatched as needed at 26^{o}C. Larvae were cultured in enamel trays with 1 L of dechlorinated water and 0.15 g of Brewer's yeast. After 6-9 days, pupae were transferred in 500 mL cups containing 350 mL of water, and the cups were then placed in 80 x 80 x 80 cm aluminum screen cages for adult emergence.

2.2 Nematode Culture

We studied the mermithid nematode, *Strelkovimermis spiculatus*.

Preparasites were cultured in larvae of *Culex pipiens pipiens* at 24 ± 1°C (Petersen and Willis, 1972). Nematodes were hatched the day before infection, with second instar mosquito larvae then were exposed at the ratio of 1:3 (host:parasite). After a 12 h infection period in 500 mL cups containing 200 mL of water, the larvae were transferred into trays (38 × 23 × 5 cm) containing 2 L of water and 0.35 g of yeast was provided every other day as food. Five days later, larvae were transferred into another container (17 x 17 x 7 cm) with 1,700 mL of water. The container has a 155-mesh screen suspended 3 cm from the bottom, which allows nematodes to move into the tray bottom after emerging, whereas host cadavers remain on the screen.

Postparasites were collected daily into glass bowls until host emergence was completed. Approximately 20–30 pairs of nematodes were inoculated into cups (60 mL) containing 1.5 cm of sterilized coarse sand (1 – 4 mm diameter particle size) and 30 mL of deionized water for molting, mating, and oviposition. To obtain eggs in diapause, water was removed from the cups 14 days after inoculation. These eggs were held at 24 ± 1°C for further experiments.

2.3 Adult Nematodes Prepare

To obtain freshly molted adult nematodes and freshly mated females with plugs, postparasites were separated by gender into different cups (60 mL) containing 30 mL of sterile water and a 3 mm layer of sterilized coarse sand to assist the molting process. Sixty molted males were merged with 60 virgin females (< 24 h post molting) in one 60 mL cup containing 30 mL of sterile water, and observed every 3 h for mating nematodes. These nematodes were then transferred into a micro plate, which was observed every 6 h to see if the mating was completed, and whether there was a plug on the female vulva. We used sterile water in our experiment to avoid nematode pathogens, and all containers were bleached and rinsed prior to every experiment.

2.4 Male Attraction Test

Chemical communication between females and males was examined in a trap apparatus. We designed an attraction assay by modifying a 90 mm petri dish. Trap wells were created by drilling four 7.5 mm diam holes in the dish bottom and hot gluing a tip cut from the bottom of a centrifuge tube (11.5 x 7.5 mm diam, 0.8 ml capacity) beneath each hole. The wells were equidistant and 10 mm from the plate edge. Any nematode entering this trap would not be able to escape. All equipment was rinsed with 70% alcohol, then washed in

deionized water prior to every test.

In each assay dish, 20 mL of sterile water and one layer of coarse sand (1 mm diameter) were added. Sand assists nematode movement in the petri dish. One virgin female was transferred into one well, and a mated female was moved into the opposite well. The other two wells, containing only 1 mL of deionized water, served as a control. After 12 h of acclimatization, one male nematode was transferred into the center of the petri dish. The position of the male nematode was examined 8 h later. The experiment was performed at 25°C, and repeated three times, using 10 replicates for each repeat.

The percentage of males attracted to virgin or plugged females was then determined.

2.5 Male attraction to female with plug removed *vs.* plug intact

Mated females, with either plugs intact or removed, were compared to determine which one was more attractive to males.

The experimental setup was identical to the previous section, except that plugs were removed from some mated females using an interdental brush. Then one plug-intact female was transferred into one well, with a plug-removed female being moved into the opposite well. The other two wells, containing

only 1 mL of deionized water, served as a control. After 12 h of acclimatization, one male nematode was transferred into the center of the petri dish. The position of the male nematode was examined 8 h later. The experiment was performed at 25°C, and repeated three times, using 10 replicates for each repeat.

The percentage of males attracted to plug-intact or plug-removed females was then determined.

2.6 Copulatory Plug Repellency Test

To determine whether the plug itself is a repellent to other males, we performed the following experiment.

The experimental method was identical to the previous sections, except that one plug removed from mated female was transferred into one of the wells. The other three wells, containing only 1 mL of deionized water, served as a control. After 12 h of acclimatization, one male nematode was transferred into the center of the petri dish. The position of the male nematode was examined 8 h later. The experiment was performed at 25°C, and repeated three times, using 10 replicates for each repeat.

The percentage of males attracted or repell to plug was then determined.

2.7 Copulatory Plug as a Physical Mating Barrier Test

Freshly mated females (< 6 h after mating completed) with their plugs removed as the treatment group. A plug-intact female, and a plug-removed female, were separately introduced into one well of a 12-well (24 mm diameter) micro plate, containing two molted virgin males and 2.5 mL of sterile water. Plug-intact females were used as the control group. Observations were made every 10 minutes, in order to determine whether males attempted to mate with females, with the total observation time being 360 minutes.

The following variables were measured:

- Number of contacts between males and females
- Number and duration of coiling (males coil onto females and move toward vulva)
- Number of copulatory plugs produced. This is considered to be indication of successful mating.
- For the control, number of attempts made by males to remove plug

2.8 Copulatory Plug as a Nutritional Gift Test

In the treatment group, the female that was mating (male grabbed onto the vulva) was selected and transferred into one well of a 12-well (24 mm

diameter) plate with 2.5 mL of sterile water. Observations were made every 30 minutes, as soon as the male released the vulva and there were plugs on it, then the plug was removed with an interdental brush to see if plug increase fecundity. A plug-intact female was used as the control. Controls were observed at 8 h intervals for 72 h, in order to determine whether the plug was absorbed. The experiment was performed at 24 ± 1°C, with 10 replicates for each treatment and control, and the experiment was repeated three times. The following variables were measured:

- Plug size (area) were measured in median longitudinal section view (from photomicrographs taken every 8 h)
- Fecundity (from the number of eggs laid by each female in the well)

2.9 Copulatory plug as a sealant to Block Sperm leakage Test

A female with the plug removed was mounted on a microscope slide with a droplet of deionized water. The slide was observed under a microscope to determine whether there was any sperm leakage from the vulva. Two h after plug removed, sampled three drop from the deionized water (0.1mL per drop), burned to fixed it on a slice and dyed by Giemsa stain (EMS company) for 15 minutes (Dilute Giemsa Stock solution at 1:10 with deionized water.) (Amer,

2001). Females with copulatory plugs intact (from cup-3, Section **2.3**) were used as the control. Each experiment was repeated three times, with three replicates for each repeat. The number of spermatids leaked from the vulva was determined for treatment and control groups.

2.10 Data Analysis

Female attractiveness, plug repellency and plug size (three days) were analyzed by ANOVA multiple comparisons, using Fisher's least significant difference (LSD) in multiple range tests among the means ($P < 0.05$). Data collected in fecundity was analyzed by student T-test among the means ($P < 0.05$). Data in the text and figures are presented as means ± S.E.

3. Results

3.1 Male Attraction Test

Twelve h after the males were transferred, 56.67 ± 3.33% of the males were found in the virgin female's well, only 16.67 ± 3.33% found in the wells of plugged females ($p = 0.0001$) (Fig. 1). Thus, virgin females were clearly more attractive for the males. The plug intact female trap was no significant difference with controls.

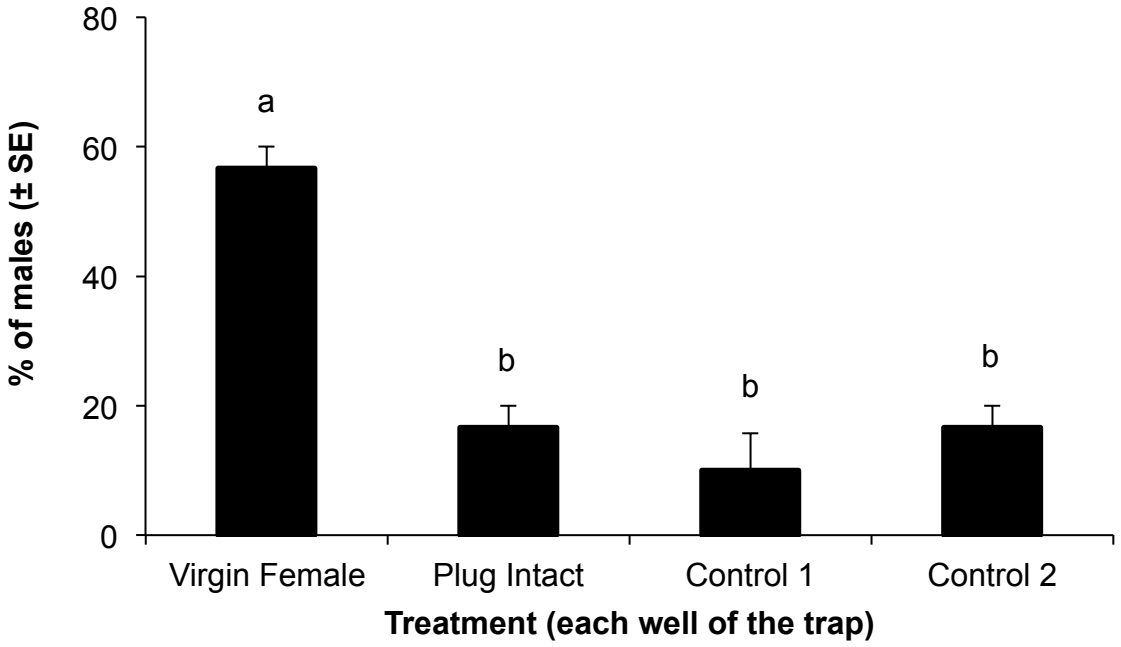

Fig. 1. Percentage of molted adult male attraction between molted adult virgin, mated female with copulatory plug and controls (sterile water). Bars with the same letter are not significantly different ($p \geq 0.05$).

3.2 Male attraction Test To Plug removed *vs.* Plug intact female

To determine whether plugged females were less attractive to males due to the plug, and not because the females had mated, plugs were removed from females, and then were compared with plugged females. 50.00 ± 0.00% of the males chose the female with the plug removed, while only 20.00 ± 5.77% of the males were found in the plugged female's trap ($p = 0.0009$) (Fig. 2). Although both females mated, it was clear that females with plugs removed

were more attractive to males.

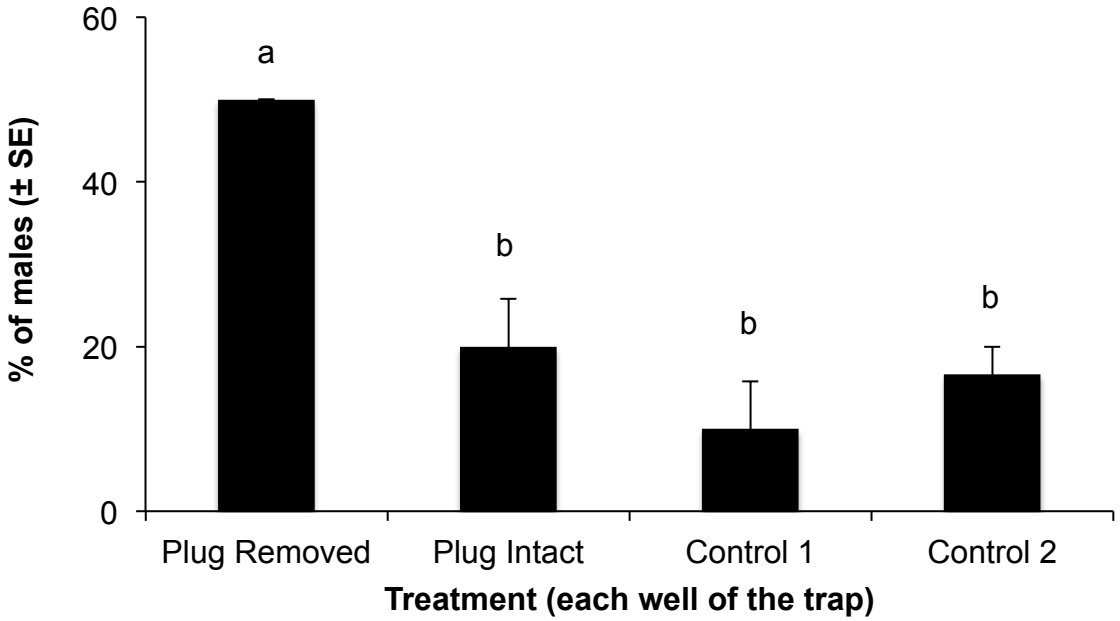

Fig. 2. Percentage of male attraction between mated but plug removed, plug intact female and controls (sterile water). Bars with the same letter are not significantly different *($p \geqslant 0.05$)*.

3.3 Copulatory Plug Repellency Test

With traps only containing plugs, only 6.67 ± 3.33% of the males were found in the plug wells. For the well that was most distant from the plug attracted 43.33 ± 3.33% of the males ($p = 0.0004$) (Fig. 3). Further from the plug, more males were found. Thus, males prefer wells containing no plugs.

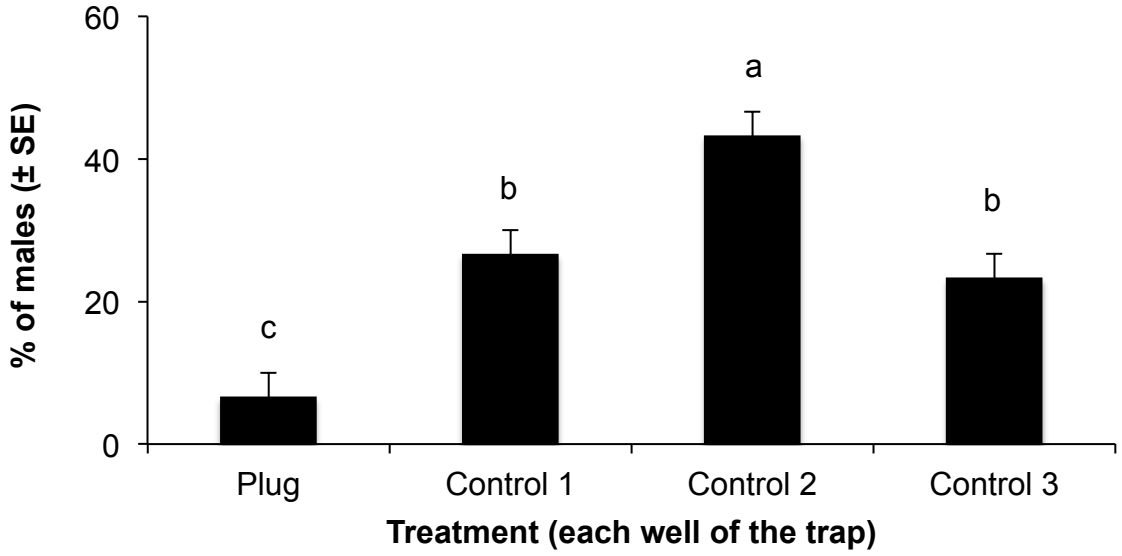

Fig. 3. Percentage of male repellency between plug and controls (sterile water). Bars with the same letter are not significantly different *($p \geqslant 0.05$)*.

3.4 Copulatory Plug as a Physical Mating Barrier Test

In the behavioral study, 48.03% of males made contact with females around the vulva, with 49.62% producing coils in this area. Neither tail (29.57%) nor head (20.81%), males coiled on females in one third of middle. Males are aware of the position of the vulva. Males were observed to coil on virgin females for average 55.26 minutes, whereas they lost interest in plugged females in average 24.78 minutes. They did not attempt to remove female plugs and subsequently mate with the females. Despite these results, in the plates, the overall mating success rate (control group) was only 2.38%. Since

males lose interests in mated female with plug, no mating attempt was observed in our experiment, so the physical mating barrier hypothesis was rejected.

3.5 Copulatory Plug as a Nutritional Gift Test

The plug area was not reduced over time, and therefore it did not appear to be absorbent. Figure 6 illustrates that there was no significant difference between days of analysis. The plug average area of Day 1 was 5,060 ± 326 μm^2, 5090 ± 545 μm^2 on Day 2 and 5100 ± 440 μm^2 on Day 3. The area sizes of plugs were not significant difference between 3 days ($p > 0.05$) (Fig. 4). Plugs came off when the female began laying eggs.

Plugged females were found to lay more eggs (2,384.90 ± 132.41 per female) than those females with plugs removed (190.46 ± 6.53 per female).

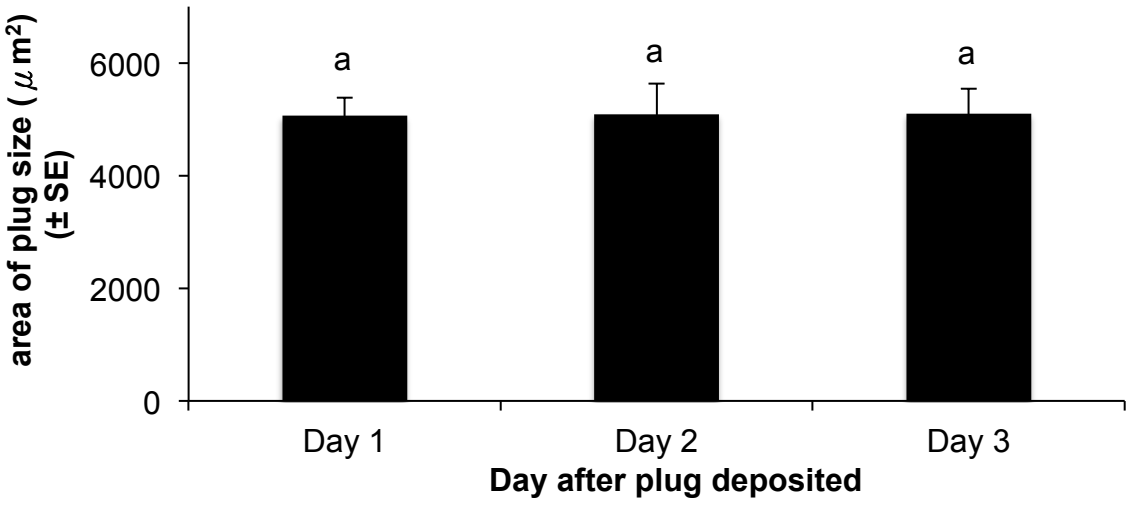

Fig. 4. Area of plug size (Lateral view) measured daily after plug placed by male.

3.6 Copulatory plug as a sealant to Block Sperm leakage Test

In our experiment, leaking was found after the plug was removed (Fig. 5). An average of 805 spermatids leaked in the first 2 h following plug removal (Fig. 6).

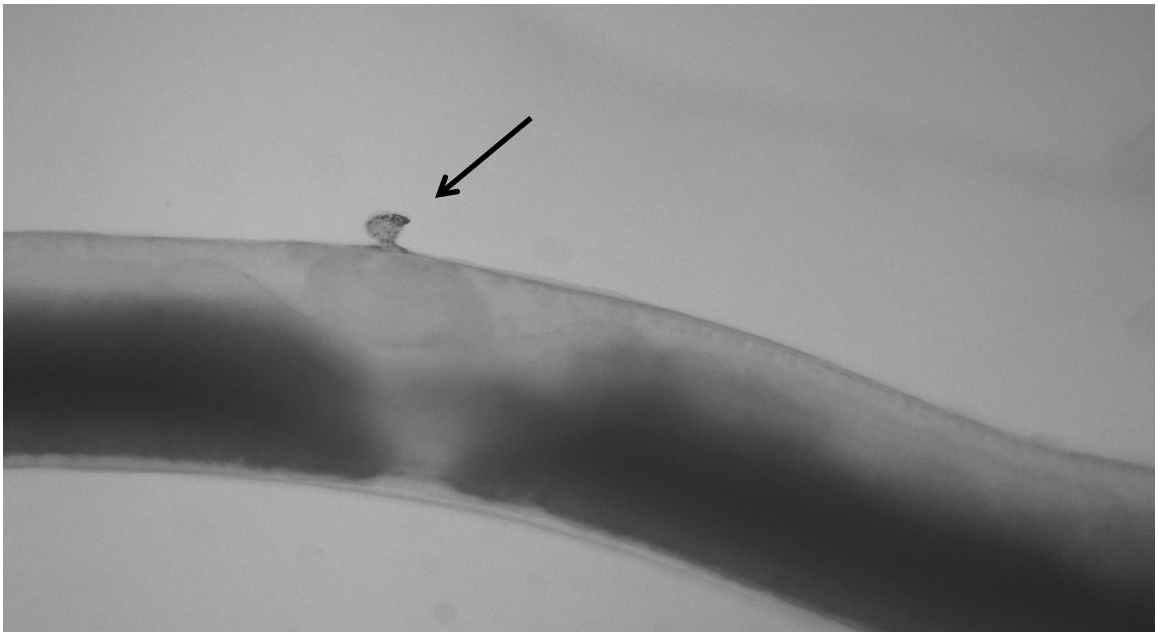

Fig. 5. Leakage (arrow) from vulva after removal of the plug.

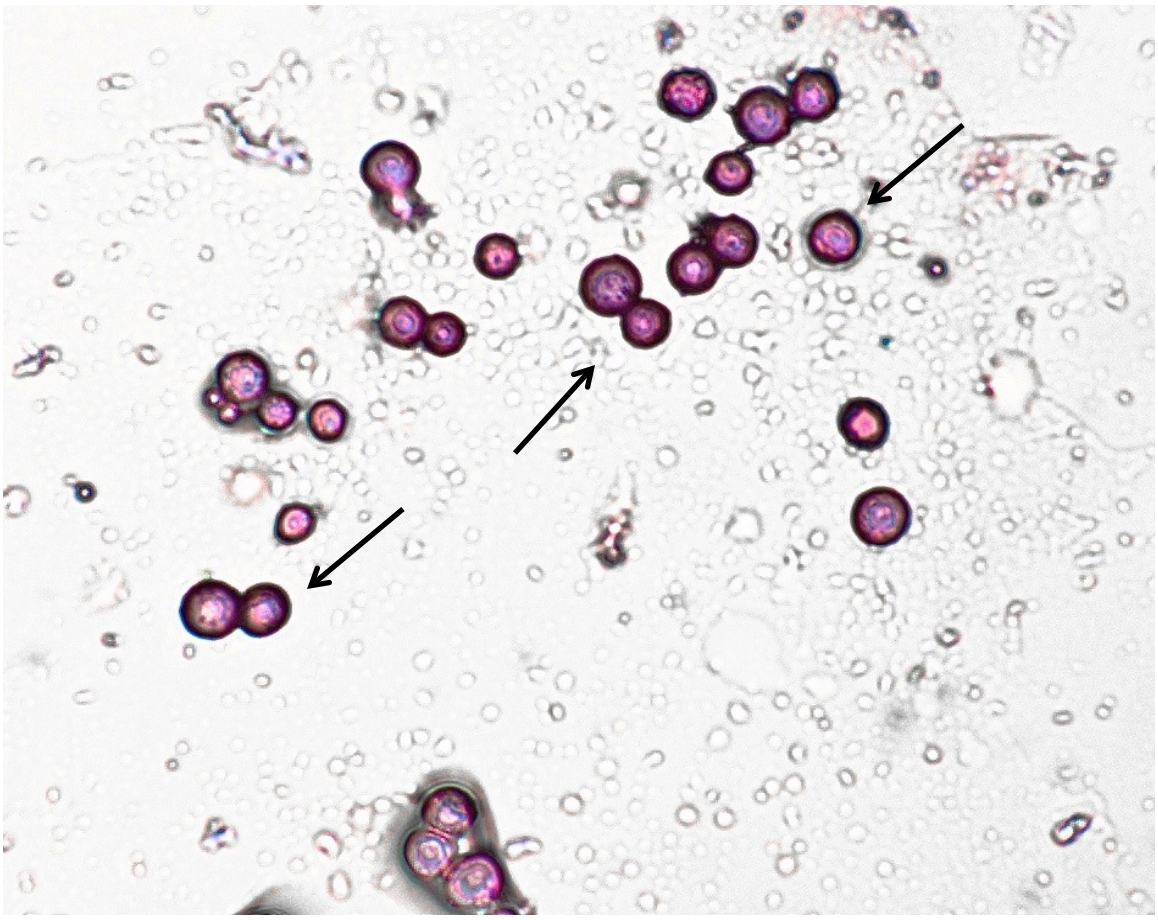

Fig. 6. Spermatids (arrows) observed after Giemsa staining in the leaked material from female genital tract after plug removal.

4. Discussion and Conclusion

Finding food or a mate is crucial for survival, particularly for those organisms of low activity, such as nematodes. Chemical cues play an important role in nematode survival (Huettel et al., 1986). Chemoreceptors known as amphids are located on both sides of the mouth in *C. elegans* (Bargmann et al., 1990). The

neurons at the ends of the amphids are sensitive, and can distinguish odorants, with some also being able to determine varying concentrations of the same chemical. Our results suggesting that males are interested in virgin females are not surprising. In *Panagrellus redivivus*, both females and males produce ascaroside-based signaling molecules to attract each other, however the attractants emitted by different sexes are distinctive, and only attracted the opposite sex (Choe et al., 2012). On the other hand, formation of mating clusters in *S. spiculatus* involves chemical cues to drive aggregation. In mermithids, both males and females are attracted to females (Dong et al., 2014). In our study, males were not attracted to all females. Males of *S. spiculatus* were interested in both virgin females and copulatory plug removed females. Males were more interested in plug removed females than plug intact ones. Moreover, our results showed that plugs alone repel other males. Males lose interest in plugged females, as they can reduce sperm competition and protect the paternity of plugger (Barker, 1994). Our result showed clearly that the presence of plug reduced contact and coiling time; male ignore those plugged females.

In this study, the mating rate for two males with one virgin female was only 2.38%. In a previous study, larger clusters were associated with increased mating rate and fecundity in *S. spiculatus* (Dong et al., 2014). To increase the successful

mating rate, mating cluster is important. In *Caenorhabditis remanei*, the presence of a copulatory plug did not decrease attractiveness of females, and the plug performs as a physical mating barrier. *Caenorhabditis remanei* males stayed longer on virgin females than plugged females (Timmermeyer et al., 2010). In contrast to *S. spiculatus*, no mating was attempted for plugged females in our study. Males lost interest in mating with such females, and did not attempt to remove the plug, and therefore, this plug does not seem to be a physical mating barrier in *S. spiculatus*. The ability for a male to identify plugged female is really important, he can look for another virgin female to mate with. The repellency of plug not only protect paternity of first mated male but also increase the opportunity that other male could mate with virgin female.

Our results suggest that plugs do not have a role as nutritional gifts for females of *Strelkovimermis spiculatus*. However, we found that fecundity in plugged females was 90% higher than in unplugged females. Compared to other studies (*C. remanei*), plugged females laid 30% more eggs than unplugged females, and no sperm leakage was found (Timmermeyer et al., 2010). It was documented in *Drosophila* that male trigger female to invest more energy into egg production (Chapman, 2001). There might be proteins or chemicals in copulatory plug that activate the sperm or oocyte.

Except this function, plug is required for maintaining ejaculate in female genital tract after mated (Avila et al., 2015). Postparasites of *S. spiculatus* are free living in water, and their internal body pressure is relatively high. Without the presence of a plug, following ejaculation, a female twisting in water would squeeze out the sperms. Whether the plug contains compounds that stimulate females to lay more eggs needs to be investigated.

In conclusion, our data support the hypotheses that copulatory plugs in *Strelkovimermis spiculatus* function not only as a chemical repellent to other males, but also as a seal that prevents leakage of spermatids. Male spends long time on placing a plug is also a guarding behavior while mating. They protect the paternity of the first mated male, and increase fecundity of the female. Copulatory plugs in *S. spiculatus* appear to benefit both sexes.

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