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Life Cycle and Mating Behavior of *Belonolaimus longicaudatus* in Gnotobiotic Culture[1]

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Abstract: The life cycle of *Belonolaimus longicaudatus* was observed in vitro on excised roots of *Zea mays*. Roots were cultured on Gamborg's B5 medium in petri dishes with 1.5% agar adjusted to pH 5.8 and incubated at 28 °C in darkness. Second-stage juveniles (J2) fed on the roots and started the second molt (M2) to the third-stage juveniles 2 days after inoculation (DAI). The third molt (M3) to the fourth-stage juveniles occurred 7 DAI, followed by the fourth molt (M4) to males 13 DAI or to females 14 DAI. Nematode gender differences were observed by the end of the fourth molt. The first male appeared 15 DAI and the first female 17 DAI, after which mating occurred. Males were attracted to females, and mating was observed. Mating was required for reproduction. Fertilized females began to lay eggs 19 DAI and continued egg laying without the further presence of males during a 90-day observation. All of the eggs hatched. Unfertilized females rarely laid eggs, and none of the eggs were able to hatch. Feeding took place between each molt and before egg deposition occurred. The first-stage juveniles molted in the eggs 4 days after deposition, and J2 hatched from eggs 5 days after egg deposition. The life cycle from J2 to J2 was completed in 24 days.

Key words: attraction, behavior, *Belonolaimus longicaudatus,* corn, excised roots, gnotobiotic culture, in-vitro culture, life cycle, mating behavior, nematode, reproduction, sting nematode, *Zea mays*.

One of the most damaging plant-parasitic nematodes in the southeastern part of the United States is the sting nematode, *Belonolaimus longicaudatus* Rau. It has a high damage potential at relatively low population densities and parasitizes a wide range of hosts among agricultural and horticultural crops (Smart and Nguyen, 1991). This obligate ectoparasite is most damaging to young plants (Perry and Rhoades, 1982). Under suitable conditions a life cycle is completed in about 28 days (Robbins and Barker, 1973). Previous in vitro studies showed that the first-stage juvenile undergoes one molt and hatches as a second-stage juvenile into the medium (Huang and Becker, 1997). Both sexes occur, with males making up approximately 40 percent of the adult population; it was suggested that the species reproduces sexually (Perry and Rhoades, 1982). It was also postulated that eggs were deposited in the soil immediately surrounding feeding sites. However, although the sting nematode has been known for more than 50 years, little has been published about details of its life cycle. The recent establishment of an in-vitro culture of *B. longicaudatus* on excised corn roots (Huang and Becker, 1997) has facilitated these studies. The objective of the current study was to describe the complete life cycle, mating, and egg-laying behavior of *B. longicaudatus* in gnotobiotic culture. A preliminary report has been published (Huang et al., 1997).

Materials and Methods

Nematode source: Belonolaimus longicaudatus was obtained from infested turf grass at a private golf club in Rancho Mirage, California, and cultured on bermudagrass (*Cynodon dactylon* (L.) Pers.) in sandy soil in the greenhouse of the Nematode Quarantine Facilities, University of California, Riverside. A population of *B. longicaudatus* was then established in petri dishes on excised corn roots (*Zea mays* cv. Golden Jubilee) supported by Gamborg's B5 medium in 1.5% agar adjusted to pH 5.8 and maintained in darkness at 28 °C (Huang and Becker, 1997). The nematodes in the following experiments originated from this culture, and all experiments were carried out under these conditions.

Nematode development: To study development, nematode eggs from the in-vitro culture of *B. longicaudatus* were aseptically

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transferred onto 1% water agar plates and incubated at 28 °C overnight. Hatched second-stage juveniles (J2) were inoculated onto 5-day-old corn root cultures. Four excised corn roots were cultured on each of four replicate culture dishes and were inoculated with 100 J2 of *B. longicaudatus*. These plates were incubated under the above conditions. Nematode development and behavior in all replicates were observed daily with dissecting and inverted microscopes. The first occurrence of each molt and development stage among the replicates was the criterion used to determine the time periods of the life cycle sequence. The presented chronology of the life cycle reflects a typical time course based on many repeated observations of each event.

Mode of reproduction: Corn root culture dishes were prepared and divided into three groups with 10 replicates each: (i) one molting female J4 in each dish, (ii) one molting female J4 and 10 males in each dish, (iii) one molting female J4 and 10 males in each dish with males removed after the first egg appeared in the medium in order to prevent further mating. Molting fourth-stage juveniles could be sexually differentiated on the basis of body morphology. After the female fourth-stage juveniles (J4) finished molting and developed into adults, they were considered virgin females and could only be fertilized by the males added to the same dish. These dishes were incubated as described above, and nematode behavior was observed daily for 90 days. Once egg deposition in a dish occurred, the eggs were transferred onto corresponding 1.5% water agar dishes and observed for hatching. Hatched juveniles were removed immediately from this agar dish to avoid being mixed with juveniles hatching later. The number of eggs that each female produced was also recorded.

Results and Discussion

The L2-to-L2 life cycle of *B. longicaudatus* was completed under gnotobiotic conditions at 28 °C in 24 days. A schematic illustration similar to previously published life cycles (Lauritis et al., 1983a, 1983b) is presented (Fig. 1). The J2 moved to the root tips and began feeding within 1 hour after inoculation. Feeding lasted for 12 to 24 hours, then the J2 became immobile and remained positioned like a ''C'' or a closed circle. The second molt (M2) started 2 days after inoculation (DAI). The most significant change during molting occurred in the esophageal region. During the first 12 to 24 hours of molting, the style shaft, esophageal lumen, and median bulb became invisible. Only the stylet cone remained discernible. Twelve hours later, the new cuticle became visible inside the old one, followed by the appearance of the new stylet shaft. Then the esophageal lumen and the median bulb emerged and gradually became more distinctive. The juvenile body progressively elongated until it was confined by the old cuticle. At that time the new stylet began to probe the old cuticle at the rate of once every 5 to 20 seconds, associated with contraction of the median bulb once every 3 to 8 probings. The nematode finally broke through the old cuticle and migrated out. This molting period (M2) lasted for 2 days. The third-stage juvenile (J3) began feeding

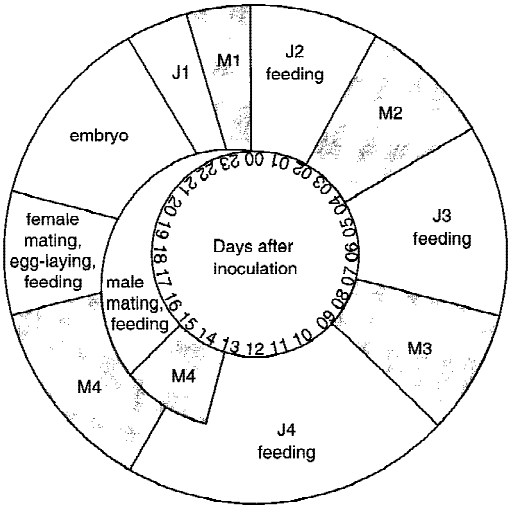

Fig. 1. Schematic representation of the life cycle of *Belonolaimus longicaudatus* on excised roots of *Zea mays*. J1 = first-stage juvenile, J2 = second-stage juvenile, J3 = third-stage juvenile, J4 = fourth-stage juvenile, M1 = first molt, M2 = second molt, M3 = third molt, M4 = fourth molt.

again. At 7 DAI the J3 entered the third molting period (M3), which lasted for another 2 days and resulted in the emergence of the fourth-stage juvenile (J4). The J4 started feeding on the roots again, followed by the fourth molting period. Juveniles that developed into males started the fourth molt (M4) 13 DAI. By the end of the 2-day molting period, the male gonad, the spicules, and the caudal alae had formed, and the male migrated out of the old cuticle 15 DAI. Juveniles that developed into females started M4 at 14 DAI, which lasted for 3 days. By the end of the molting period, the female gonads and the vulva had formed. The female migrated out of the old cuticle 17 DAI. Faster development of males than females has been observed with other nematodes, e.g., *Heterodera schachtii* both in potted cultures (Raski, 1950) and under gnotobiotic conditions (Johnson and Viglierchio, 1969).

Males approached females soon after the females finished the last molt. Often two or more males surrounded a female, which caused more competition for mates than the sex ratio of approximately 3:2 in favor of the females (Huang and Becker, 1997; Perry and Rhoades, 1982; Todd 1989) would have predicted. The males seemed to be directly attracted by the females and gathered around them quickly. The males moved around the female and began to intensely rub the side of the female body with the lateral side of their lip region. The rubbing movement of the male head was perpendicular to the axis of the female body. In the meantime one of the males moved toward the female head so that its bursa finally touched the female body. This male would move farther ahead, continuously rubbing the female body until its bursa reached the vulva region of the female. The male then moved back and forth, and the female also twisted its body until finally the spicules penetrated through the vulva with the bursa covering the area around the vulva. Then the body movement of both nematodes slowed down. Mating in this manner lasted for 6 to 10 minutes, during which fertilization presumably took place. The male then withdrew its spicules, and both nematodes moved away. This mating behavior was observed at least 10 times, and each time the mating occurred on the surface of the culturing medium. It was not determined whether a female nematode mated more than once during its life.

After mating, both females and males fed on the roots. Meanwhile, eggs began forming within female uteri and were clearly visible. Before females began to lay eggs, they stopped feeding and moved slowly within or on the surface of the medium. The eggs in the uterus were pushed toward the vagina. The eggshell was very flexible and was squeezed to pass the shallow lumen of the vagina and delivered through the vulva. Egg deposition was completed in approximately 1 minute, during which the female did not move. The egg resumed its shape outside the female body. The first eggs were laid 19 DAI. No preference was observed for depositing the eggs at a particular location. The egg consisted of one cell after deposition, and cell division occurred soon after, becoming a two-celled egg within 3 hours and a four-celled egg within 5 hours. The first-stage juvenile (J1) appeared in the egg 3 to 4 days after egg deposition. The first molt (M1) occurred 4 days after egg deposition and lasted for 1 day. The J2 of the second generation hatched 5 days after egg deposition.

Perry and Rhoades (1982) found no evidence that females of sting nematodes could reproduce in the absence of males. The present study allowed examination of this hypothesis in more detail and confirmed that sexual reproduction was obligatory. In the treatment with only one female in each of 10 dishes, the females produced in total only 0.4 ± 0.3 eggs during 90 days incubation, and none of these eggs hatched. When males were always present in the medium, each female produced an average of 124 ± 16 eggs in 90 days, and all the eggs hatched. When the males were removed after the first eggs appeared, each female still produced an average of 133 ± 20 eggs in 90 days, and all the eggs hatched. The numbers of eggs in treatments with fertilized females were

not significantly different from each other (Tukey's test, $P = 0.01$). Therefore, fertilized females produced eggs without the continued presence of males during the 90-day observation. Females collected from field locations almost always have spermathecae filled with sperm (Perry and Rhoades, 1982). Under the present culture conditions, each fertilized female produced an average of 1.43 ± 0.14 eggs per day for 90 days. It was not determined how many eggs a female can produce throughout its life time.

The in-vitro culture of plant nematodes on their host tissues allows continuous observation of the nematodes and has been utilized in nematological studies since the 1950s (Zuckerman, 1971). It has proven to be helpful in studying nematode life cycles and host-parasite relationships. By means of this technique, the life cycle of *Heterodera glycines, Heterodera zeae,* and *Helicotylenchus multicinctus* have been described in detail (Lauritis et al., 1983a, 1983b; Orion and Bar-Eyal, 1995). However, only a few strictly ectoparasitic phytonematodes such as *Criconemella xenoplax* (Westcott and Hussey, 1992), *Helicotylenchus multicinctus* (Orion and Bar-Eyal, 1995), and *Tylenchorhynchus claytoni* (D. Harshman, pers. comm.) have been successfully cultured on excised roots. Our *B. longicaudatus* population has been in monoxenic culture for more than 2 years. This technique not only provided sterile nematode inocula for well-controlled host-nematode relationship studies but also allowed direct observation of the nematode behavior without the interference of soil flora and fauna. Results must be interpreted with caution, however, since the metabolic response of the host might be quite different from that of an intact host. However, no obvious changes were observed during the course of this study in terms of behavior and parasitism. Compared to previously reported generation times of about 28 days (Perry and Rhoades, 1982) and approximately 1 month (Todd, 1989) in field studies, the 24-day generation period under our experimental conditions suggests that the gnotobiotic culture environment was well-suited for the nematode.

All juvenile stages as well as the adult stages of both genders fed on the host. As previously reported (Huang and Becker, 1997), all stages fed strictly as ectoparasites. Congregation of juveniles and adults of both sexes of this parasite was observed with strong feeding preference at or very near root tips. Apparently, attraction to this metabolically active area is strong in all stages. In addition, searching for newly hatched females seemed to be aided by sex attractants. Similar observations have been reported with many other amphimictic nematode species, and in several cases it has been demonstrated that nematode females secrete sex pheromones that attract males (Clemens et al., 1994; Green, 1980; Jaffe et al., 1989; Riga et al., 1997). Studies in nematode chemotaxis and sensory recognition have not only advanced the understanding of complex signaling between nematode genders (Zuckerman and Jansson, 1984) but may ultimately contribute to advances in integrated pest management. The identification of a sex pheromone of the soybean cyst nematode (Jaffe et al., 1989) and results from recent field studies suggest the potential of using such attractants or their synthetic analogs for practical pest management (Meyer et al., 1997).

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