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Mating and Sexual Communication by *Steinernema carpocapsae* (Nemata: Steinernematidae)

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Abstract: Entomopathogenic nematodes are lethal insect parasites that reproduce exclusively inside their hosts in nature. Infection decisions made by the free-living infective-stage juveniles have an impact on reproductive success, but it is likely that mating decisions are made by adults while inside their host. We investigated sexual communication between male and female adult stages of *Steinernema carpocapsae* (Rhabditida: Steinernematidae) to assess whether mating is chemically mediated during the adult stage or results from incidental encounters between adults inside the insect host. To assess chemical communication, we measured the behavioral response of adult male *S. carpocapsae* to several different potential sources of chemical information. Male *S. carpocapsae* responded to virgin females only and were not influenced by mated conspecific females, conspecific males, or heterospecific females. These results show that species-specific communication takes place between adult entomopathogenic nematodes within the host cadaver just prior to mating.

Key words: behavior, entomopathogenic nematode, insect, mating, parasite, pheromone, reproductive isolation, *Steinernema*.

Parasitic nematodes of several species produce species, and sex-specific chemicals that influence the behavior of the opposite sex (Cheng and Samoiloff, 1971; Chin and Taylor, 1969; Shrazian et al., 1986). These sex pheromones are the primary mechanism involved in reproductive isolation among different species of many parasitic nematodes (Cheng and Samoiloff, 1971; Huettel et al., 1982).

Infective juvenile entomopathogenic nematodes find an insect host and penetrate to the hemocoel. Once inside, they release symbiotic bacteria, *Xenorhabdus nematophilus*, which kill the host within about 48 hours. The established nematodes feed on the bacteria and degrading host tissue, develop into adults, mate, and reproduce for up to three generations inside a single host cadaver (Kaya and Gaugler, 1993). Infection decisions are made by the non-reproductive infective-stage juveniles. Infection of the same host by more than one species of entomopathogenic nematode can occur because multiple species of entomopathogenic nematodes have been isolated from a single soil sample (Gaugler et al., 1992; Stuart and Gaugler, 1994). However, multiple-species infections giving rise to more than one species of infective juvenile are unusual (Kopenhöffer et al., 1995). Multispecific infection may lead to a mechanism that would enable adult nematodes to recognize conspecific mates inside the host cadaver.

We investigated sexual communication between male and female adults of the entomopathogenic nematode *Steinernema carpocapsae* (Rhabditida: Steinernematidae). Our objective was to determine whether mating in an entomopathogenic nematode is chemically mediated or results from incidental encounters between adults inside the insect host. Species recognition or assessment of the fitness of a potential mate (i.e., whether or not a female has previously mated) is likely made immediately prior to mating inside the host cadaver. We tested the possibility that secretory products of conspecific and heterospecific adult female nematodes influenced the search and mating behavior of adult males, and determined what information was gained by male nematodes from the female-produced pheromone.

Materials and Methods

Nematode culture: Adult nematodes were obtained by in vitro culture. Infective juveniles were taken from stock cultures, sterilized with bleach (0.5%), and transferred to lipid agar plates containing a monoxenic culture of the nematode species' symbiotic bacteria: *Xenorhabdus nematophilus* for *S. carpocapsae* and *Photorhabdus luminescens* for *Heterorhabditis bacteriophora*. Plates were maintained at 25 °C for 48 to 72 hours. Virgin females were obtained by placing juveniles individually on 12- or 6-well tissue culture plates containing lipid agar with a bacterial culture. Males and mated females used in the experiments were obtained by placing 100 to 150 juveniles onto lipid agar in 100-mm-diam. petri dishes inoculated with bacteria. Seventy-two to 96 hours after the inoculation of a bacteria plate with juveniles, mated females were collected. Females assumed to be mated were held for 2 days after behavioral testing, and progeny production was confirmed. For tests of male response to mated females, only data collected from experiments using females that subsequently produced viable progeny were retained and analyzed. Hermaphroditic *H. bacteriophora* were raised in similar fashion following the methods of Zioni et al. (1992).

We examined females immediately after copulation to determine whether a mating plug was inserted by males as a physical barrier to additional matings (Barker, 1994). A drop of Ringer's solution was placed in the center of a microscope slide and a recently mated female was deposited into it and examined for a mating plug.

Nematode/Nematode interactions: We constructed an

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ethogram of male *S. carpocapsae* mating behavior, based on the frequency and duration of each of the behavioral categories listed and defined in Table 1 to illustrate the order of events in a mating episode. For each of the treatments, we recorded the time spent in contact between the test nematode (the male whose behavior was observed) and the treatment nematode, and whether or not the two nematodes coiled. These two behaviors were the most reliably measured responses by the male to the treatment nematode.

All tests for sexual communication were conducted on 35-mm-diam. petri dishes filled with a 2-mm layer of lipid agar on which either *X. nematophilus* or *P. luminescens* had been cultured. Five combinations of nematodes were tested. In the first test, *S. carpocapsae* male behavioral response to conspecific virgin females was documented in detail. In addition to males' response to virgin conspecific females, we measured their response to conspecific mated females and to *H. bacteriophora* hermaphrodites. Conspecific males and heat-killed conspecific virgin females served as additional controls. One treatment nematode (i.e., females and controls) was placed in the center of a petri dish and left for 3 hours at room temperature. Because males are mobile, when they were used as the treatment they were kept under observation and returned to the initial location with a probe when necessary. An individual male was then placed in each petri dish approximately 2.5 mm from the treatment nematode. The frequency and duration of the behavioral events of males were recorded for a total of 180 minutes using a stereomicroscope. Each pair was observed for 2 minutes at 10-minute intervals. Eight observations were conducted for each treatment. Data were compared with analysis of variance (SAS Institute, Cary, NC). Means were separated by the Student-Newman-Keules test ($P = 0.05$).

Response assay: The presence of a relevant chemical cue can be recognized by changes in the movement patterns of animals when they contact the area where the suspected cue exists. These behavioral changes usually include slowed movement and increased turning, which result in a decreased search area (Bell, 1991). Giving-up-time, which is the duration of the animal's contact with the cue, or the area where the cue is suspected to be, was used as a measure of the changes in searching behavior described. We employed a giving-up-time assay to determine whether females secreted a pheromone, which would be left behind on the surface of the agar arena, that would affect male movement. Experiments were performed with adult male and female *S. carpocapsae* obtained as described above. Tests were conducted on double-chambered microscope slides (2.5 ml volume and 21 × 20 mm per chamber) with a 2-mm layer of lipid agar inoculated with *X. nematophilus*. After the bacteria developed for 24 hours, a 2-mm-diam. circular enclosure was made with plastic tubing in the center of each chamber. A virgin female was placed in the center of one enclosure. The enclosure in the other chamber was left empty, to be used as a control. After 3 hours, the female and both enclosures were removed and one male was placed in the center of each circle. Time of residence in the circle, or giving-up-time, was recorded. Each treatment was replicated 10 times. Data were analyzed with a paired t-test ($\alpha = 0.05$).

Results

Nematode/Nematode interactions: We depict the behavior of *S. carpocapsae* males in response to *S. carpocapsae* virgin females with an ethogram (Fig. 1) Males moved randomly for approximately 1 to 5 minutes and then, in 80% of the cases, crawled directly toward the female. Males approached the middle region of the female's body (the location of the vulva) 56.25% of the time, the head in 37.5% of the cases, and the tail in only 6.25% of the cases. A period of continuous contact between the sexes always followed the first contact. In 44% of the cases of contact, males subsequently coiled around the middle of the female's body. This position was maintained during copulation. Copulation (as determined by subsequent observation of progeny) was recorded in 25% of the nematode pairs where coiling occurred. The apparent low level of fertilization efficiency could be due to the artificial conditions imposed by the two-dimensional experimental arena, which are quite different from the three-dimensional conditions inside the host cadaver.

Table 1. List of behaviors of male *S. carpocapsae* in response to virgin, conspecific females.

Behavior	Definition
Crawling randomly	Moving on the agar surface without any direct objective
Crawling toward the female	Moving in direction of the female from a 2.5-mm distance
Approaching head, middle, or tail	The different parts of the female's body that males approached
Contact	The period following the approach by the male where the pair remained in contact with each other
Coiling	The male curled around female's body before and during copulation (Greete, 1964)
Copulation	Copulation was reported only in the cases in which the virgin females used in the experiment developed progeny after 2 days' incubation
Stopping	Males stopped for more than 10 minutes after contact or coiling (this signaled the end of the observation)

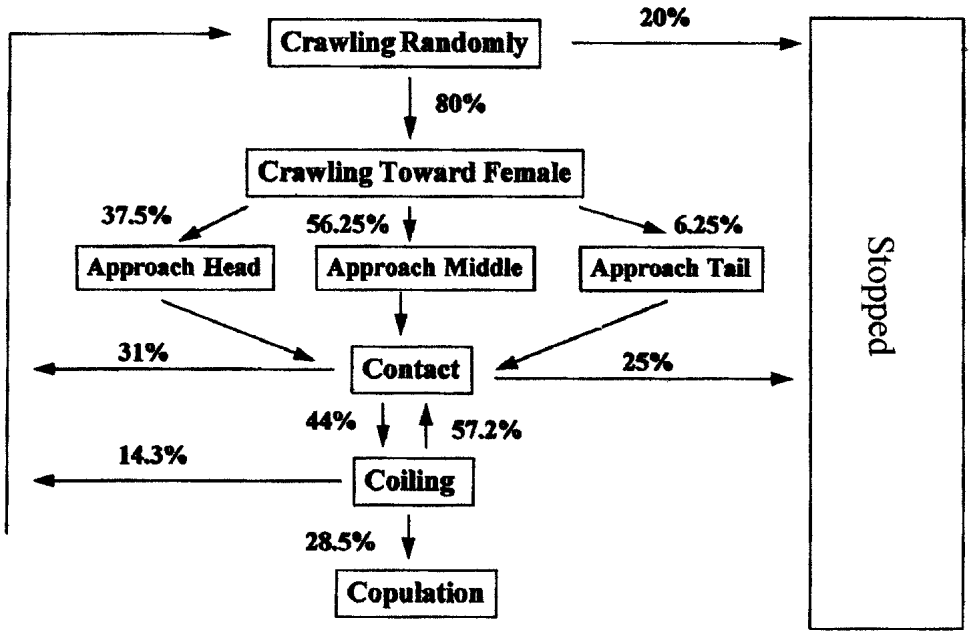

FIG. 1. Ethogram describing the events leading to copulation between male and female *S. carpocapsae.*

We gauged degree of male stimulation by females by the duration males remained in contact with the females during the observation period described above. Several treatments of females and "sham females" were compared. The time spent by males in contact with females differed among treatments. *Steinernema carpocapsae* males were in contact with virgin conspecific females for an average of nearly 50 minutes, compared with less than 3 minutes on average for all other treatments; no differences were found among treatments other than with the virgin conspecific female (Fig. 2). Interestingly, *S. carpocapsae* males spent little time in contact with mated conspecific females.

Males demonstrated coiling behavior exclusively with live conspecific females. The average time spent coiling by male *S. carpocapsae* was 4.5 ± 1.6 minutes with virgin females and 1.4 ± 1.2 minutes with mated females. These durations are statistically different at $\alpha = 0.06$. Considering the relatively small sample size, high degree of variability, and corroborating data, this level of difference is likely to be biologically relevant.

Giving-up-time experiments: Previous occupation of the test arena by a conspecific virgin female nematode stimulated *S. carpocapsae* males to remain within the perimeter of the arena significantly longer than the control (nearly 160 minutes vs. less than 100 min) ($P = 0.025$) (Fig. 3). No other treatment (either conspecific males or any heterospecific treatment) differed significantly from the control (bacteria alone) (data not shown).

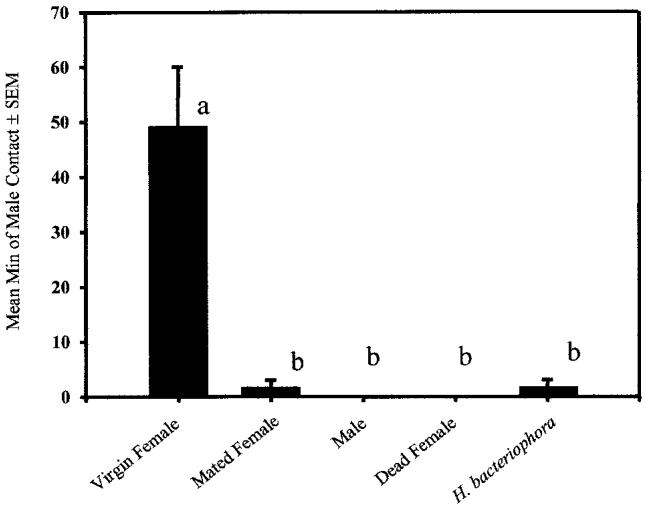

FIG. 2. Average (± SEM) duration (min) of contact between male *S. carpocapsae* and various female and sham treatments. Bars with different letters are significantly different ($\alpha = 0.05$).

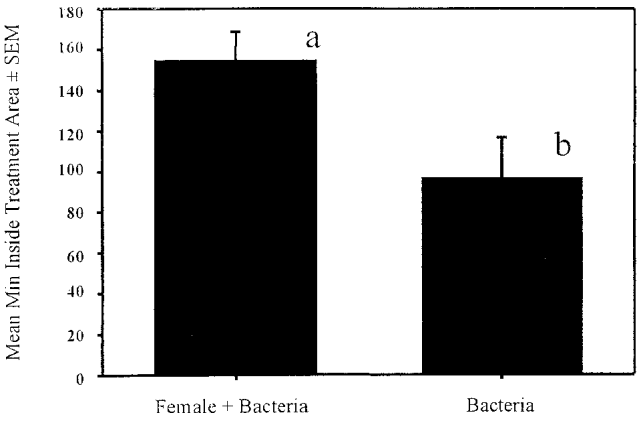

FIG. 3. Average (± SEM) duration (min) of giving-up-time by male *S. carpocapsae* in response to exudates produced by conspecific virgin females. Bars with different letters are significantly different ($\alpha = 0.05$).

Discussion

Reproductive decision-making, based on assessment of species identity and relative fitness of potential mates, is among the primary functions of adult animals. These data show that for *S. carpocapsae*, adult males are stimulated by chemical cues produced by virgin conspecific females. The attractant produced by adult female entomopathogenic nematodes was species-specific, at least between this pair of species, because attraction was not observed between *S. carpocapsae* males and *H. bacteriophora* hermaphrodites. However, these two genera are not closely related phylogentically, and pheromonally based mate discrimination could be less reliable between species that are more recently diverged. This phenomenon has been shown with some sister species of insects (Lewis and Cane, 1990).

The difference in attractiveness between virgin and mated females for *S. carpocapsae* diverges from the conclusions reported in previous studies on parasitic nematodes (Cheng and Samoiloff, 1971). According to Bone (1987), this difference could be important for locating unmated females by the males, thereby enhancing reproductive success. Because no morphological barriers to mating were observed after mating (e.g., a mating plug, Barker, 1994), distinguishing virgin from mated females may be accomplished via chemical cues. Alternatively, the production of the attractant chemical by females may be diminished after mating.

The role of a female-produced pheromone for sexual communication by entomopathogenic nematodes seems more likely to be one of recognition rather than location. Although 80% of *S. carpocapsae* males responded strongly to conspecific virgin females, this response occurs only in the female's immediate proximity (2.5 mm after 3 hours of exposure to the source). The attractant's sphere of activity is restricted. Perhaps diffusion is limited by the artificial substrate used in our assay chamber. Alternatively, considering the limited volume inside the insect host where the substance operates, perhaps a pheromone is most effective when limited to short-range activity.

The relationship among mating behavior, infection decisions by infective juveniles, and infection dynamics of entomopathogenic nematodes is complex and difficult to interpret. All infection decisions are made by the non-reproductive infective stage juvenile and are affected by the infection status of the prospective host. For example, Grewal et al. (1997) found that infective juvenile entomopathogenic nematodes were highly attracted to hosts already infected by conspecific entomopathogenic nematodes, and were repelled by hosts infected by some heterospecific species of entomopathogenic nematodes. Obviously, infection decisions directly impact reproductive decisions and are therefore difficult to treat individually. We have shown that adult male *S. carpocapsae* respond to substances associated with conspecific females. Infection strategies, mating strageties, and dispersal of entomopathogenic nematodes have received a great deal of attention in the literature, and have been reviewed by Kaya and Gaugler (1993). Their field ecology, which is influenced by these factors to a great extent, has been investigated at some length and summarized by Lewis et al. (1998). The information available about entomopathogenic nematode field ecology, however, remains largely descriptive. Understanding the chemical ecology of pheromonally based communication systems in general has led to great advances in the ecology of many animal groups, most notably the insects. We once again emphasize the importance of entomopathogenic nematode chemical ecology to understanding these organisms and their interactions with other subterranean fauna and flora.

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