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Environmental Factors Affecting Sexual Differentiation in the Entomopathogenic Nematode *Heterorhabditis bacteriophora*

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ABSTRACT The present study was aimed at determining the influence of various environmental factors on sex differentiation (SD) in the entomopathogenic nematode *Heterorhabditis bacteriophora* HP88 strain, under in vivo and in vitro culture conditions.

Injection of individual nematodes into last instars of *Galleria mellonella* resulted in development of a similar number of females and hermaphrodites (35–40%) and 20–25% males. Increasing the number of nematodes injected into the insect did not change these proportions. In smaller insects (0.7–1.5 cm long), an increase in the proportion of hermaphrodites was recorded as compared with larger size cadavers (2.4–2.7 cm long). When individual hermaphrodites were placed on NGM, the proportion of hermaphrodites, females and male progeny was 63%, 31%, and 6%, respectively. Rearing on richer medium ("Dog-food" agar) resulted in reduction in the proportion of hermaphrodites.

Nematodes introduced to the symbiotic bacterium obtained from other nematode strains (IS-5 and IS-33) developed similarly to the culture reared on the HP88 bacteria. Rearing the nematodes at a temperature range between 21°C to 30°C also did not have a significant effect on the sexual differentiation among nematodes cultured on NGM. The proportion of hermaphrodites increased as the starvation period of hatching nematode juveniles lengthened (>6 hr).

The data obtained in the present study strongly suggest that the main factor affecting sex differentiation in *H. bacteriophora* is the nutrition source. The practical and biological implications of the results are discussed. *J. Exp. Zool. 287:158–166, 2000.* © 2000 Wiley-Liss, Inc.

The mechanisms of sex determination and sexual development have fascinated many biologists and are extensively studied in a variety of organisms. The sex ratio is particularly important for distribution and persistence of parasites (Downes, '95). For higher organisms, it has been established that sexual development is determined by either chromosomal factors or by environmental factors (Bull, '85; Adams et al., '87; Korpelainen, '90). For example, temperature-dependent sex determinations have been extensively studied (Bull, '85).

For more than two decades, nematodes have been used as model organisms for such studies. While the chromosomal mechanism was studied extensively, particularly in the free-living nematode *Ceanorhabditis elegans* (Hodgkin, '85, '90; Meyer, '97), little is known about the environmental factors affecting sex determination. Sex determination in *C. elegans* is governed entirely by a chromosomal mechanism, whereas it has been shown that under stressed conditions, i.e., crowding, depletion of food source, or high temperature, the number of males increases in species belonging to the heteroderidae (Santos, '72; Triantaphyllou and Hirschmann, '73) and Mermithidae families (Poinar and Hansen, '83; Tingley and Anderson, '86).

Recent studies (Strauch et al., '95) have indicated that the sex ratio of the entomopathogenic nematode *Heterorhabditis bacteriophora* may also be influenced by environmental factors. This nematode is a facultative parasite of insects (Kaya and Gaugler, '93) and is associated with the symbiotic bacterium *Photorhabdus luminescence.*

The life cycle of this nematode occurs within the insect cadaver; only the infective juvenile (IJ) stage can persist outside of the host. In this IJ stage, which is the third stage juvenile, the nematode searches for the host and invades it through natural openings or soft cuticular parts. It reaches the hemolymph where it releases the symbiotic

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bacterium carried in its intestine. The bacteria multiply and kill the insect within 24–48 hr. Once the bacteria are released into the hemolymph, the infective stage of the nematode molts and resumes development to the fourth-stage juvenile. These will continue to develop to adult hermaphrodites that will then lay eggs. Unlike the parental generation, which consists solely of hermaphroditic adults, the F_1 progeny develop to either adult males, females, hermaphrodites, or cease development at the infective juvenile third stage. The life cycle of this nematode species has been thoroughly described (Zioni et al., '92; Johnigk and Ehlers, '99a). Johnigk and Ehlers ('99a) characterized the morphological differences between the juveniles of the F_1 generation that develop to the various sexual phenotypes. They reported that the rate of development varies between the sexes, whereby males reach maturity first, followed by females. The last to reach maturity are hermaphrodites.

Varying reports have been published on the number of males in strains of *Heterorhabditis*. The ratio among the F_1 progeny of *H. bacteriophora* cultured in vivo was 1:1 (Poinar and Hansen, '83). In the HSH strain of *H. bacteriophora*, originated from northwest Europe, 38% of the F_1 generation cultured in vitro were males (Strauch et al., '94). In the HP88 strain, the proportion of males in the F_1 progeny was found to be lower; in one study 4–5% (Zioni et al., '92) and in another, 8–10% (Dix et al., '94).

Strauch et al. ('94) were the first to hypothesize that the sex ratio is affected by environmental factors, particularly nutrition. They showed that when hatching juveniles were deprived from a food source for 24 hr, all developed to hermaphrodites and IJs. Whereas, when food was available from the moment of hatching, the sex ratio was 32% hermaphrodites, 30% females, and 38% males.

The present study was aimed at determining the influence of various environmental factors on sex differentiation in the nematode *H. bacteriophora* strain HP88, under in vivo and in vitro culture conditions.

MATERIALS AND METHODS

Nematode culture

The nematode *H. bacteriophora* strain HP88, used in the present study, has been maintained in our laboratory for the past 10 years. It was reared on last instar larva of the greater wax moth *Galleria mellonella* according to the method described by Stock and Kaya ('97). The infective juveniles were routinely stored, in water suspensions, at 10–12°C for 3–4 weeks prior to use in all experiments.

Bioassays

For in vitro tests, the nematodes were cultured in 12-well dishes (Bibby Sterilin, Staffordshire, England) on "Nematode Growth Medium" (NGM) (Sulston and Hodgkin, '88), and pre-seeded with suspension of the symbiotic bacterium *P. luminescence* according to the procedure described by Zioni et al. ('92). The nematodes were allowed to initiate development from IJ stage, concentrated on a 5 cm petri dish, and when they reached late fourth juvenile stage or young hermaphrodite adult, they were placed in the wells.

In both in vivo and in vitro cultures, sexual differentiation was determined in the second- and third-stage juveniles of F_1 progeny that originated from parental hermaphrodites developed from IJs. We followed the detailed description made by Johnigk and Ehlers ('99b), as well as our own experience, to distinguish between larval stages that developed to the various sexes. At the second stage, the sex specific structures can be distinguished. The male pregonad develops asymmetrically, whereas in females and hermaphrodites, the pregonads always develop symmetrically. The female second juvenile is shorter (398 ± 47 μm) and broader (25 ± 1.8 μm) than the hermaphrodite juvenile, which at this stage remains thin (21 ± 1.6 μm) but elongated (440 ± 11 μm) and stores fat reserves in the intestine cells. The measurements were obtained from Johnigk and Ehlers ('99a). The timing of juvenile development was predetermined in both in vivo (approximately 120 hr from injection) and in vitro (approximately 96 hr from placement in the well) cultures. At the appropriate time, the infected insects, in the in vivo experiments, were dissected in saline (0.85% NaCl w/w in distilled water) and the nematodes that migrated out of the cadavers were observed under an inverted microscope at 300 × magnification for sexual discrimination. In the in vitro cultures, the developing juveniles were removed from the wells for examination by suspending them in 10 ml saline.

For the in vivo experiments, two parameters were evaluated: crowding and host size. To determine the effect of the first parameter 1, 5, or 20 IJs were injected to individual *G. mellonella* larvae in 20 μl of sterile saline using a 1-ml syringe (Plastipak, Becton-Dickinson, Madrid, Spain). For the second parameter, one nematode was injected to two sizes of *G. mellonella* larvae, young (0.7–1.5 cm long) or

mature (2.4–2.7 cm long). In both experiments, control treatment consisted of injection of nematode-free saline solution. Each treatment consisted of 25 insect larvae. The experiments were repeated three times.

To establish a basis for determination of the effect of various factors on sex differentiation in vitro, we used a "standard" culture procedure that consisted of the following conditions: NGM medium, *P. luminescence* bacterium, one hermaphrodite per well, and 25°C incubation temperature. We then modified each growth parameter at a time as follows:

a. Culture medium—The NGM was compared with, Dog Food Agar (Zioni et al., '92) and with NGM+Yeast extract (Riddle, '88).
b. Symbiotic bacterium—The hermaphrodites were placed on a lawn of bacteria obtained from the HP88 strain as well as from the IS-5, IS-´ and IS-33 strains of *Heterorhabditis* spp. The bacteria were isolated from infected *G. mellonella* instar according to Poinar and Thomas ('65).
c. Crowding effect—In each well 1, 5, or 20 hermaphrodites were placed.
d. Temperature—The wells were placed at 20 ± 0.5, 25 ± 0.5,or 30 ± 0.5°C incubation temperature. Longer (120 hr) incubation periods were required to obtain the appropriate developmental stages (J_2 and J_3), at the lower temperature, and shorter periods (36 hr) at the high temperature treatment.

The different treatments, in all the experiments, were replicated 24–35 times. Each replicate consisted of one well.

The following procedure was employed to determine the effect of juvenile starvation on sexual differentiation: Developed eggs were obtained from gravid hermaphrodites by cutting them with a sharp tip of a needle in sterile saline solution. The eggs were allowed to hatch and the young juveniles were transferred to a bacterial lawn in a well after 0, 3, 6, 12, 16, and 24 hr. Sexual differentiation was recorded among the developing larvae 48 hr from transfer.

Statistical analysis

The proportion of different sexual stages in the population was expressed as a percentage. An arcsine of square root transformation was used on these data. The General Linear Model (GLM) Procedure of SAS ('88) was used for analysis of variance. Significance between treatments was determined using Scheffe's test at $P < 0.05$.

RESULTS

Injection of individual nematodes into last instars of *G. mellonella* resulted in development of a similar number of females and hermaphrodites (35–40% from the F_1 progeny that were examined) and 20–25% males (Fig. 1). Increasing the number of nematodes injected into the insect did not change the proportion significantly ($P < 0.05$) (Fig. 1). Under these culture conditions, no IJs were

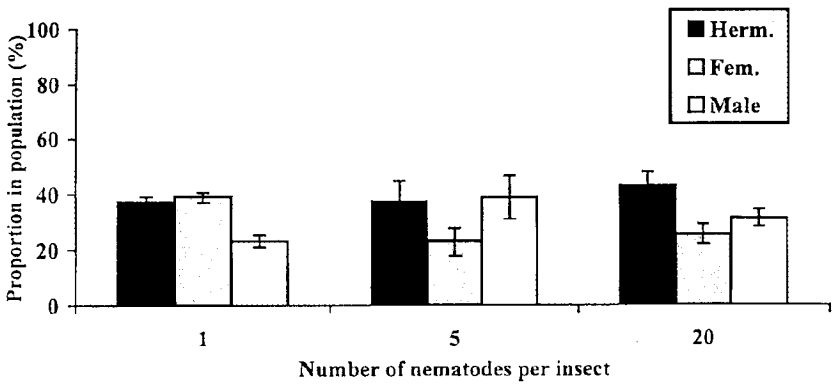

Fig. 1. Effect of number of parental entomopathogenic nematode *Heterorhabditis bacteriophora* HP88 on the proportion of hermaphrodites (Herm.), females (Fem.), males, and infective juveniles (IJs) among F_1 progeny in the cadaver of last instar larvae of the greater wax moth *Galleria mellonella*. The infective juveniles of the nematodes were injected into the insect larvae and the proportion of the different sexes was recorded after 120 hr incubation at 25°C among the second- and third-stage juveniles. Error bars are standard error of the mean.

found among F_1 progeny. In smaller insects, a significant ($P < 0.05$) increase in the proportion of hermaphrodites was recorded (Fig. 2) as compared with larger size cadavers. In this treatment, a low percentage of IJs also was found (Fig. 2).

When individual hermaphrodites were placed on NGM, the proportion of hermaphrodites, females, and male progeny was 63%, 31%, and 6%, respectively (Fig. 3). Addition of yeast extract to the medium did not change the proportions significantly, whereas rearing on DF medium resulted in significant ($P < 0.05$) reduction in the proportion of hermaphrodites (Fig. 3). In this culture, a threefold increase was recorded among the proportion of males while the female proportion increased moderately (Fig. 3).

The secondary phase of the HP88 symbiotic bacterium *P. luminescence* and the primary form of IS-´ did not support any development of individual nematodes on NGM in the multi-well system (data not shown). Nematodes introduced to the symbiotic bacterium obtained from other nematode strains (IS-5 and IS-33) developed similarly to the culture reared on the HP88 bacteria. These strains did not have any effect on the proportion of the different sexes (Fig. 4) as compared to the natural symbiotic bacterium of *H. bacteriophora* HP88. Rearing the nematodes at a temperature range between 21°C to 30°C also did not have a significant effect on the sexual differentiation among nematodes cultured on NGM in the multi-well system (Fig. 5).

A moderate, but significant ($P < 0.05$), increase in the proportion of hermaphrodites was observed among progeny in the multi-well system when each well was inoculated by more then five individuals (Fig. 6). In the higher inoculum size treatments (> 5 nematodes per well) the phenomenon of "Endotokia matricida," i.e., hatching of eggs and development of nematode juveniles within the hermaphrodites/female body, was observed. The vast majority of these juveniles developed to IJs, which eventually develop to hermaphrodites. Thus, the actual increase in the proportion of hermaphrodites is higher by 10–15% (data not shown). The increase in the inoculum size reduced the proportion of female progeny but did not effect the males' proportion in the population (Fig. 6).

Larval starvation had a considerable effect on sexual differentiation among *H. bacteriophora* progeny (Fig. 7). In all treatments, survival was greater then 90% for the juveniles transferred to the culture wells after the different period of starvation. Maintaining the hatching J_1 larvae for up to 6 hr in saline did not affect the proportion of sexes. Above 6 h the proportion of hermaphrodites increased as starvation period lengthened. In parallel, the proportion of females and males was sharply reduced (Fig. 7).

DISCUSSION

The data obtained in the present study strongly suggest that the nutrition source is the main factor affecting sex differentiation in *H. bacterio-*

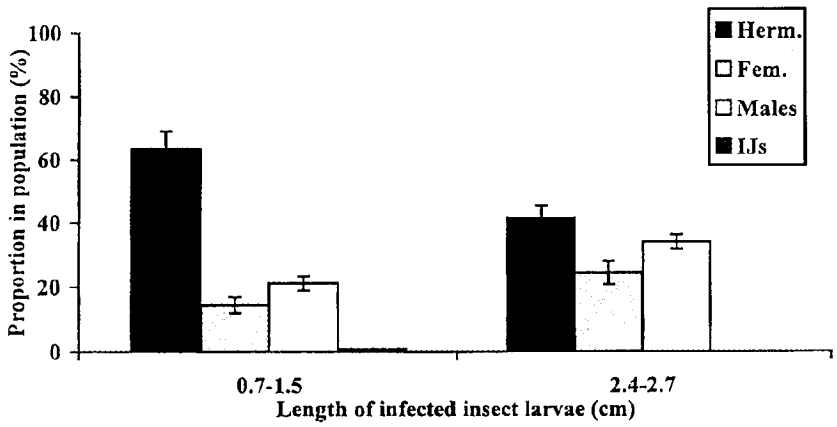

Fig. 2. Effect of host size on the proportion of hermaphrodites (Herm.), females (Fem.), and males among progeny of the entomopathogenic nematode *Heterorhabditis bacteriophora* HP88 in the cadaver of instar larvae of the greater wax moth *Galleria mellonella*. The infective juveniles of the nematodes were injected into the insect larvae and the proportion of the different sexes was recorded after 120 hr incubation at 25°C among the second- and third-stage juveniles of F_1 progeny. Error bars are standard error of the mean. Columns at different treatments annotated with the same capital letter are not significantly different according to Scheffe's test ($P = 0.05$).

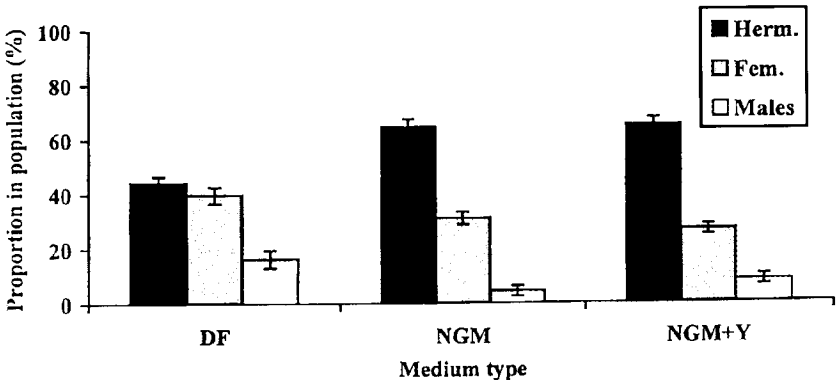

Fig. 3. Effect of culture medium on the proportion of hermaphrodites (Herm.), females (Fem.), and males among progeny of the entomopathogenic nematode *Heterorhabditis bacteriophora* HP88. Individual fourth-stage juveniles, developed from infective juveniles were placed on the different media in a plastic dish (multi-well dishes). Each medium was pre-seeded with suspension of the symbiotic bacterium *Photorhabdus luminescence*. The different sexes were recorded after 96 hr incubation at 25°C among the second- and third-stage juveniles of F_1 progeny. DF = dog food agar; NGM = nematode growth medium, Y = yeast extract. Error bars are standard error of the mean. Columns at different treatments annotated with the same letter are not significantly different according to Scheffe's test ($P = 0.05$).

phora. The wax moth *G. mellonella* is known to be highly susceptible to nematode infection and a suitable host for their development and reproduction (Dutky et al., '64). It was demonstrated here that in this host the sex ratio is roughly 1:1:1 for hermaphrodites: females:males. The increase in the proportion of hermaphrodites in smaller insects (Fig. 2) is attributed to nutrition depravation, either due to lower amounts of food or different, and less nutritional value, of the younger instar as compared to the last instar stage.

In our "standard" in vitro culture medium (NGM), the proportion was 60%, 30%, and 10% for hermaphrodites, females and males, respectively. The NGM has been developed for culture of *C. elegans* and contains minimal nutritional substances that mildly support bacterial propagation (Brenner, '74). Shifts from a "standard" ratio between sexes was recorded among F_1 progeny, in in vitro culture, only where food availability was altered either by increasing the number of parent hermaphrodites in the well (Fig. 6), caus-

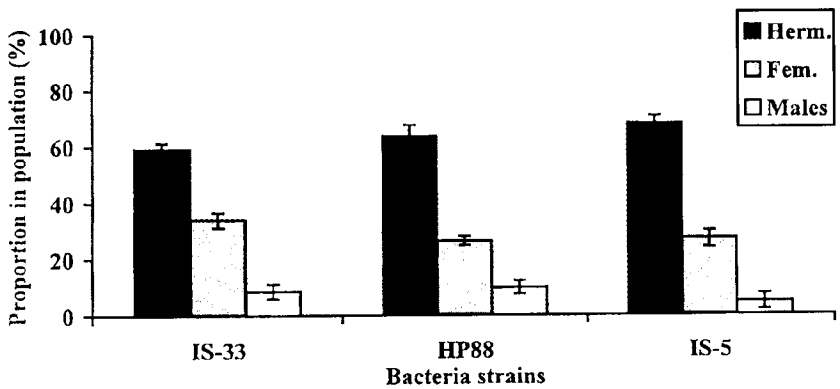

Fig. 4. Effect of symbiotic bacterium on the proportion of hermaphrodites (Herm.), females (Fem.), and males among progeny of the entomopathogenic nematode *Heterorhabditis bacteriophora* HP88. Individual fourth-stage juveniles, developed from infective juveniles, were placed on nematode growth medium, in a plastic dish (multi-well dishes). The medium was pre-seeded with suspension of the symbiotic bacterium *Photorhabdus luminescence*. The different sexes were recorded after 96 hr incubation at 25°C among the second- and third-stage juveniles of F_1 progeny. IS-33 and HP88 are symbiotic bacterium of different *H. bacteriophora* strains and IS-5 is a symbiotic bacterium of *Heterorhabditis* sp. IS-5 strain. Error bars are standard error of the mean. Columns at different treatments annotated with the same letter are not significantly different according to Scheffe's test ($P = 0.05$).

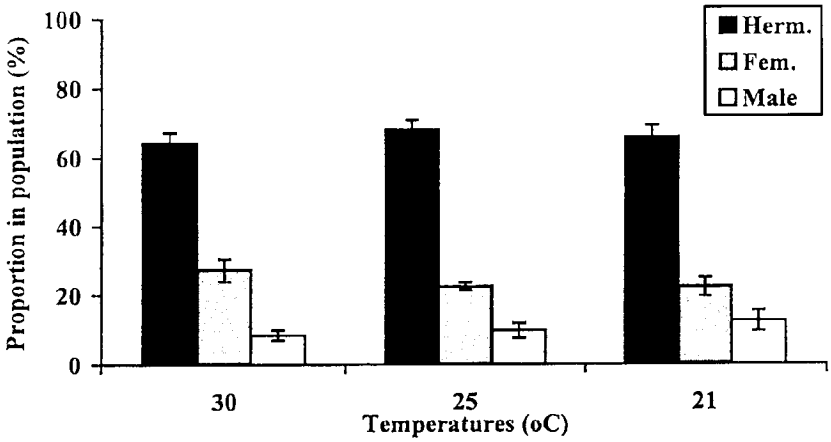

Fig. 5. Effect of incubation temperature on the proportion of hermaphrodites (Herm.), females (Fem.), and males among progeny of the entomopathogenic nematode *Heterorhabditis bacteriophora* HP88. Individual fourth-stage juveniles, developed from infective juveniles, were placed on nematode growth medium, in a plastic dish (multi-well dishes). The medium was pre-seeded with suspension of the symbiotic bacterium *Photorhabdus luminescence*. The different sexes were recorded after incubation at the various temperatures among the second- and third-stage juveniles of F_1 progeny. Error bars are standard error of the mean.

ing crowding effect of the progeny, or by starving the J_1 progeny (Fig. 7). The impact of food availability on the sex ratio also was evident when the effect of culture medium on sex differentiation was determined (Fig. 3): The DF medium, which is considered a 'rich' medium, supported higher proportion of males and females among the progeny, as compared to the NGM medium. In all cases, reduction of food accessibility or quality resulted in a substantial increase in the proportion of hermaphrodites among the F_1 progeny.

One of the most common environmental sex-determining factors in many animals, especially vertebrates, is extreme temperature (Bull, '85; Adams et al., '87; Korpelainen, '90). Within the temperature range tested in the present study, it seems that this factor does not have any effect on sex determination in *H. bacteriophora*. This temperature range was chosen because it is characteristic to the *H. bacteriophora* optimal development temperature (Grewal et al., '94). Extreme temperature conditions would

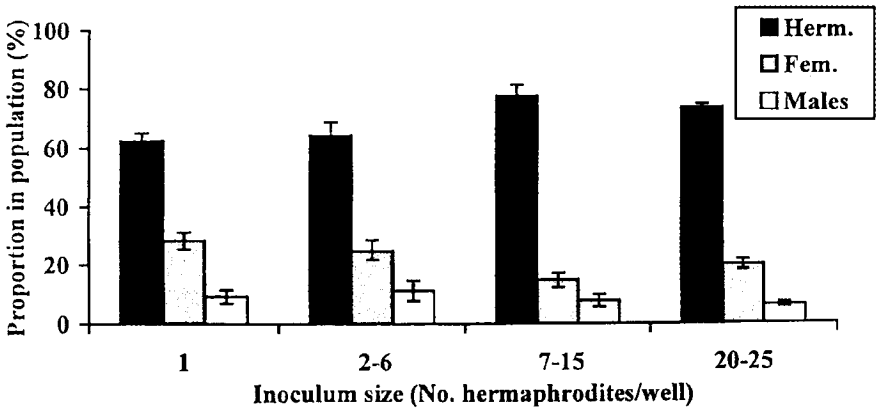

Fig. 6. Effect of nematode inoculum density on the proportion of hermaphrodites (Herm.), females (Fem.), and males among progeny of the entomopathogenic nematode *Heterorhabditis bacteriophora* HP88. Different numbers of fourth-stage juveniles, developed from infective juvenile, were placed on nematode growth medium, in a plastic dish (multi-well dishes). The medium was pre-seeded with suspension of the symbiotic bacterium *Photorhabdus luminescence*. The different sexes were recorded after incubation at the various temperatures among the second- and third-stage juveniles of F_1 progeny. Error bars are standard error of the mean. Columns at different treatments annotated with the same letter are not significantly different (P = 0.05) according to Scheffe's test (P = 0.05).

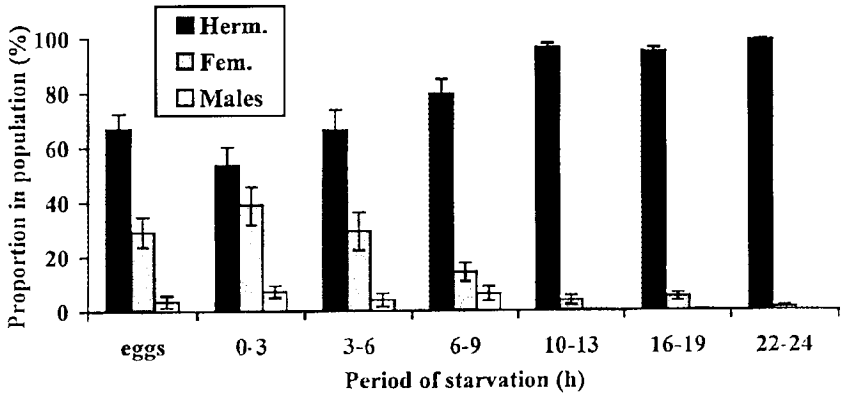

Fig. 7. Effect of first-stage juvenile starvation, of the entomopathogenic nematode *Heterorhabditis bacteriophora* HP88, on the proportion of hermaphrodites (Herm.), females (Fem.), and males. Eggs originated from gravid hermaphrodites that developed from infective juveniles were allowed to hatch in saline. The juveniles were placed on nematode growth medium, in a plastic dish (multi-well dishes) at different time ranges from attaching. The medium was pre-seeded with suspension of the symbiotic bacterium *Photorhabdus luminescence*. The different sexes were recorded after 48 hr incubation among the second- and third-stage juveniles. Error bars are standard error of the mean. Columns at different treatments annotated with the same letter are not significantly different according to Scheffe's test ($P = 0.05$).

have hampered the overall development of the nematode.

The development of heterorhabditid nematodes is highly dependent on the symbiotic bacteria (Gerristen and Smits, '93; Frost et al., '97). As expected, the secondary form of *P. luminescence* did not support development of young hermaphrodites, and consequently did not affect sexual differentiation. It was previously demonstrated (Shapiro et al., '97) that the symbiotic bacterium of the heterorhabditid strain IS-5 can support development and reproduction of *H. bacteriophora* HP88. The IS-´ and IS-33 strains were tested here for the first time. The fact that the latter had a similar effect on sexual differentiation as the HP88 strain suggests that it is compatible for symbiotic relation with the HP88 nematode strains. The test used in the present study may be applied to evaluate other symbiotic bacteria compatibility for heterorhabditid culture.

Stress conditions usually result in an increase in the proportion of males in the population among other nematode species in which the environmental condition affects sexual differentiation. This is true for the mosquito *Romanomermis culicivorax* (Tingley and Anderson, '86), the free-living nematode *Dipletron potokikus* (Clark, '78), and the root-knot-nematode *Meloidogyne* spp. (Santos, '72). These changes are also associated with crowding effects and depletion of nutrition resources. For *D. potokikus* and *Meloidogyne* spp. male development is favored, under stress conditions, over parthenogenic females (Clark, '78). It has been implied that the evolutionary advantage to the increase in males under stress conditions is in the ability to expand the heterogeneity of the population and thus to enhance the ability of individual progeny to withstand the stress (Poinar and Hensen, '83; Tingley and Anderson, '86).

The phenomenon described in the present study of increasing the proportion of self-fertilizing individuals due to nutritionally related stress conditions is unique. It was suggested by Downes ('95) that reproduction by self-fertilization is sometimes seen as a necessary adaptation because of the difficulty of finding a mate, particularly under stress conditions. Furthermore, the selfing process serves as a "filter" for deleterious recessive alleles (Downes, '95).

Another interesting phenomenon observed in the present study is the occurrence of "endotokia matricida" among hermaphrodites under crowding conditions. Although this phenomenon has been previously described among heterorhabditids (Poinar, '76), as well as among other nematodes (Luc et al., '79), the genetic and physiological mechanisms involved are unknown. Recently, Johnigk and Ehlers ('99b) described the "endotokia matricida" among heterorhabditids in great detail and demonstrated that low food supply significantly promoted this phenomenon. Johnigk and Ehlers ('99b) observed that almost all juveniles developed to the infective stages and suggested that the "endotokia matricida" secure the

development of IJs when external food supply is reduced.

The data obtained in the starvation experiment (Fig. 7) indicate that the critical stage in which the sexual development is determined is the first-stage juvenile (J_1). Strauch et al. ('94) have shown that 24 hr starvation of J_1 progeny results in a complete shift of the populations towards hermaphroditism (IJs and adults). We demonstrated here that 6–9 hr of post hatching are sufficient for the developing juvenile to sense the environment and initiate the appropriate development pathway toward hermaphrodites/infective juveniles, females, or males. The fact that high survival (>90%) was recorded in the different treatments eliminates the possibility that the shift toward hermaphroditism is caused by differential survival rather than a sex determination signal.

Although the genetic and physiological mechanisms of this process are unknown, we propose that the initial decision is between male and female/hermaphrodite, and at the second stage between female and hermaphrodite. This suggested scenario is based on the observation that males develop more quickly, then the females, and finally the hermaphrodites (Johnigk and Ehlers, '99a, plus personal observations). It is also supported by the assumption that the morphogenesis of male organs is completely different from that of female or hermaphrodite, while the latter are very similar. They differ only by the fact that hermaphrodites produce sperms in the germ-line tissue at the early stage of development. Under nutritionally stressed conditions, the options for development toward male and female are eliminated and the default option remains the hermaphrodites, either directly or via an IJ arrested stage. The ratios between the different sexes highly depend on the intensity of the stress.

Understanding and manipulating the mechanisms involved in sexual development of *H. bacteriophora* has practical implications. The ability of this organism to initiate clonal as well as cross-breeding populations raises questions about adaptation to laboratory/commercial production conditions. This is especially relevant under large-scale production conditions in which the nematodes are reared in crowding condition, thus produced mainly by selfing.

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