Male insect-parasitic nematodes: a colonizing sex

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Abstract. An adaptive strategy enhancing reproductive success is described for parasitic nematodes. Male infective juveniles of insect-parasitic nematodes, *Steinernema* spp. (Rhabditida: Steinernematidae) are dispersers, and take greater risks than females during the host-finding phase of parasitism. They disperse, locate, and establish in distant live hosts before females. Parasitism by male infective juveniles renders the infected hosts suitable for nematode development, and more attractive to female infective juveniles. Such 'recruitment' may be a strategy to protect the nematodes against uncertainties of mate finding, thereby enhancing reproductive success.

Key words. Nematoda; insect parasites; reproductive strategies; host-finding; mate recruitment; reproductive success; risk factors.

Males of many animal species are dispersers, and take greater risks than females to enhance their reproductive success[1-11]. A male's genetic contribution to the next generation often depends on the number of females fertilized, whereas females are restricted by the number of eggs produced. It follows that males take the more active role in mate-acquisition behavior, and will consequently encounter more risks[7]. We provide the first evidence of such an adaptive phenomenon in the parasitic Nematoda.

The soil-inhabiting infective juveniles of insect-parasitic nematodes in the families Steinernematidae and Heterorhabditidae (Nematoda: Rhabditida) locate and parasitize suitable insect hosts in which they develop to adults, mate, and reproduce[12]. This group of nematodes has a mutualistic association with the bacteria of the genus *Xenorhabdus*[13]. After gaining access to host's hemocoel, the bacteria harbored by the infective juveniles are released, killing the insect within 24–48 h and providing optimum conditions for nematode growth and reproduction[12-14]. As development and mating of these insect-parasitic nematodes can occur only within hosts, selection pressure should operate to evolve means of successful infections wherein mating and reproduction is maximized[15-16]. These insect parasites have evolved two distinct reproductive strategies. Infective juveniles of all the known species of *Heterorhabditis* develop into hermaphrodites (thus not requiring mates) in the first generation, the progeny of which consist of male and female (monoecious) individuals[14]. The members of *Steinernema* spp. are dioecious, and require at least one infective juvenile of each sex to invade a host to allow mating and reproduction. We describe here how *Steinernema* spp. may have evolved a means of enhancing their likelihood of successful reproduction.

Materials and methods

Third-stage infective juveniles of the following insect-parasitic nematode species were reared[17] in last-instar *Galleria mellonella* L. larvae at 25 °C: *Steinernema anomali* (Kozodoi), *S. carpocapsae* (Weiser), *S. feltiae* (Filipjev), *S. glaseri* (Steiner), and *S. scapterisci* (Nguyen and Smart). As it is difficult to distinguish morphologically the sex of juvenile nematodes[16,18], we used a sand column bioassay to isolate the first infective juveniles to locate and establish in distant host insects. One host (last-instar *G. mellonella*) larva was placed at the bottom of a 10 cm sand column (10% moisture content) and 100 infective juveniles of one nematode species were introduced at the top. After 24 h exposure at 25 °C, the host larvae (N = 12 per species) were removed, rinsed with distilled water, and incubated at 25 °C for 48–72 h. Cadavers were then dissected, and the male and female nematodes counted. Sex ratios of control populations were determined by exposing larvae individually to 100 infective juveniles of one nematode species (N = 12 per species) on filter papers in well plates, where contact was assured. After 48–72 h, the parasitized hosts were dissected as above.

To eliminate the effects of host factors on the sex ratio of nematodes, an agar host-finding bioassay[19] was used. Briefly, 1.5% agar was poured onto a glass plate to which rubber tubing had been cemented along the margins. A plastic lid was secured tightly onto the tubing with rubber bands to form a closed chamber. The lid held a pair of plastic pipette tips fixed through holes on opposite sides from, and equidistant from, a central access port sealed with plastic film. The pipette tips rested 2 mm above the agar surface. The pipette tips served as 'holsters' to secure the experimental pipettes described below. Two hours before testing, two host larvae (200–300 mg each) were placed into an Eppendorf-style disposable pipette tip (101–1000 µl) designated as the source. The tip was loosely packed with steel wool at each end to prevent insect escape, and between insects to prevent conflict. The top end was sealed with parafilm. A control tip was similarly packed but did not contain larvae. These pipette tips were inserted into the larger holster tips fixed to the plate lid, suspending the insects 3 cm above the agar.

The assay plate, complete with hosts, was transferred to 25 °C for 1 h before testing to permit an attractant gradient to form. About 10,000 infective juveniles were then brushed onto the agar surface of each plate via the access port. The infective juveniles that aggregated within 0.5 cm of the host larvae within 30 min ('responders') ($N = 10$ plates per species) were collected and placed on lipid agar medium[20] pre-inoculated with the respective symbiotic bacteria for each nematode species. About 1000 'responders' were collected for each species except for *S. scapterisci*, where only 105 infective juveniles were collected due to their poor response to the host. For controls, 1000 infective juveniles were inoculated, without previous exposure to host cues, to bacteria on the lipid agar medium ($N = 10$ plates per species). Sex ratios were assessed after nematodes developed for 48–72 h at 25 °C.

Using the agar host-finding bioassay, the effect of exposure duration and distance from the host on infective juvenile sex ratios was evaluated. Infective juvenile *S. glaseri* were exposed to hosts located 6 cm from the inoculation point for 15, 30, or 60 min ($N = 10$ plates per treatment). In another experiment, the distance of the nematodes from the host was varied and the sex of the juveniles that located the hosts in 30 min at 3, 6, or 12 cm was determined as described above.

Sex-related differences in the dispersal behavior of nematodes in the absence of host cues were determined. Five thousand infective juveniles were placed on agar in the centre of a 12-cm diameter Petri dish. After 20 min, the nematodes that had moved out of a 9 cm circle were collected and their sex ratios determined after growing on lipid agar medium pre-inoculated with their respective symbiotic bacteria.

To evaluate the response of female infective juveniles to the hosts parasitized by males, we separated infective juvenile *S. glaseri* into potential males and females using the 12-cm dispersal assay dish (see above). Each host larva was exposed for 0, 4, 8, 24, or 48 h on filter paper to 100 male *S. glaseri* infective juveniles, and then rinsed with sterile distilled water. Response of potential female infective juveniles to the larvae previously exposed to male nematodes was determined using the agar host-finding bioassay. Response was scored after 1 h by counting the infective juveniles that either located, or initiated movement towards the host, those that initiated movement away from the host, and those that did not move.

Proportional data from the sand column, agar-host finding, and random disperser bioassay were analyzed by t-test. Percent data from the exposure duration and distance experiments, and on the response of nematodes to parasitized hosts were arcsine transformed and analyzed by analysis of variance. Significance was tested by Tukey's mean separation test at 0.05%. Data in the text are reported as mean ± standard error.

Results and discussion

In four of the five species studied, 58–100% of infective juveniles that penetrated and established in hosts located 10 cm away in the sand column within 24 h were males (fig. 1). Mean number of nematodes that established per host in sand columns were 37 ± 2.7, 3.2 ± 1.0, 10.8 ± 2.6, 32 ± 5.2, and 2 ± 0 for *S. anomali*, *S. carpocapsae*, *S. feltiae*, *S. glaseri*, and *S. scapterisci*, respectively. With the exception of *S. feltiae* ($t = 0.534$, $p = 0.29$), the sex ratios of nematodes recovered from the hosts deviated significantly ($P < 0.001$) from that of control population for all the species studied. The proportion of males in control populations of different species ranged between 38–42%. Mean numbers of nematodes established per host were 42.5 ± 3.4, 38.8 ± 2.4, 33.5 ± 2.8, 46.7 ± 3.6, and 17.5 ± 3.8 for *S. anomali*, *S. carpocapsae*, *S. feltiae*, *S. glaseri*, and *S. scapterisci*, respectively.

Deviations in sex ratio occur due to stress-related factors within the host for some plant (Tylenchidae)[21] and

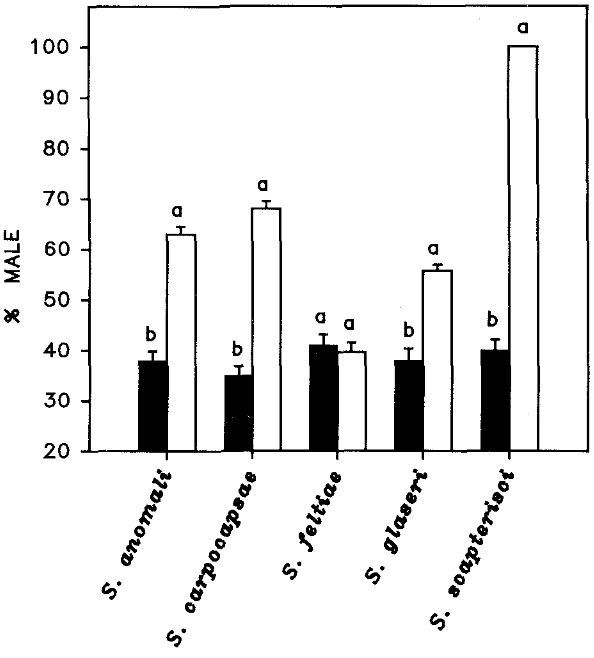

Figure 1. Proportion of males (mean ± SE) of different species of insect-parasitic nematodes (*Steinernema* spp.) among control populations (in contact with host) (■), and individuals that first located and established in their insect hosts in a sand column (□). Values for the same species with the different letter are significantly different at $P < 0.001$ (t-test).

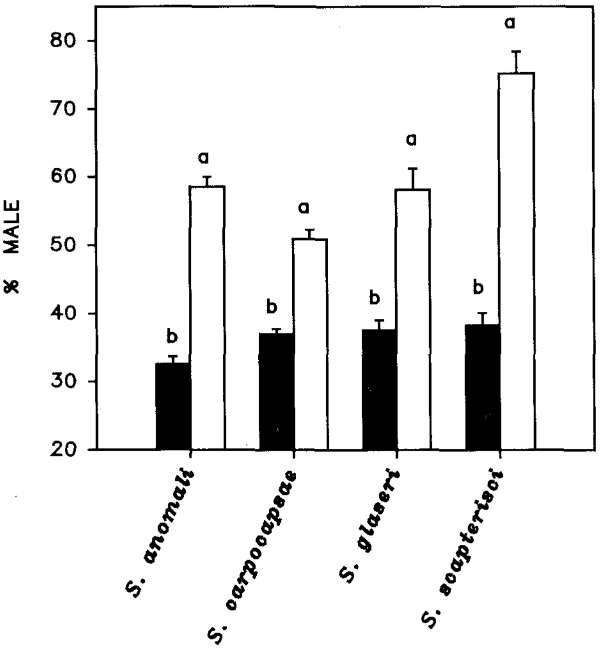

Figure 2. Proportion of males (mean ± SE) of different species of insect-parasitic nematodes (*Steinernema* spp.) among control populations (■) and individuals that first located their insect hosts on agar plates (□) and subsequently developed into adults on lipid agar plates pre-seeded with their respective bacteria. Values for the same species with the different letter are significantly different at $P < 0.05$ (t-test).

insect-parasitic (Mermithidae)[22] nematodes. In both of these cases, over-crowding skews the sex ratios in favor of males. This is not the case with steinernematids. When inoculated onto lipid agar medium, thus eliminating host or crowding effects, the sex ratios of nematodes that located their hosts from 6 cm away from the inoculation point in 30 min on agar plates were similarly skewed toward males (fig. 2). Furthermore, sex determination in *Steinernema* spp. occurs at the chromosomal level ($2n = 10$ for female and $2n = 9$ for male)[23] so is unlikely to be affected by environmental factors during the life-span of an individual.

By varying the duration of exposure and distance of nematodes from the host, we demonstrated that male *S. glaseri* infective juveniles located distant hosts much more quickly than their female counterparts (fig. 3). The proportion of male *S. glaseri* infective juveniles that located hosts increased with decreasing duration of exposure (fig. 3A). An increase in the distance between the nematodes and the hosts resulted in an increase in the proportion of male *S. glaseri* infective juveniles successfully locating the hosts (fig. 3B).

Even in the absence of host cues, there were differences in the dispersal behavior of male and female infective juveniles (fig. 4). Significantly more male than female infective juveniles of all nematode species studied dispersed out of a 9 cm circle from the inoculation point on agar plates. This suggests that males are more active, and their host finding behavior may involve increased mobility. This follows that behavior performed to acquire a mate or mating site may require increased mobility during searching activities[5,6]. Because of their dispersal behavior, male infective juveniles are more likely to encounter host cues[24,25] and may, therefore, be more effective in locating new hosts.

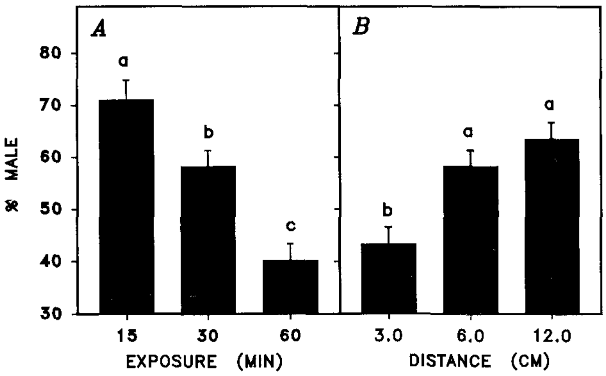

Figure 3. Effect of exposure period *A* and distance *B* on the proportion of male infective juvenile *S. glaseri* (mean ± SE) that located the hosts on agar plates. Values with the different letter are significantly different at $P < 0.05$ (Tukey's test).

When infective juveniles emerge from cadavers (infected hosts), males may disperse quickly and colonize suitable insect hosts before females. All species in this study showed similar behavior with the exception of *S. feltiae*, a species that is adapted to parasitize the larvae of bibionid flies (Diptera: Bibionidae)[26]. Because of the small size (last instars are 5–10 mm long) of these fly

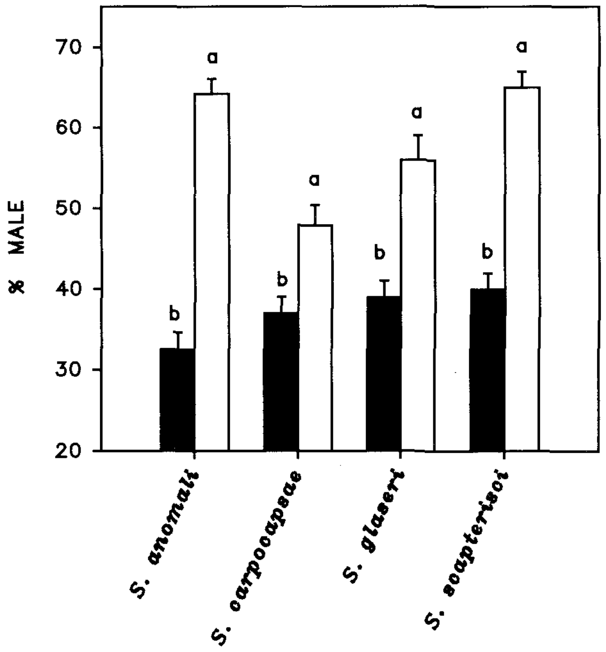

Figure 4. Proportion of males (mean ± SE) in *Steinernema* spp. among control populations (■) and dispersing individuals (□). Values for the same species with the different letter are significantly different at $P < 0.001$ (t-test).

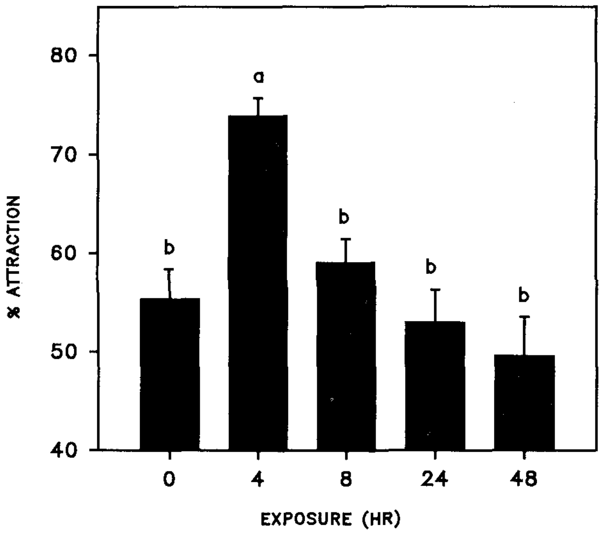

Figure 5. Proportion of female *S. glaseri* infective juveniles (mean ± SE) attracted to hosts parasitized by conspecific male infective juveniles for varying duration. Values with same letter are not significantly different at $P > 0.05$ (Tukey's test).

larvae, only a few infective juveniles can complete their life cycle within the host[27]. The limited food resource and the distribution of hosts may be responsible for the observed differences in *S. feltiae.*

Male colonization would only make sense, from an evolutionary standpoint, if females are likely to be recruited following successful infections. Significantly more female infective juvenile *S. glaseri* were attracted to hosts exposed to male infective juveniles for 4 h than to the hosts exposed to nematodes for either a longer duration or not exposed (fig. 5). Lower attraction of nematodes to the hosts previously exposed for longer than 4 h may be a means to avoid interspecific competition. Although it was not possible to separate juvenile nematodes into males and females completely, our results demonstrate the occurrence of mate recruitment. We have also found that this 'recruitment' is species-specific and may be a means of reproductive isolation in Steinernematidae[28].

First invaders are subject to stronger insect defenses (e.g., immune responses)[29] than individuals entering after the defenses are exhausted and the insect is moribund. For instance, some of the infective juvenile *S. carpocapsae* that first enter into the hemocoel of Japanese beetle larvae are melanized and encapsulated[30]. Males of most animal species are known to take risks to inseminate as many females as possible[1,8-10]. This is viewed as a type of parental investment by males[8-11]. Male bark beetles[11] prepare nuptial chambers in tree trunks and produce a sex pheromone to attract females. It appears that male infective juveniles of *Steinernema* spp. locate a host, invade and develop first. Consequently, females more easily find and enter the host, develop, and are inseminated. Such a strategy would enhance reproductive success. Our results suggest that parental investment of males lies in the risks undertaken to colonize and subdue healthy hosts.

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