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Life Cycle and Reproductive Potential of the Nematode *Heterorhabditis bacteriophora* Strain HP88[1]

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Abstract: Development of the entomopathogenic nematode *Heterorhabditis bacteriophora* strain HP88 was studied in vivo with larvae of the greater wax moth, *Galleria mellonella,* as host and in vitro. At 25 C in vivo, the duration of the life cycle from egg hatch to egg hatch was 96 hours. Juvenile development took 48 hours, with the duration of each juvenile stage ranging from 8 to 12 hours. Under crowded conditions, development proceeded to the infective juvenile (IJ) stage instead of the third juvenile stage (J3). Life-cycle duration and proportion of the various developmental stages in the population were similar in in vitro and in vivo cultures. When in vivo or in vitro development was initiated from the IJ stage, only hermaphrodites developed in the first generation and males appeared only in the second generation. The average (±SD) number of progeny per hermaphrodite was 243 ± 98. The ratio of males to hermaphrodites in the second generation was 1:9.4 ± 6.8.

Key words: development, *Heterorhabditis bacteriophora,* life cycle, nematode, reproduction, sex ratio.

Entomopathogenic nematodes from the families Steinernematidae and Heterorhabditidae, associated with the *Xenorhabdus* bacterium, are potentially useful agents for biological pest control (1,7,13, 14). However, the sensitivity of these nematodes to extremes of the physical environment (e.g., high temperature, solar radiation, and desiccation) prevents exploitation of their maximal potential as bioinsecticides under field conditions (2,8, 11). Genetic improvement has been suggested as a means of increasing the tolerance of such nematodes to environmental parameters (4–6,10). Recently, we have initiated this genetic approach using the entomopathogenic nematode *Heterorhabditis bacteriophora* (Heterorhabditidae: Nematoda) strain HP88 (9), which is commonly used against soil-inhabiting insect pests (19,20).

A comprehensive knowledge of the nematode's life cycle and potential for reproduction is needed as a prerequisite for genetic studies aimed at enhancing the nematode's performance under field conditions. It has been suggested that in this species the adults of the first generation reproduce hermaphroditically, whereas their progeny (F1 generation) are amphimictic (12,15). However, details such as duration of each developmental stage, sex ratio, and number of progeny per hermaphrodite are not known.

This study describes the life cycle and reproduction of the nematode *H. bacteriophora* HP88 in vitro and in vivo.

MATERIALS AND METHODS

Life cycle: Nematodes were reared in 5-cm-d plastic petri dishes containing a nematode growth medium (NGM) originally devised for culture of the free-living nematode *Caenorhabditis elegans* (3). The NGM is transparent and allows continuous observation of nematode development on the medium surface. The NGM plates were preinoculated with a lawn of the *Xenorhabdus luminescens* bacterium associated with the HP88 strain. The bacteria were isolated from the nematodes and propagated (17). The plates were seeded with nematode eggs (ca. 200 eggs per plate) obtained from the bodies of gravid hermaphrodites (18).

Nematode development was monitored at 5-hour intervals for a period of 96 hours, using a stereoscopic microscope. At every time point, the number of nema-

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todes at each developmental stage was recorded and ten plates were examined.

In addition, nematode development was examined in NGM medium in "Lab-Tek chamber slides (Nunc, Naperville, IL). This installation allowed observation of the nematodes using a compound microscope.

Population development: The rates of nematode development in vitro and in vivo were compared by recording the number of individuals at each developmental stage in time-lapse intervals. The various developmental stages, particularly the juvenile stages, were identified by their relative size and morphological characteristics.

Dog Food Agar (DFA) was used to study the development of *H. bacteriophora* HP88 nematode in vitro. Infective juveniles (IJ) were surface-sterilized with 1% methylbenzethonium chloride (Sigma, St. Louis, MO) (14) and seeded on 5-cm-d petri dishes containing 5 ml of sterile DFA (8% w/w sterile dog food, Gaines Gravy Train, Gaines Foods, Kankakee, IL) mixed with 1.6% w/w agar (Difco-Agar, Difco, Detroit, MI) in distilled water. The DFA plates were preinoculated with *X. luminescens* subspecies HP88. Approximately 40 IJ were seeded on each DFA plate. For examining development, the nematodes were rinsed from 10 plates with saline into empty petri dishes every 10 hours for 250 hours.

The rate of *H. bacteriophora* HP88 development in vivo was determined by periodic dissection (every 10 hours during 250 hours postinfection) of *G. mellonella* larvae that had been preinoculated with 50 IJ/insect larva in 5-cm-d petri dishes padded with a moist filter paper. Seven insect larvae were examined at each time point.

The number of individuals in each developmental stage was recorded at each time point for both in vitro and in vivo cultures. At early stages of development, all the nematodes were counted. Once reproduction had yielded thousands of individuals at each replicate, however, their numbers were estimated by counting three 1-ml samples obtained from serial dilutions of the original nematode suspension.

Reproductive potential: Surface-sterilized IJ of *H. bacteriophora* HP88 were seeded on a bacterial lawn grown on the DFA medium in 5-cm-d petri dishes. Following 50-hour incubation at 25 C, hermaphrodites that developed on the plates were transferred individually to separate 3-cm-d petri dishes containing NGM preinoculated with the symbiotic bacterium. The number of eggs in the nematode body, the number of eggs laid on the plate surface, and the number of progeny were recorded periodically during the 96-hour period. Once the data were obtained at each interval, each hermaphrodite parent was transferred to a fresh NGM plate that had been seeded with the bacterium. Eighty-seven hermaphrodites were examined at every time point.

Sex ratio: Infective juveniles of *H. bacteriophora* were seeded on a bacterial lawn in DFA plates. Following 48-hour incubation at 25 C, 72 J4 and young adults were transferred individually to separate fresh DFA plates and allowed to develop and reproduce for an additional 120 hours. Thereafter, the number of males and hermaphrodites were recorded on each plate.

Results

Life cycle: The life cycle described here was initiated in vitro from fertilized eggs. These eggs gave rise to both hermaphrodites and males. On the other hand, when development was initiated from IJ only hermaphrodites developed in the first generation (12).

The entire life cycle (from egg hatching to F1 egg hatching) was completed in 96 hours at 25 C (Fig. 1). Juvenile development took 48 hours, with the duration of each juvenile stage ranging from 8 to 12 hours. More than 60% of the life cycle involved adult development and maturation (both hermaphrodites and males), including egg laying and hatching.

The eggs were oval, and the developing juveniles could be observed within mature eggs (Fig. 2A). The first-stage juveniles

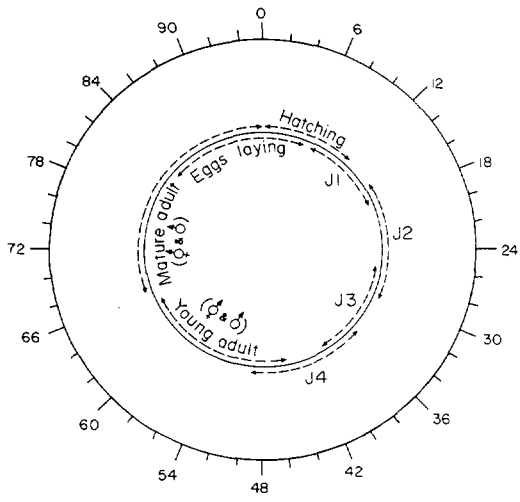

FIG. 1. Diagrammatic representation of the life cycle of the nematode *Heterorhabditis bacteriophora* strain HP88, showing the duration of each developmental stage at 25 C. Numbers on the outer circle indicate hours after seeding NGM plates with eggs obtained from gravid hermaphrodites. J1 = first-stage juvenile; J2 = second-stage juvenile; J3 = third-stage juvenile; J4 = fourth-stage juvenile.

(J1) were transparent and 8–15 μm long (Fig. 2B). Their movement was slow, but a rapid pharyngeal pumping activity was initiated immediately upon hatching. The J1 were found within the first 10 hours after egg laying. The J2 were 35–50 μm long, and the accumulation of storage material was evident in their partially transparent bodies (Fig. 2C). The J2 were observed on the surface of the NGM plates within 9–22 hours following egg hatching. The J3 were 1.5 times as long as J2 and their bodies were filled with storage material (Fig. 2D). The J3 were also characterized by a transparent area initially near midbody (Fig. 2D), which is likely the genital primordium. The J3 were found within 18–30 hours post egg hatching (Fig. 1). The J4 were characterized by a relatively long body in comparison with the younger juveniles, ranging from 140–210 μm, and the development of reproductive organs was noticeable in them (Fig. 2F). The J4 were found within 28–42 hours of the beginning of the life cycle (Fig. 1).

Young hermaphrodites were found from the 46th hours after the beginning of egg hatching (Fig. 1). They were characterized by the conspicuous reproductive organs (ovaries, oviducts) visible in their semitransparent bodies and the undeveloped vulva (Fig. 2G). When the hermaphrodite had matured, it was full of eggs and its vulva was protruding (Fig. 2H). A period of 12–14 hours was required for hermaphrodite maturation (Fig. 1). Under suitable growth conditions (i.e., availability of food and uncrowded population), the hermaphrodites reached a length of 1–3 mm. Once crowding occurred due to the sequential development of new generations on the same plate, hermaphrodites were shorter, with an average length of 500 ± 85 μm.

Males could be recognized on the plate surface by virtue of their spicules (Fig. 2I). They were also shorter than the hermaphrodites (200–285 μm) and moved more rapidly.

Population development: Because in vivo population development could be initiated only from IJ, it was compared to in vitro development, which was also initiated from IJ. In this case only hermaphrodites developed from the IJ, whereas males appeared only in the second generation.

The initiation of IJ development to J4 occurred in vitro 25 hours after inoculation, whereas in vivo it started after 35 hours. A pattern of synchronous development was noticeable both in vivo and in vitro within the first 105 hours after inoculation: IJ: 0–35 hours (Fig. 3A), J4: 35–45 hours (Fig. 3B), hermaphrodites: 50–85 hours (Fig. 3C), J1–2: 85–100 hours (Fig. 3D) and J3: 105–115 hours (Fig. 3E). The entire life cycle of the second generation took approximately 60–70 hours, which was shorter than for the first generation (80–85 hours, starting from IJ development to J4, which began at 25 hours after inoculation, Fig. 3A).

Male development occurred both in vivo and in vitro only at the second generation (Fig. 3C). The average proportion of males found in the in vivo population (10 ± 3%) was 2.7 times higher ($P = 0.05$) than that found in the in vitro culture (3.7 ± 2.5%).

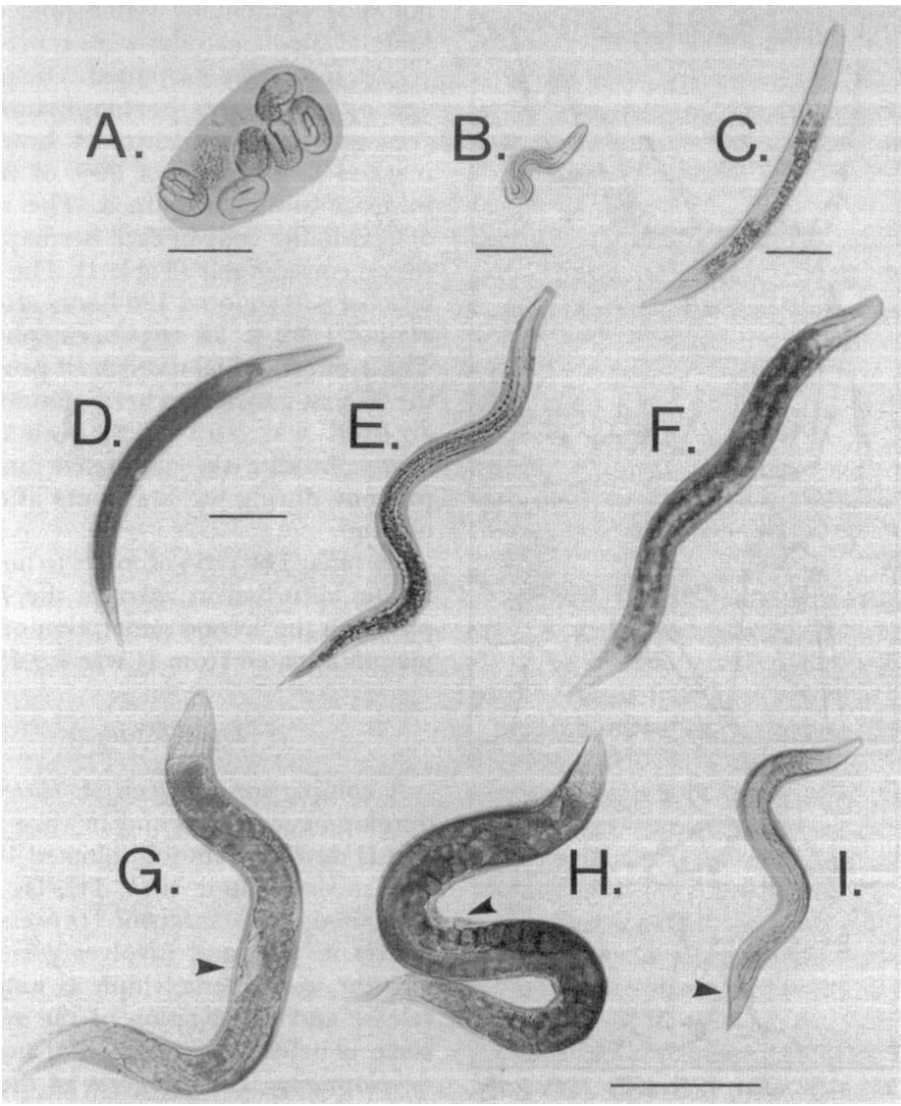

FIG. 2. Photomicrographs of different developmental stages of the nematode *Heterorhabditis bacteriophora* strain HP88. A) Eggs. B) First-stage juvenile. C) Second-stage juvenile. D) Third-stage juvenile. E) Infective juvenile. F) Fourth-stage juvenile. G) Young hermaphrodite (arrow denotes undeveloped vulva). H) Mature hermaphrodite (arrow denotes protruding vulva). I) Male (arrow denotes spiculi). Scale bars = 12 μm for A; 25 μm for B; 10 μm for C; 30 μm for D–F; and 100 μm for G–I.

Although some of the nematodes that developed in vitro continued for a third or even a fourth generation, development in vivo ceased at the third generation. In both cultures, about 160 hours after inoculation, eggs hatched inside the hermaphrodites and the resultant juveniles destroyed their mothers as they developed to IJ.

A rapid increase in the percentage of IJ in the in vivo population began 185 hours after inoculation (Fig. 3A), reaching a level of 95–100% at 250 hours. During the same period, the proportion of IJ in the in vitro culture reached only 60% of the population, whereas the rest of the nematode population consisted of 4% J4 stage (Fig. 3B) and 36% females (Fig. 3C).

Because the data are presented in relative values (percentage of the population), it is noteworthy that the first generation consisted of 40–50 individuals per plate, whereas the subsequent generations com-

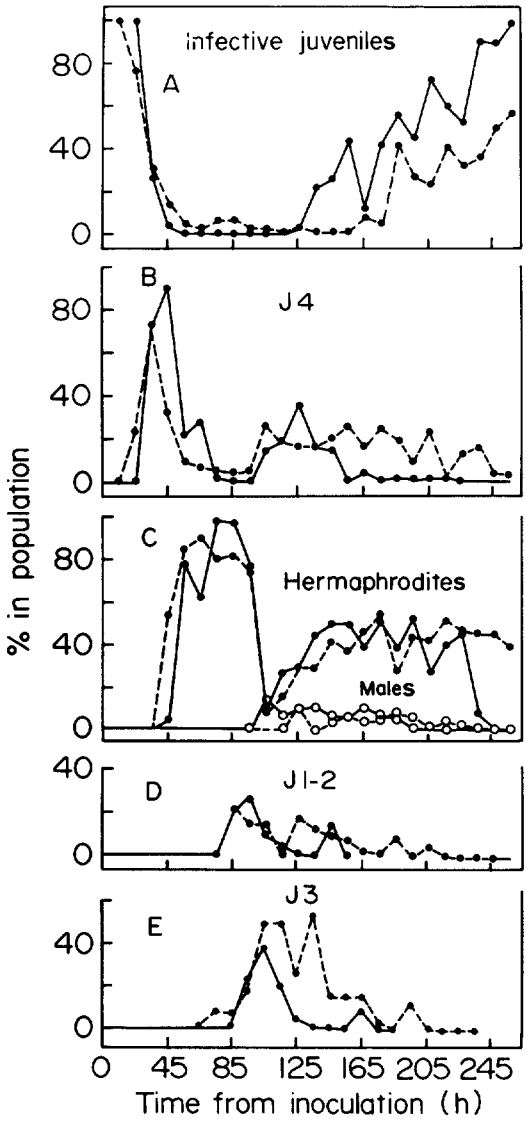

FIG. 3. The relative number (percentage) of different developmental stages of the nematode *Heterorhabditis bacteriophora*, strain HP88, reared in vivo (solid line) on the last instar of the greater wax moth, *Galleria mellonella* and in vitro (broken line) on DFA plates. Both cultures were inoculated with infective juveniles and incubated at 25 C.

posed thousands of worms per plate (8–10 $\times$ 10^3 in the second generation and 5–7 $\times$ 10^5 in the third generation).

Reproductive potential: Egg laying by first-generation hermaphrodites, developed from IJ in vitro, started approximately 77 hours after the beginning of incubation (Table 1). Because the populations were not developmentally synchronized, only some of the nematodes were reproductive at each time point examined. The percentage of egg-laying hermaphrodites increased twofold within 24 hours and reached a maximum of 66% of the total hermaphrodites examined. The number of eggs in the body of each hermaphrodite varied considerably (Table 1). The highest number was counted 120 hours after inoculation (102 ± 53 eggs/hermaphrodite). The average (±SD) number of progeny of the 87 first-generation hermaphrodites examined was 243 ± 98 nematodes/hermaphrodite (the cumulative number of progeny during 86–144 hours after inoculation).

Sex ratio: The ratio of males to hermaphrodites recorded in vitro on the 72 DFA plates, in the second generation of a population initiated from IJ was 1:9.4 ± 6.8.

DISCUSSION

A comparison between *H. bacteriophora* development in vivo and in vitro showed that IJ development was initiated 10 hours later in vivo than in vitro. This lag may be attributed to the "infection" process, which occurs in vivo and involves penetration into the insect hemolymph as well as the release and propagation of the symbiotic bacteria before the initiation of nematode development. The progress of the development of all stages of the life cycle in vivo was delayed to some extent during the first generation as compared with the rate of development in vitro. Nematodes cultured in vitro were smaller in size and produced fewer eggs than those previously grown in vivo (12). The reasons for these differences are not known, but the overall similarity in the pattern of development both in vivo and in vitro, particularly during the first generation, indicates that the in vitro culture techniques used in this study support essentially normal development and are, therefore, suitable for further studies relevant to genetic improvement of these nematodes.

The rapid increase in the proportion of

TABLE 1. Average number of eggs developed, laid, and hatched from individual hermaphrodites of the entomopathogenic nematode *Heterorhabditis bacteriophora* strain HP88, reared on NGM plates preseeded with the bacterium *Xenorhabdus luminescens*.

Time (hour after inoculation)	% egg-laying hermaph.†	No. eggs in the body (±SD)	No. eggs laid (±SD)	No. progeny‡ (±SD)
48	0	0	—	—
72	26	28 ± 7	—	—
86	39	50 ± 27	52 ± 5	9 ± 5
96	57	26 ± 19	97 ± 40	83 ± 56
120	66	102 ± 53	65 ± 21	86 ± 46
144	22	0	0	65 ± 32

† Percentage of 87 hermaphrodites examined. Following progeny counts, the mothers were transferred individually to fresh DFA plates at each time point.
‡ Including all developmental stages.

IJ 185 hours post in vivo inoculation is a result of crowding and depletion of the food source (18). Yet, at the same time, some development still continues in vitro on the DFA plates, indicating that during this period the nutritional resources have not been exhausted completely. The corresponding but moderate increase in proportion of IJ on the DFA plates is due to the hatching and development of juveniles within their mothers bodies. It is suggested, therefore, that the DFA medium is nutritionally richer for the development of the symbiotic bacterium, and hence of *H. bacteriophora,* than the hemolymph of *G. mellonella* larvae. Physical factors such as space per nematode and ventilation might also contribute to the differences in the development of the population between the two culture systems.

Nutritional as well as physical factors most likely also account for the difference in the proportion of males between the in vivo and in vitro cultures of *H. bacteriophora.* Poinar and Hansen (16) noted that adverse environmental conditions affect sex determination by increasing the proportion of males in amphimictic as well as hermaphroditic nematode species. Apparently, the enriched nutritional and physical conditions in vitro are the cause of the lower number of males in the population compared with in vivo culture. We demonstrated recently (21) that under similar in vitro conditions (i.e., DFA plates), second-generation adults reproduce hermaphroditically and the presence of males is not necessary for reproduction, whereas in vivo amphimictic reproduction occurs in the second generation (15). It is likely that the in vivo conditions stimulate the increase of the proportion of males in the population.

In the present study, the NGM as well as DFA media were used. Although the NGM medium is transparent and therefore convenient for direct observation of nematode development, it is not as rich in nutrients as DFA. Hence, due to depletion of the food source on the NGM medium, most nematodes developed to IJ faster, within 1–2 generations. Therefore, we suggest that the NGM medium be used only for short-term (1–2 generations) observations; for prolonged culture (>2 generations), the DFA medium is more suitable.

The information gained in the present study about the life cycle and reproductive potential of *H. bacteriophora* HP88 constitutes crucial baseline information for studies directed at genetic improvement of this nematode.

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