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Behavior manipulation of mosquitoes by a mermithid nematode

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ABSTRACT

We examined manipulation of mosquito behavior by the parasitic mermithid nematode, *Strelkovimermis spiculatus*. This nematode species typically infects early instar host larvae and emerges after parasitic development to kill last-instar larvae. Parasitized adults, however, have occasionally been reported from field collections. We obtained low rates (1.7–11.5%) of parasitized adults in laboratory exposures only when *Culex pipiens pipiens* fourth-instar larvae nearing pupation were exposed to infective nematodes. This did not allow an adequate interval for parasitic development in immature host stages. Parasitized adult females in a multiple-choice assay were three times more likely to seek water than a blood source (63.1 vs. 20.5%), whereas uninfected females were twice as likely to seek blood than water (64%3.9 vs. 32.6%). This altered host behavior benefits the parasite by providing the only mechanism for dispersal and colonization of new host habitats while concurrently avoiding risks from the defensive behaviors associated with blood-feeding. Behavioral alternation in *Cx. p. pipiens* larval hosts was also examined using larvae infected as second instars to allow for a normal duration of parasitic development. As larvae neared pupation and parasite emergence, parasitized larvae became more spatially aggregated than unparasitized larvae. This altered host behavior benefits the parasite by providing a corresponding increase in post-parasite aggregation, which facilitates formation of large mating clusters and concomitantly reproductive success. Parasites derive fitness gains by overriding host autonomy, whereas hosts have zero fitness once parasitism is established, suggesting a coevolutionary response is inoperative and that the behavioral modifications may be adaptive.

1. Introduction

The mosquito-parasitic mermithid nematode *Strelkovimermis spiculatus* (Mermithidae: Nematoda) was first isolated from *Aedes albifasciatus* (Macquart) (Poinar and Camino, 1986) and subsequently from *Culex pipiens* (Garcia and Camino, 1990). The nematode's parasitic phase begins with the newly hatched pre-parasites (second-stage juveniles) seeking out and penetrating mosquito larvae. After 7–10 days of parasitic development, nematodes exit from fourth-instar mosquito larvae as post-parasites (i.e., third-stage juveniles which must molt twice more to become adults). Mermithid emergence invariably kills the host. This lethality, coupled with mermithid specificity for mosquitoes and ease of in vivo culture, have inspired inoculative biological control efforts with encouraging results (Achinelly and Micieli, 2013; Platzer, 1981).

Although larval mosquitoes are characteristically parasitized by *S. spiculatus*, parasitized adults have occasionally been reported from field collections (Camino and Reboredo, 1994; Campos and Sy, 2003; Di Battista et al., 2015). Because parasitism is lethal, an exclusively larval parasitic mermithid should have an exceptionally limited geographical distribution. Parasitism of adult hosts is the sole means for *S. spiculatus* to escape mosquito pools, which are often ephemeral, and colonize new host habitats. This generates a conflict of interest between the fitness needs of: (1) an anautogenous host to take a blood meal for egg development before locating a habitat for oviposition and (2) the parasite to return to water without the risky intermediate step of blood-feeding.

Host behavioral manipulation is one of five infection processes (i.e., host habitat location, host finding, host acceptance, host suitability and host manipulation) (Vinson, 1976). According to the host manipulation hypothesis, some parasites modify host behavior to benefit their own fitness (Moore, 2002). This hypothesis has been confirmed for an array of host-parasite interactions. For example, the protozoan parasite

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Toxoplasma gondii modifies host mice's aversion to cats, the intermediate host, to increase the likelihood of being captured and ingested (Webster, 2007). Thus, absence of fear in *Toxoplasma*-infected mice increases parasite fitness (Flegr, 2007). The 'death grip' behavior characteristic of carpenter ants infected by the parasitic fungus *Ophiocordyceps unilateralis* has no apparent purpose other than to enhance parasite reproduction and transmission (de Bekker et al., 2014).

Aggregation is a social behavior reported for many nematode species (Gaugler and Bilgrami, 2004), including *S. spiculatus* post-parasites which seek to form mating clusters following emergence. Female molting, mating and fecundity are optimal in large mating clusters (Dong et al., 2014). Males participating in mating clusters may similarly maximize fitness by having access to multiple females. Mermithid post-parasite aggregation is clearly an important behavior.

We address two hypotheses for host behavioral manipulation by *S. spiculatus*. First, parasitized adult hosts are more likely to seek water than to blood feed, thereby increasing parasite fitness by facilitating dispersal while avoiding parasite risk from host defensive behaviors during blood-feeding. Second, the distribution of parasitized mosquito larvae nearing pupation is more likely spatially aggregated than that of unparasitized larvae, thereby increasing parasite fitness by facilitating the formation of nematode mating clusters.

2. Methods

2.1. Mosquito colony

A colony of the anautogenous species and *S. spiculatus* host, *Cx. pipiens pipiens*, was established at Rutgers University from eggs collected in New Brunswick, New Jersey, USA in 2016. The colony was maintained at 26 °C, 75% RH and a 16L:8D photoperiod. Egg rafts were collected from an oviposition container (400 ml black cup) containing 250-ml dechlorinated water. Larvae were held in enamel trays with 1-L dechlorinated water replaced on alternate days. Food (0.15-g Brewer's yeast) was supplied daily. Pupae were transferred into 400 ml plastic cups in 80 × 80 × 80 cm mesh cages for eclosion. Adults were supplied with 10% sucrose solution on cotton wicks. Northern bobwhite quail (*Colinus virginianus*) were used for blood-feeding adult mosquitoes. Animal care, maintenance, and blood-feeding were in accordance with approved Rutgers University Animal Use Protocol #86–129.

2.2. Nematode colony

The mermithid *S. spiculatus* was reared in *Cx. p. pipiens* larvae according to methods developed by Petersen and Willis (1972). Briefly, nematode eggs were hatched by flooding overnight with deionized water to provide pre-parasites. Second-instar *Cx. p. pipiens* larvae were exposed to mermithid pre-parasites at a parasite:host ratio of 3:1 in a beaker holding 200-ml of water for 12 hrs. Mosquito larvae were then removed by sieve, rinsed, and transferred into trays holding 1-L deionized water. Emerged mermithid post-parasites were maintained at 25 °C in a 5.5 × 7 × 4.5 cm container with 20-g sand and 50-ml water for mating and oviposition. Free water was drained after 14 d and the moist sand containing mermithid nematode eggs stored in a container sealed with plastic film to maintain high humidity at 25 °C. Only eggs stored for no longer than two weeks were used in the experiments.

2.3. Adult host parasitism

A replicate of either twenty early or twenty late fourth-instar mosquito larvae were introduced into 30-ml of water at parasite:host ratios of 5:1, 10:1, 15:1, 20:1 or 40:1. Twelve hours after exposure to pre-parasites, mosquitoes were transferred by pipette to an enamel tray containing 1-L water. Mosquitoes were observed daily, and dead specimens removed for microscopic dissection to determine parasitic status. Eclosed adult mosquitoes were transferred into 50-ml centrifuge tubes individually with 10-ml water to allow for nematode emergence. Dead adults were dissected to determine parasitism. Each experiment was replicated three times and the experiment was repeated twice. Proportions of parasitized and unparasitized mosquitoes were recorded for each infection ratio.

2.4. Adult host preference

Our hypothesis that parasitized adult mosquitoes are likely to seek water over blood was tested in a choice assay. Parasitized adult mosquitoes for the assay were obtained by exposing late fourth-instar larvae to a parasite:host ratio of 10:1 as determined to be optimal from the experiments described in section 2.3. These infections were initiated in 30-µl water droplets holding 10 pre-parasites and a single *Cx. p. pipiens* larva. After a 12-h exposure, mosquito larvae were transferred into enamel trays. Emerged adult mosquitoes were held in a 38 × 29 × 30 cm mesh cage with a 10% sucrose solution provided on a cotton wick.

A three-choice preference assay was constructed using three connected cages: a center or neutral cage (66 × 41 × 34 cm) was linked to two opposing 3.8-L attraction cages (26 × 16 × 14 cm), one baited with a 250-ml cup of water and the second one with a restrained quail (Fig. 1). Unidirectional funnels connected the center neutral cage to the attraction cages which served as traps for adult mosquitoes entering the cages. A 1 cm diameter opening in the center cage lid was provided for mosquito inoculation. A 3-cm diameter meshed hole at distal ends of each attraction cage allowed air movement. Female mosquitoes (2–4 d post-eclosion) were starved for 15 h before an assay was initiated.

A restrained quail and a water cup were placed in opposing attraction cages 10 min before introducing mosquitoes into the neutral cage via the inoculation port. The assay was repeated three times with 73, 144 and 108 female mosquitoes held at 25 °C, 78% RH and no light.

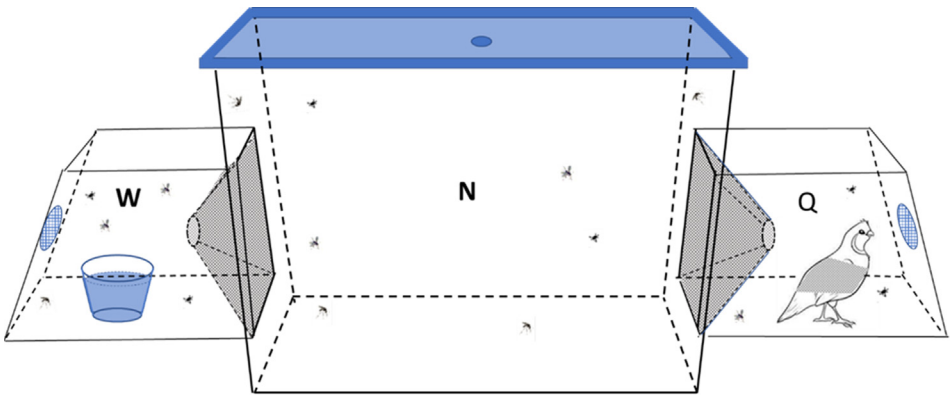

Fig. 1. Apparatus for adult female *Culex pipiens pipiens* preference assay. N: neutral cage for mosquito inoculation; W: water-baited cage; Q: quail-baited cage.

Attraction cages were reversed between replicates. Mosquitoes were collected from each of the three cages after 100 min and dissected to determine which mosquitoes had been parasitized. Proportions of parasitized or unparasitized mosquitoes preferring water, host or neutral cages were compared to establish preference. All data are represented as mean ± SE.

2.5. Larval host aggregation

Our hypothesis that parasitized mosquito larvae nearing pupation are more spatially aggregated than unparasitized larvae was tested in a 78 × 31 × 15 cm plastic tray holding a 5-cm depth of water. The tray was gridded with a marker on the bottom into 160 squares each measuring 3.9 × 3.9 cm. One hundred second-instar mosquito larvae were infected at a 3:1 parasite:host ratio as described previously. Mosquito larvae in the control (i.e., unparasitized) group were prepared the same as the treatment larvae but were not exposed to mermithids. Trays were maintained at 26 °C and 16L:8D photoperiod. The number of mosquito larvae in each square was determined from photographs taken on the day of mermithid emergence. The experiment was repeated four times.

Spatial aggregation of parasitized (treatment) and unparasitized (control) mosquito larvae nearing pupation was analyzed using Lloyd's mean crowding index (Lloyd 1967, Reiczige 2005). Lloyd's mean crowding, m*, is the mean number per individual of other individuals in the same quadrat. The samples were bootstrapped 9999 times to estimate 95% confidence intervals using package "boot" in R statistical software (R Core Team, 2017). To assess statistical significance, the differences between the bootstrapped indices of parasitized and unparasitized groups in each of the four experiments were calculated and a single sample *t*-test conducted to determine 95% confidence intervals. The difference was considered significant if 95% confidence intervals did not include zero.

3. Results

3.1. Adult host parasitism

Exposing early fourth-instar *Cx. p. pipiens* larvae to *S. spiculatus* preparasites did not result in adult parasitism at any parasite:host ratio tested (data not shown), whereas exposing late fourth-instar larvae to ratios of 10:1 and 15:1 resulted in 11.2 ± 3.6 and 11.52 ± 0.9% parasitized adults, respectively (Fig. 2). Extreme exposures of 20:1 and 40:1 yielded nominal adult parasitism (1.7% each). All other exposed hosts either were resistant to infection (range, 43.5–71.6%) or died as parasitized larvae (range, 10.0–47.8%) or pupae (range, 4.4–23.3%). For example, at the 5:1 ratio, 56.5 ± 1.8% of exposed host larvae became infected, yet only 4.4% ± 0.5 became adults, as most either failed to pupate (47.8 ± 2.1%) or failed to eclose (4.4 ± 0.7%). Infected mosquito larvae could survive up to nine days post-infection, typically with live but incompletely developed nematodes that failed to exit the host. Arrested nematode development was frequently observed again in pupae that failed to eclose. Dissection of these pupae revealed well-developed parasites which nevertheless were unable to exit via the tough pupal integument. Cuticles of parasitized adults became increasingly translucent due to the depletion of fat, reproductive and other host tissues so that fully developed parasites on the threshold of emergence were frequently observable through the cuticle (Fig. 3).

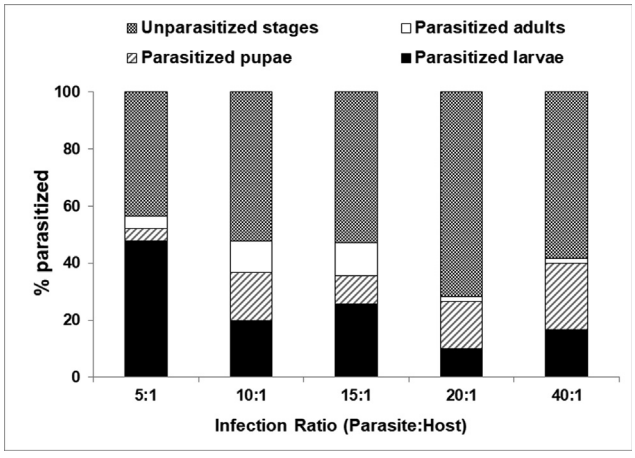

Fig. 2. Parasitic status of the mosquito *Culex pipiens pipiens* at different life stages exposed as late 4th-instar larvae to the mermithid nematode *Strelkovimermis spiculatus* at five different parasite:host ratios. Parasitized mosquito larvae and pupae did not survive infection to become adults.

Fig. 3. *Culex pipiens pipiens* adult mosquito with a distended, translucent abdomen from carrying the parasitic mermithid nematode *Strelkovimermis spiculatus*.

3.2. Adult host preference

Parasitized female mosquitoes presented with a choice of water or a blood meal (i.e. quail) were three times more likely to choose the water cage (63.1 ± 10.5% vs. 20.5 ± 6.6%; $P = 0.03$) (Fig. 4). There was no difference between the likelihood of parasitized adults migrating to the quail-baited cage as in remaining within the central neutral cage (20.5 ± 6.6% vs. 16.4 ± 4.4%, $P = 0.64$). In contrast, unparasitized adults were twice as likely to choose the quail than the water-baited cage (63.9 ± 4.1% vs. 32.6 ± 5.4%; $P = 0.01$), and they rarely failed

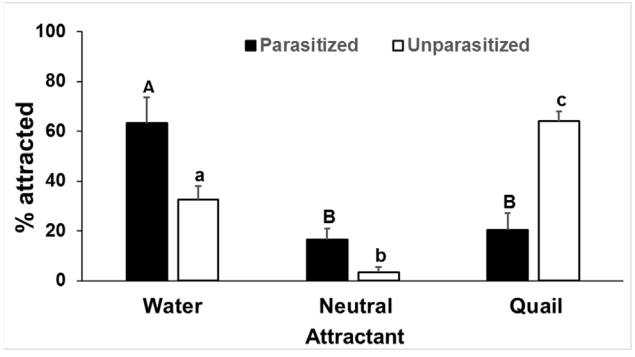

Fig. 4. Attraction response in preference assay of *Strelkovimeris spiculatus* parasitized vs. unparasitized *Culex pipiens pipiens* adult females to chambers baited with water vs. a restrained quail. Same letters in the same case indicate no significant difference ($P \geq 0.05$).

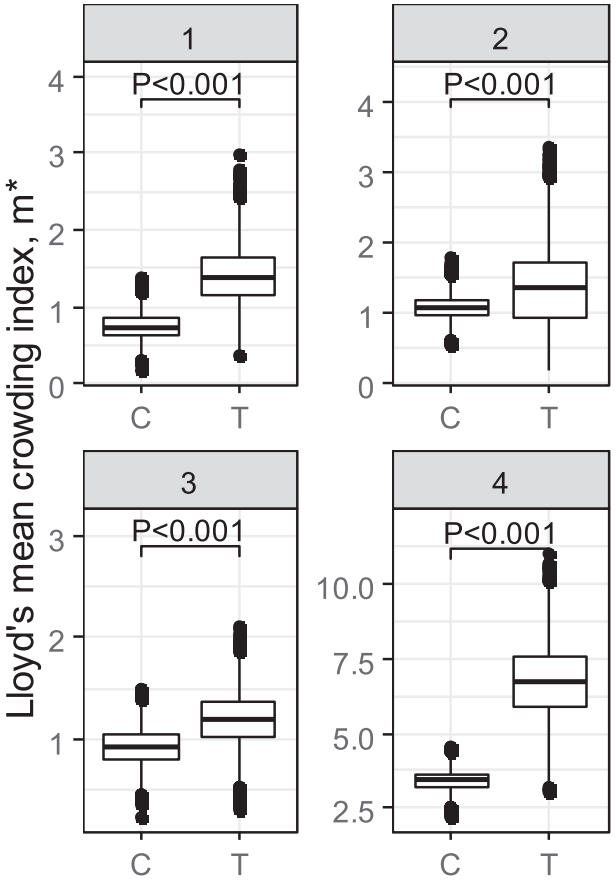

Fig. 5. Mean crowding comparison between parasitized and unparasitized mosquito larvae for four experiments. Bootstrapped sample distributions are presented as boxplots for parasitized (T = treatment) and unparasitized (C = control) groups for each experiment. To assess statistical significance, the differences between the bootstrapped means of parasitized and unparasitized groups in each experiment were calculated and a single sample *t*-test conducted to determine 95% confidence intervals. The difference was considered significant if 95% confidence intervals did not include zero. Differences for all four experiments were statistically significant at $P < 0.001$.

to migrate from the neutral cage (3.5 ± 1.8%).

3.3. Larval host aggregation

In each of the four experiments, parasitized (treatment) larvae on the threshold of nematode emergence were more aggregated than unparasitized (control) larvae (Fig. 5). Mean crowding index comparisons for each experiment [95% CI estimated by bootstrapping] resulted in the following, treatment vs. control: $m^*_t = 1.4$ [95% CI 0.9–2.5] vs. $m^*_c = 0.8$ [95% CI 0.5–1.1] in experiment 1; $m^*_t = 1.4$ [95% CI 0.7–3.2] vs. $m^*_c = 1.1$ [95% CI 0.8–1.5] in experiment 2; $m^*_t = 1.2$ [95% CI 0.8–1.8] vs. $m^*_c = 0.9$ [95% CI 0.7–1.3] in experiment 3; and $m^*_t = 6.8$ [95% CI 4.9–9.8] vs. $m^*_c = 3.4$ [95% CI 2.8–4.2] in experiment 4. Analysis of the mean difference between the two groups, parasitized versus unparasitized, indicated that in each experiment the difference was statistically significant at $P < 0.001$ with bootstrapped 95% CI not overlapping zero.

4. Discussion

Strelkovimermis spiculatus occasionally infected *Cx. p. pipiens* larvae nearing pupation and persisted through to adult hosts. Parasitism of adult hosts is a dispersal mechanism as suggested by Campos and Sy (2003) and Di Battista et al. (2015). Campos and Sy (2003) provided support for this idea by suggesting that flight ability is likely unhindered by mermithid parasitism. Nevertheless, adult infection is a high-risk strategy as parasitism usually concluded in host and therefore parasite death.

The opportunity for mermithids invading larval mosquitoes to be carried over into adult hosts has a narrow window of time as few hosts infected as they neared pupation survived to eclosion. Exposure of early fourth-instar mosquito larvae failed to produce parasitized adult mosquitoes. This is consistent with earlier studies on other mermithid species. Kurihara and Maeda (1980) did not obtain parasitized adults when exposing 3-day old fourth-instar *Culex pipiens molestus* to *Romanomermis culicivorax,* whereas exposing 4-day old fourth-instars did subsequently produce adult parasitism. Our findings resolve Di Battista et al.'s (2015) question as to whether mermithids found in adult mosquitoes were infected during earlier or later instar host larvae.

Delayed host development is a common response in insects infected by pathogens (Gaugler and Brooks, 1975) and parasitoids (Shaw, 1981). Host physiological regulation may be responsible as hypothesized by Vinson and Iwantsch (1980) for insect parasitoids and Gordon and Webster (1971) for the terrestrial mermithid *Mermis nigrescens*. Destroying or altering the host endocrine system to disrupt metabolism and hormonal status tends to favor parasite over host growth and development (Gordon, 1981). We submit that pre-parasites initiating infection in hosts nearing pupation have insufficient time to alter host hormones. Consequently there is insufficient time to complete parasitic development in host larvae and these mermithids must pass into pupae for further development. Pupae appear impenetrable to parasite escape, and therefore nematodes persist into adult hosts.

Parasitized adult mosquitoes offer a means for mermithids to colonize new host larval habitats. Adult hosts must seek water for *S. spiculatus* to complete its life cycle, since water is the exclusive habitat for free-living stages of mosquito-parasitic mermithids. This assumes that parasites obtain adequate nutrition from hosts for full development. Parasitized adult mosquitoes in our study, however, were sometimes collected in the quail-baited cage. These hosts may carry a heavy parasite load that depletes host resources to a point where nutritional supplement for further parasitic development is needed, driving the host to blood for protein initially instead of water or nectar. Petersen et al. (1967) observed field collected *Aedes sollicitans* parasitized by as many as 23 *Agamomermis culicis* attempting to take blood meals. Poinar (1977) suggested *Empidomeris cozii* required the host to take a blood meal to complete parasitic development. Blood feeding would otherwise not serve the parasite's interests as this behavior is inherently risky since host defense behaviors may damage or kill blood-feeding mosquitoes (Walker and Edman, 1985; Edman and Scott, 1987). Host-seeking also poses an indirect risk (Nayar and Van Handel, 1971) since energy expenditure in a nutrient exhausted mosquito contravenes the parasite's priority of emergence into water.

Our mermithid-mosquito system of host behavior manipulation closely parallels that of horsehair worms parasitizing crickets. These parasites similarly alter adult host behavior to seek out water where worms emerge to form mating clusters (Thomas et al, 2002). Parasitized crickets also reduce calling behavior which promotes predator avoidance (Barquin et al., 2015) and thereby reduces needless risk to the parasite. Biron et al. (2007) identified proteins that act on the cricket central nervous system with differential expression of proteins linked to neurogenesis, circadian rhythm and neurotransmitter activities. Neuroparasitology mechanisms of behavioral manipulation have not been studied in mermithids. Whether parasite-induced water-seeking behavior is a result of increased hemolymph osmolality (Williams, 2004), alteration of opioids (Thompson and Kavaliers, 1994) or neurotransmitter systems (Øverli et al., 2001) is unknown.

Host larvae parasitized by *S. spiculatus* showed increased aggregation on the day of nematode emergence, a behavioral change which should boost parasite reproductive success. Aggregation upon emergence reduces the distances necessary for low mobility post-parasites to

traverse to conspecifics and assemble into mating clusters. Large clusters assist molting, mating, and fecundity thereby enhancing fitness (Dong et al., 2014). Our finding is dissimilar from Di Battista's (2019) report that *S. spiculatus* parasitism did not modify the behavior of *Ochlerotatus albifasciatus* larvae; however, aggregation was not one of the eight larval behaviors examined.

Once host larval defenses have been breached and parasitism has been initiated, *S. spiculatus* manipulates host larval and adult behaviors to increase nematode fitness. This is not a true parasite-host evolutionary struggle because hosts have zero fitness as soon as parasitism is established, and therefore no conceivable outcome that is beneficial to mosquitoes. Hosts are usually killed as larvae and occasionally as pupae, but even survival to the adult stage under the narrow test parameters of late fourth-instar hosts and high parasite densities offers no evolutionary advantage. These rare adult hosts cannot contribute to subsequent generations due to parasitic castration. Because parasites derive fitness gains via enhanced reproduction, reduced risk, and greater prospects for dispersal and colonization, there is intense evolutionary pressure to override adult host autonomy, for which hosts have no evolutionary answer.

In conclusion, our results support our two proposed hypotheses. Behavioral changes of mosquitoes parasitized by *S. spiculatus* were observed in both larval and adult stages. These changes benefited parasites, not hosts. Avoiding blood-feeding during the host adult stage is a behavior beneficial to parasite dispersal and risk avoidance, whereas spatial aggregation during the host larval stage facilitates the formation of parasite mating clusters.

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