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Superparasitism and Population Regulation of the Mosquito-Parasitic Mermithid Nematodes *Romanomermis iyengari* and *Strelkovimermis spiculatus*

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Abstract: Superparasitism is a common phenomenon in mosquito-parasitic mermithid nematodes. Multiple nematodes are needed in a single host to produce males. Host selection behavior and intraspecific competition among *Romanomermis iyengari* and *Strelkovimermis spiculatus* were investigated against their host, *Culex pipiens pipiens* in laboratory experiments. In a choice assay between previously infected and uninfected host larvae, infectious preparasites of both nematode species could distinguish not only between infected and uninfected hosts, but even between different parasite loads in showing a strong preference for uninfected hosts or hosts with a low parasite load. Host heart rate declined briefly immediately after parasitism. Superparasitism resulted in increased parasite mortality. Scramble competition within mosquito larvae for limited host nutrients, coupled with a skewed sex ratio favoring males, is assumed to lead to parasite population decline and subsequently toward host-parasite population equilibrium. The ability of mermithid preparasites to accurately assess parasite load likely plays an important role in host population dynamics and regulation.

Key words: heart rate, host selection, population regulation, *Romanomermis iyengari*, *Strelkovimermis spiculatus*, superparasitism.

Superparasitism occurs when multiple conspecifics parasitize a single host. Once regarded as a mistake, this behavior is now recognized as an adaptive approach to population regulation and stability in parasitoids (van Alphen and Visser, 1990). Parasite density has multiple effects on the population and survival of both host and parasite (Lanciani, 1975). Ecosystems only thrive when equilibrium is achieved between the host and parasite (May, 1977). Manipulation of this equilibrium, therefore, is central to population regulation. Suboptimal host–parasite densities, however, can lead to male-dominant populations in some species, as is most evident for mosquito-parasitic mermithid nematodes (Petersen, 1980; Tingley and Anderson, 1986; Sanad et al., 2013), which serves to dampen parasite populations and reestablish host–parasite equilibrium.

Mermithids (Nematoda: Mermithidae) are long, slender roundworms that are parasites of invertebrates, particularly insects (Petersen, 1985; Platzer, 2007). Eggs of aquatic mermithid nematodes are deposited in the external environment and the newly hatched preparasites (second-stage infective juveniles) search for hosts. Preparasites initiate infection using a needle-like stylet to inject a "venom" (Shamseldean and Platzer, 1989). This causes a reduction in host heart rate and a concurrent temporary paralysis which facilitates nematode entry via a cuticular wound. Although the host immune system rapidly recognizes and encapsulates most invading parasites, mermithids secrete an extracellular surface coat which aids in immune evasion (Shamseldean et al., 2006, 2007). The coat serves as a disposable, renewable barrier between parasite and host that is intermittently shed to cleanse the nematode of adhering host immune products. After molting once and completing parasitic development, free-living postparasites emerge, killing the host. The postparasitic juveniles then undergo two additional molts to become adults, mate, and lay eggs.

Mermithids have received attention as biological alternatives to chemical insecticides because of their host lethality, potential for mass rearing (Alavo et al., 2015), and narrow host specificity (Platzer, 2007). Although many mermithid species parasitize arthropods, few have been studied extensively. Because of a host range that includes mosquitoes of public health importance, *Romanomermis culicivorax* has received intensive study of their pathogenicity, ecology, mass production, specificity, and biology (Petersen, 1985; Platzer, 2007). Additional mosquito-parasitic mermithids examined for biological control include *R. iyengari* Welch (1964) and *S. spiculatus* Poinar and Camino (1986). Field releases have demonstrated the ability of mermithids to reduce mosquito populations (Pérez-Pacheco et al., 2005; Achinelly and Micieli, 2009; Abagli et al., 2012).

Sex determination in mosquito mermithids occurs postinfection within the host and is dependent on nematode density (Tingley and Anderson, 1986; Sanad et al., 2013). Sex ratios are female biased at low parasite loads and male biased at high parasite loads. Superparasitism in mermithid nematodes is essential for male production because single infections invariably produce a female (Sanad et al., 2013).

Unlike insect parasitoids where ovipositing females make infection decisions, preparasitic mermithids must make the decision themselves whether to penetrate and infect an already parasitized host. Whether a single preparasite can assess a host to identify earlier conspecific parasitism and what is the impact of superparasitism on parasite and host are the central questions for our study.

Materials and Methods

Second instar mosquito larvae were used in all infections and experiments were conducted at 26 ± 2°C.

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Each experiment contained 10 replicates and was repeated three times for each nematode species.

Mosquito culture: Culex pipiens pipiens Linnaeus larvae obtained from a colony established from eggs collected in Mercer County, NJ were used as the mosquito host. The colony was maintained at 26°C, 75% RH, and 16L:8D photoperiod. Adults were held in 0.51-m^3 aluminum screen cages and supplied with 10% sucrose solution on cotton wicks. Restrained adult quail were used to blood-feed female mosquitoes (Rutgers Animal Use Protocol #86-129). Egg rafts were collected from a 400-ml container, and the larvae were transferred to enamel trays with 1 liter of dechlorinated water after hatching. Larvae were fed 0.15 g Brewer's yeast:lactalbumin (50:50) daily, and the water was replaced on alternate days.

Nematode culture: R. iyengari and *S. spiculatus* parasites cultures were initially obtained from Prof. Edward Platzer, University of California, Riverside, CA. Cultures were maintained in 21 × 14 × 6 cm containers containing moist sand (1.4 to 2.0 mm diam particle size). Eggs were stored in sand for at least 6 wk at 26 ± 2°C. As needed for experiments, sand was flooded with water to induce egg hatch.

Infection time assay: To assess whether infective preparasites assess parasite load, one 2nd instar *Cx. p. pipiens* was transferred in a 0.1 ml droplet of water to a Petri dish. Up to 15 *R. iyengari* or *S. spiculatus* preparasites were individually introduced into the host droplet sequentially, one-by-one, immediately after each successful host penetration was observed under the stereomicroscope. That is, a single fresh preparasite was exposed to the host only after the previous one had completed penetration, so the host: preparasite ratio in the droplet was always 1:1. The average infection time for each preparasite in the sequence was recorded. Our hypothesis was that as parasite load increased, preparasites would require additional time to assess hosts before making an irrevocable decision, therefore providing a measure of penetration reluctance. Water loss in the droplet because of evaporation was replenished as needed during the experiment. For each nematode species, there were 10 replicates (one host per replicate) for each experiment, and the experiment was repeated three times.

Host heart rate assay: Because heartbeat rate decreases temporarily in parasitized hosts, we tested the hypothesis that this parameter is used by preparasites to assess parasite load and thereby is a mechanism to reduce conspecific parasitism. That is, as parasite load increases this would be reflected in heart rate reductions and thereby would serve as a mechanism to reduce excessive conspecific parasitism. One mosquito larva was transferred into a 0.1 ml water droplet in a petri dish and preinfection host heart rate (beats/min) was recorded for 5 min under the stereomicroscope for each host immediately before treatment. Preparasites (0, 1, 3, 5, 10, or 15) of each parasite species were then added to the droplet. Water was replenished as needed to compensate for evaporation. Postinfection heart rate was recorded for 5 min immediately after the last preparasite for each exposure had penetrated a host (i.e., all preparasites had initiated infection). At each parasite load, the heart rate was also recorded by visual observation for 5 min in the control group. For both control and treatment groups, there were 10 replicates (one host per replicate) for each experiment, and the experiment was repeated three times.

Host preference assay: A choice experiment was conducted to determine whether preparasites recognized and discriminated among potential hosts based on infected, noninfected, or parasite load status. One uninfected host larva and one larva preinfected with 1, 3, 5, 10, or 15 parasites from each mermithid species were concurrently exposed to a single preparasite in a water droplet to assess discriminatory ability. Preinfected hosts had been for held individually for 24 hr in 4 ml of water with food to allow for recovery before testing was initiated. Preparasites penetrating each host were observed and counted through the translucent cuticle using a stereomicroscope. Water was added as needed to replenish the droplet. After infection, each pair of larvae was transferred into individual containers with 60 ml of water and fed as previously described.

Parasite survival: Six days postpenetration, fourth-instar mosquito larvae from the host heart rate assay were transferred individually to 23-mm diam plate wells with 4 ml water until postparasite emergence. Nematode survival during the infection phase (i.e., completion of parasitic development and emergence from the host) was determined by recording the difference between initial parasite load and the number of emerged nematodes.

Statistical analysis: All data were analyzed by one-way analysis of variance using Fisher's least significant difference in multiple range tests among the means ($P \leq 0.05$). Nematode survival was correlated with parasite load using Pearson's correlation coefficient. The same method was used to analysis the correlation coefficient between parasite load and infection time, parasite load (1 to 15) and host heart beat rate, as well as heart rate and infection preference (percentage parasites that penetrated). Data are presented as mean ± SE.

Results

Infection time: There was a major difference between the two mermithid species in the time required for a first preparasite (i.e., infective-stage juvenile) to locate and penetrate a *Cx. p. pipiens* larva (Fig. 1). The first *R. iyengari* preparasite required 3.45 times more time to complete the initial infection than *S. spiculatus* (22.7 ± 1.23 vs. 6.58 ± 0.76 min). The time for successive nematodes to penetrate an already parasitized host showed a steady increase with increasing parasitic load for both species; however, *S. spiculatus* was faster than *R. iyengari* up to the fourth penetration (35.39 ± 42.69 vs.

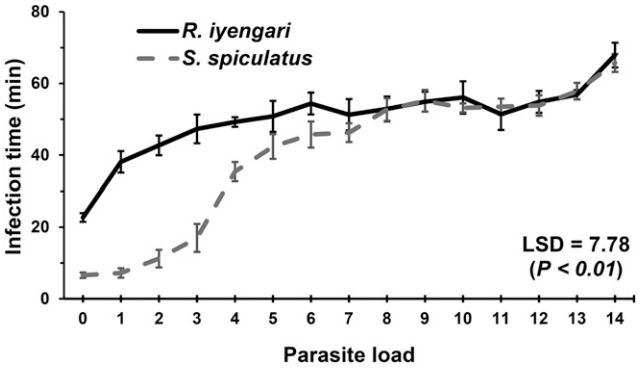

FIG. 1. Time required by individual *Romanomermis iyengari* and *Strelkovimermis spiculatus* preparasitic juveniles to search for and penetrate *Culex pipiens pipiens* larvae at different parasitic loads.

49.24 ± 1.35 min; $P = 0.01$). Differences in penetration time between the two mermithid species remained significant ($P < 0.01$) until parasite load reached five per host, where the nematodes required similar durations to infect heavily parasitized hosts. A strong positive relationship between parasite load and infection time was detected for *R. iyengari* ($r = 0.91$, $P < 0.01$) and for *S. spiculatus* ($r = 0.93$, $P < 0.01$), respectively. Infection time increased 10-fold for *S. spiculatus* as the load increased from 0 (6.58 ± 0.67 min) to 14 (65.72 + 2.52 min) per host, whereas *R. iyengari* increased only threefold from 0 (22.70 + 2.13 min) to 14 (69.72 + 7.99 min).

Host heart rate: A reduction in host heart rate was recorded immediately after a preparasite entering the host regardless of mermithid species (Fig. 2). Regression analysis showed that reduced heart rate was correlated with parasite load as the load increased from 1 to 15 (*S. spiculatus*: $r = -0.871612$, $P < 0.005$; *R. iyengari*: $r = -0.932715$, $P < 0.005$). Heart rate averaged 119.5 ± 2.68 beats/min in preinfection hosts, but postinfection by a single infective stage induced a heart rate decrease of more than one-third irrespective of parasite species (78 + 4.09 and 74.6 + 3.27 beats/min for *R. iyengari* and *S. spiculatus*, respectively). Heart rate declined further as parasite load increased, dropping to one-half of the preinfection rate after penetration by 15 preparasites of either species (52.20 ± 0.92 and 51.80 ± 2.80 beats/min for *S. spiculatus* and *R. iyengari*, respectively).

Host preference: Both mermithid species discriminated against previously infected mosquito larvae in our host choice experiment, demonstrating a strong preference for uninfected hosts (Fig. 3). Moreover, as parasitic load increased, superparasitized larvae were increasingly avoided. For example, *S. spiculatus* infections declined from 54.39% ± 2.3% in uninfected hosts to 9.19% ± 2.33% for hosts with 15 parasites, whereas *R. iyengari* declined from 47.37% ± 3.08% to 5.97% ± 1.93%. Parasite load and host preference were negatively correlated for *R. iyengari* at a confidence level of 95% ($r = -0.85$, $P = 0.03$), and for *S. spiculatus* at 90% ($r = -0.75$, $P = 0.08$).

Parasite survival: Mermithid survival within the host steadily decreased as parasite load increased in both nematode species ($r = -0.905$, $b = -4.41$, $P = 0.034$ for *R. iyengari* and $r = -0.92$, $b = -5.16$, $P = 0.027$ for *S. spiculatus*) (Fig. 4). For *S. spiculatus*, 88.89% ± 2.94% of invading nematodes survived to complete development and emerge from hosts with a single nematode load. This decreased further, by more than one-half to 38.67 ± 2.96 when the load increased to five and to only 14.67% ± 1.54% at a load of 15 ($P = 0.01$). For *R. iyengari*, survival declined from 78.89% ± 4.01% to 12.89% ± 2.99% as parasite load increased from 1 to 15. There were small differences between species in mermithid survival at the lowest parasite loads ($P < 0.05$), but these differences diminished at loads of five and above ($P > 0.05$).

Each preparasite penetrated the host cuticle at a different site, resulting in hosts with multiple wounds.

DISCUSSION

The ability of preparasitic mermithids to assess and discriminate among potential hosts during the host selection process has been previously reported only to

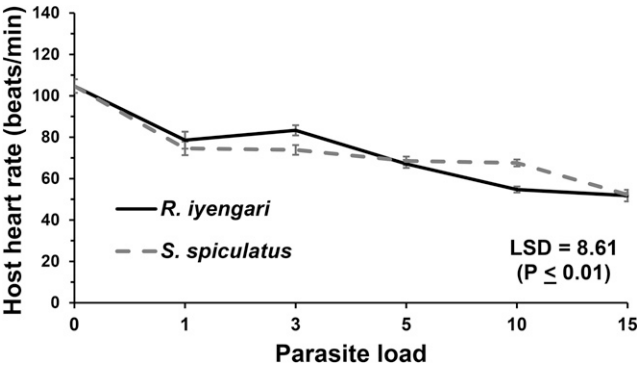

FIG. 2. Heart rate of *Culex pipiens pipiens* larvae infected with *Strelkovimermis spiculatus* or *Romanomermis iyengari* at different parasite loads.

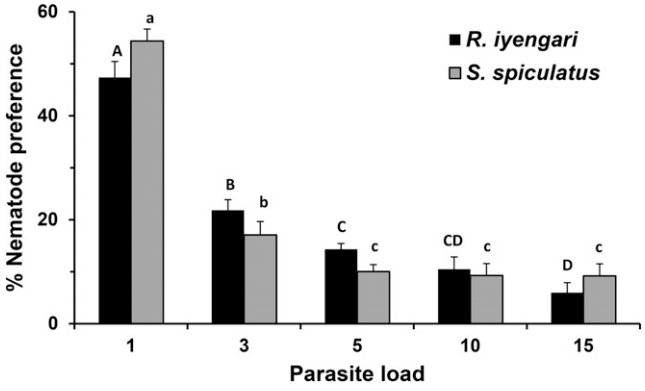

FIG. 3. Host choice experiment showing *Romanomermis iyengari* and *Strelkovimermis spiculatus* preparasite preference for *Culex pipiens pipiens* larvae at different parasite loads. Bars with the same letters of the same case are not significantly different ($P \leq 0.01$).

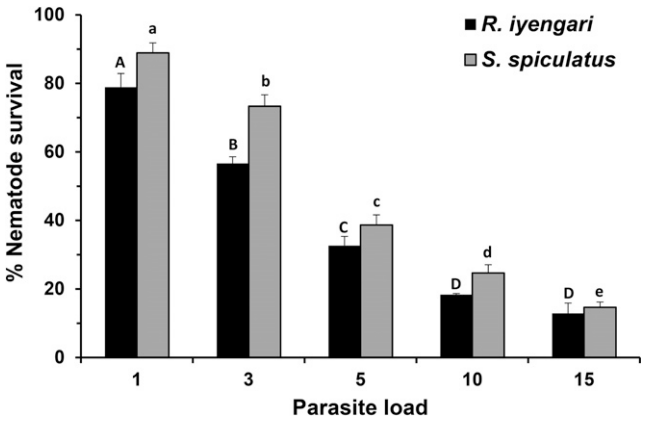

FIG. 4. Survival of *Romanomermis iyengari* and *Strelkovimermis spiculatus* during infection in *Culex pipiens pipiens* larvae at different parasite loads. Bars with the same letters of same case are not significantly different ($P \leq 0.01$).

the extent that preparasites show a preference for early instar hosts (Petersen, 1975; Camino and Reboredo, 2000; Wang et al., 2012). Our study extends our understanding of this discriminating ability by demonstrating that preparasites can also distinguish between infected and uninfected hosts and even among larvae with different parasite loads. The detection mechanism is not clear. A strong negative correlation between host heart rate and infection preference was noted for both mermithid species as parasite load increased. Although preparasite penetration caused a decrease in heartbeat, presumably because of venom injection (Shamseldean and Platzer, 1989), it seems unlikely the preparasites use the change in host heart rate to evaluate host condition because of the heart rate quickly recovered after each penetration (data not shown). Accordingly, we reject our hypothesis that preparasites assess heartbeat in making infection decisions. Penetration wounds, shedding parasite surface coat, or adhesive residues left by the preparasites after penetration could more plausibly serve as indicators for host evaluation and need investigation.

Our data indicate that the mermithid species, *S. spiculatus* and *R. iyengari*, assess host status and prefer those hosts with zero or low parasite loads to reduce the risks associated with excessive superparasitism and to maximize fitness. Even when presented with no alternative host, preparasites of both species showed increasing reluctance to penetrate hosts as the number of penetrants accumulated. Although preparasites attempt to avoid excessive superparasitism, superparasitism must occur as this is necessary for male production. The operative word here is "excessive." A parasite load of two yields a balanced sex ratio—42.9% and 53.9% males for *R. iyengari* and *S. spiculatus*. respectively (Sanad et al., 2013). But as parasite load increases, host nutrients become increasingly depleted, so there is an upper limit. Superparasitism clearly plays an important role in the regulation of mermithid populations and in improving host–parasite synchrony.

Density-dependent variation in the sex ratio is a key determinant in the regulation of mosquito-mermithid populations. When mosquito population density is low, the outcome may be more frequent superparasitism and a skewed mermithid sex ratio favoring males. Superparasitism provides mermithid population reduction, regulation, and stability by relaxing pressure on the host population (Tingley and Anderson, 1986; Sanad et al., 2013). Conversely, when mosquitoes are abundant, the already reduced mermithid population encounters less competition, less superparasitism, more nutrients and consequently produce more females to boost their population to track an expanding host population. This type of scramble competition in insect parasitoids was regarded by Taylor (1988) as having an unstable trajectory due to excessive competition. Tingley and Anderson (1986), however, suggest that mermithid nematodes may not closely follow this pattern because of their longer life cycle relative to the host. In a 3-yr field study, Micieli et al. (2012) observed that the frequency over time and the level of infection by *S. spiculatus* were key parameters in regulating populations of *Ochlerotatus albifasciatus*.

Our data support Tingley and Anderson's (1986) concept for mermithid scramble competition as diverging from that of insect parasitoids because preparasites continue to penetrate hosts even when parasite loads exceeded 10. The preparasites may have little choice because this short-lived infectious stage has a small window to find and penetrate a host (Platzer, 2007). Although our experiments were conducted under laboratory conditions which may not reflect the natural situation, excessive superparasitism nevertheless likely exists because of a concentrated nematode population resulting from aggregation. Superparasitism plays an important role in population stability, and this is especially important when aggregation is a common behavior for parasitoids (Hassell and May, 1974; May, 1977). Aggregation is a fundamental behavior in mermithid nematodes where postparasites form mating clusters and lay eggs (Dong et al., 2014). Mating aggregations result in an aggregated egg distribution which in turn results in an aggregated preparasite distribution once the temporary mosquito pool floods (Poinar and Camino, 1986). The outcome is often superparasitism and therefore greater male nematode production, which would cause a decline in the parasite population in the subsequent generation. When parasite load reaches a threshold the nutrients available become inadequate for development. This density-dependent reduction mechanism helps stabilizes host–parasite populations and explains why as load increases, the mermithid sex ratio favors males, which are smaller and so require less nutrients (Petersen, 1972; Platzer, 2007; Sanad et al., 2013).

Scramble competition is characterized by resources that are shared by all competitors, which can lead to

group starvation. This is why mermithid sex determination occurs only postinfection; that is, after the parasites have assessed resource availability and made optimal sex ratio apportionment. Males of *S. spiculatus*, for example, are less than half the length of females (Poinar and Camino, 1986) and therefore require less nutrients enabling the host to support a greater parasite load. Progeny production in the hymenopteran parasitoid *Nasonia vitripennis* is similarly density dependent, with decreasing female production as parasite load increases, albeit this is determined by the density of competing ovipositing females rather than the number of eggs laid in each host (Walker, 1967). When mermithid population density is low or hosts are abundant, a greater quantity and quality of females are produced because competitive pressure is released.

Despite the density dependent regulation of sex ratio, we show inordinate parasite mortality resulted when superparasitism was excessive. Fewer mermithids completed their parasitic development successfully as parasite load increased; that is, later arrivals penetrating hosts with high parasite load encountered high risk because of greater intraspecific competition for limited host resources. This allows individual parasites to compete for hosts when hosts are scarce, which is considered as an adaptive strategy in insect parasitoids (van Alphen and Visser, 1990). In short, sex ratio regulation facilitates competition in mermithids.

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