Environmentally Controlled Sex Expression in *Meloidodera floridensis*[1]

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Abstract: Larvae of *Meloidodera floridensis* develop as females after feeding on pine roots, but become males under conditions of starvation. Seventy to 80% of the larvae kept in tap water at 23 C for 4 months underwent one or two molts, developing as males, and more than 50% became adult males. Ninety-six percent of the larvae that entered pine roots became females and only 4% developed as males. There is evidence that the latter did not feed on the roots. In comparison with tap water, solutions of cholesterol, testosterone propionate and β-estradiol did not significantly affect the percentage of larvae that developed into males. Larvae kept in soil without a host plant did not develop into males. Most of them exhausted their energy supply and died without undergoing any development. We conclude that sex expression in *M. floridensis* is to a large extent controlled by environmental factors. Under natural conditions of feeding on a host plant, larvae develop as females according to their genetic constitution (thelytokous organism). Under conditions of starvation, however, sexual differentiation proceeds toward the male direction, probably as a result of alteration of the hormonal balance of the larvae and the subsequent activation of different sites of genetic function. *Key Words:* postembryogenesis, development, hormones.

Unbalanced sex ratios are known to occur in various members of the family Heteroderidae. Males may be very rare or absent in some cases and abundant in others. In the genus *Meloidogyne* Goeldi, second-stage larvae have the potentiality of developing into adult females when the conditions during post-infection development are favorable, but undergo sex reversal and develop into males or male intersexes under adverse environmental conditions (10).

In the genus *Heterodera* Schmidt, unbalanced sex ratios have been explained in two different ways. Sex of *H. rostochiensis* Wollenweber is reported to be environmentally controlled (3, 9, 12). Under crowded conditions, female larvae change course of sexual differentiation and develop into males. Unbalanced sex ratios in *H. schachtii* Schmidt and *H. glycines* Ichinohe are believed to be the result of differential death rate of male and female larvae under adverse environmental conditions (6, 7, 8).

Males of *Meloidodera floridensis* Chitwood, Hannon and Esser appear to be very rare in natural populations. No males were observed in the original population from which the species was described (2). Some males were reported later from incubated pine roots (1) and from soil collected around the roots of slash pine from various localities (5). Before the present studies were initiated, we had observed incidentally that many males of *M. floridensis* could be obtained from pine roots incubated in a moist chamber at room temperature for a period of 1-2 months. Pursuing this subject further, we learned that males could develop even from freshly hatched larvae kept in tap water for a period of 1-3 months. It appeared that some larvae had the capacity to develop and become males without feeding. This was rather unexpected, because it was well known that second-stage larvae of the related genera *Meloidogyne* and *Heterodera* require at least a short period of feeding on a host plant, before they can start developing. Without feeding, they remain as preparasitic second-stage larvae until they die.

Although early observations, and the results of preliminary tests, established beyond doubt that some second-stage larvae of *M. floridensis* developed to adult males without feeding, the percentage of larvae that had this capacity and the environmental conditions which promoted or supported such development were not known. Neither did we understand why no males, or so few males, are found in the soil surrounding infected pine roots under field and greenhouse conditions. The fundamental question, of whether sex differentiation of these larvae was genotypically or phenotypically controlled, also remained unresolved. To clarify these questions was the objective of the studies included in this paper. Understanding the anatomical aspects of

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development of both males and females also became necessary, and the results of this second study are reported in a separate paper (4).

MATERIALS AND METHODS

Population 21-NC of *Meloidodera floridensis* used in this study was collected on loblolly pine (*Pinus taeda* L.) from Eastern North Carolina in 1964 and has been maintained in the greenhouse on pine seedlings. Cytologically, this population appears to be a triploid and reproduces exclusively by mitotic parthenogenesis (11).

Second-stage larvae used throughout these studies were hatched from embryonated eggs which had been obtained by dissecting gravid females.

Meloidodera larvae are very sensitive to the chlorine in tap water, to various disinfesting agents and, to some extent, to mechanical handling. Therefore, larvae were handled always in chlorine-free tap water, and were transferred to the various media with a glass pipette. Care was taken not to allow excessive build-up of bacterial and fungal populations in the various media, but no sterile conditions requiring nematode disinfestation were employed.

The normal development of *M. floridensis* was studied by inoculating 3-month-old loblolly pine seedlings grown in 10-cm clay pots with 200 second-stage larvae each. Following inoculation, the pots were kept in a 24-26 C growth chamber. Seedlings were washed free of soil at the designated time intervals (Table 1), and the roots were stained for 2 min with boiling acid fuchsin in lactophenol. All nematodes found in each root system were dissected out and examined microscopically to determine their stage of development and sex.

To study the development of larvae under conditions of starvation, 3000 second-stage larvae were placed in each of three petri dishes with 15 ml tap water in a 23±2 C air-conditioned room. At weekly intervals for a period of 120 days, dead second-stage larvae, dead molting larvae and adult males were removed, and the water was changed.

To study the possible effect of steroid hormones on the development of larvae as compared to tap water, 200 second-stage larvae were placed in each of three petri dishes under the same conditions described for tap water. Hormone solutions were prepared by dissolving the chemicals in an appropriate solvent (ether for cholesterol, acetone for testosterone propionate and β-estradiol). Each solution was added slowly, and under constant stirring with an electric stirrer, to tap water in which a few drops of the detergent Tween 80® had been added. The final concentrations were 0.4 mg of cholesterol, 0.4 mg of testosterone propionate and 0.5 mg of β-estradiol per ml of water. Some crystalization occurred in the final solutions following evaporation of the solvents, and, therefore, the actual concentrations were lower than those shown above.

For the study of the development of larvae in soil, 100 second-stage larvae were added to each of 24 vials containing 10 ml of methyl bromide-fumigated, sandy soil. Twelve of the vials were kept in a 21-C constant temperature incubator, the others were kept at 26 C. Larvae were extracted from soil at the indicated time intervals (Table 2) by the sieving and decanting procedure, combined with the sugar flotation technique. Every effort was made to recover as many larvae as possible. Nematodes recovered from the soil were examined to determine the

TABLE 1. Development of *Meloidodera floridensis* in loblolly pine seedlings at 24-26 C.

Days after inoculation	Total number of nematodes recovered	Stages of development and sex of nematodes (%)							
			Females			Males			Total males (%)
		2nd-stage larvae	3rd-stage larvae	4th-stage larvae	Adults	3rd-stage larvae	4th-stage larvae	Adults	
5	70	100.0	0	0	0	0	0	0	0
10	114	96.5	0	0	0	3.5	0	0	3.5
15	404	81.7	17.3	0	0	1.0	0	0	1.0
20	244	58.7	37.6	1.2	0	2.5	0	0	2.5
25	326	31.3	43.3	20.9	0	3.0	1.2	.3	4.5
30	338	22.5	31.4	40.1	1.2	1.5	1.8	1.5	4.8
35	246	12.2	28.5	51.7	1.6	1.6	1.2	3.2	6.0
40	191	13.0	19.9	42.0	18.8	1.0	2.1	3.2	6.3
47	124	16.9	3.2	30.6	43.7	0	1.6	4.0	5.6
55	113	13.3	2.6	8.8	70.9	0	0	4.4	4.4

TABLE 2. Development of 100 *Meloidodera floridensis* larvae kept in soil for different periods of time at two temperatures.

Days in soil	Number of nematodes recovered from soil[a]						Further development of the live second-stage larvae extracted from soil and kept in tap water for 60 days	
	Total No.	Live nematodes			Dead nematodes			
		2nd-stage larvae	Molting larvae	Adult males	2nd-stage larvae	Molting larvae	Molting larvae (No.)	Adult males (No.)
At 21 C								
20	79.7	78.7	.3	0	.7	0	15.3	8.7
40	74.3	70.0	.7	0	2.7	.1	10.3	4.7
60	68.0	52.0	1.3	1.3	11.3	.2	6.7	1.3
80	58.7	14.0	.3	2.0	40.3	.2	5.0	.3
At 26 C								
5	83.7	81.3	0	0	2.3	0	2.7	.3
15	77.0	76.7	0	0	0	.3	2.0	0
30	67.3	2.7	0	0	63.3	1.3	0	0
45	42.3	1.0	0	0	41.3	0	0	0

[a]Average of three replicates.

stages of development and the general state of appearance. Dead nematodes, and live, developed specimens were discarded, whereas live second-stage larvae were transferred to chlorine-free tap water and were observed for 2 months at weekly intervals to determine whether they were undergoing molts.

RESULTS

Development in pine roots: Most of the nematodes recovered from the pine roots were females (Table 1). Males varied between 1 and 6.3% of the nematodes of each sampling date, with a general average of 4%. Males appeared to develop slightly faster than females. All male nematodes had become adults 55 days after inoculation. The increased numbers of second-stage larvae found in the 47- and 55-day samplings probably represent new infections by second-generation larvae.

Microscopic examination showed that males were always associated with the outer layers of the root cortex, whereas females had their heads embedded deeper in the cortex and in the vascular parenchyma.

Development in tap water: At the end of a 120-day period in tap water, starting with 3000 larvae, the average numbers of nematodes per dish in the various developmental stages were as follows: adult males 1765, molting specimens 693, second-stage larvae 542. Some of the molting larvae and the second-stage larvae were still alive. Adult males represent 59% of the larvae added to the dishes, and this is significantly higher than 50% (total χ^2=468 with 1 d.f.).

Parallel anatomical and developmental studies showed that all molting larvae were developing as males, although some of them died before they became adults (4). If molting larvae are counted as males, then the total number of males will represent 82% of the nematodes tested, which similarly is significantly higher than 50% (total χ^2=3697 with 1 d.f.).

Development in various steroid hormone solutions: At the end of a 120-day period more than 50% of the larvae had become adult males in all treatments, but none of the hormone solutions had an effect significantly different from that of tap water. When molting larvae were added to the number of adult males, the percentage of males became significantly higher than 70 in all treatments, but again, it was not significantly different from that of tap water (χ^2 <1.76 with 1 d.f.).

Development in soil: At 21 C, most of the nematodes recovered from the 20-, 40- and 60-day soil samples were still live second-stage larvae (Table 2). Those recovered from the 80-day samples were mostly dead second-stage larvae. A small proportion of the larvae recovered from the 20- and 40-day soil samples had undergone one or two molts. Slightly higher numbers of developing larvae were recovered from the 60- and 80-day soil samples

in which some had become adult males. Not all developing larvae became adult males, however. Some had died before reaching adulthood.

At 26 C, live nematodes were recovered in large numbers only from the 5- and 15-day soil samples. Most of the nematodes from the 30- and 45-day soil samples were dead second-stage larvae. A very small number of larvae had undergone one or two molts at this temperature, but had died before reaching adulthood. Dead nematodes disintegrated in the soil, and this probably accounts for the reduced recovery of total numbers of nematodes in older samples at both temperatures.

When the live second-stage larvae extracted from the soil were placed in tap water at 23±2 C, many began to molt and some became adult males (Table 2). In the 21-C test, second-stage larvae extracted from the soil were in good condition with plenty of food supply stored in the intestine, and many of them began to molt. In contrast to this, larvae recovered from soil kept at 26 C were transparent, indicating very little food stored in the intestine, and very few began to molt.

CONCLUSIONS AND DISCUSSION

From the data on the development of *Meloidodera floridensis* in tap water and in various steroidal solutions, we concluded that more than 50% of the second-stage larvae of this nematode can develop to adult males without feeding. Many more larvae start developing by undergoing one or two molts, but die before they reach maturity. Anatomical studies have shown that the latter larvae are developing as males (4) and therefore, they can be classified as such. Consequently, about 70 to 80% of the second-stage larvae of *M. floridensis* can develop as males under conditions of starvation.

Contrary to this, only 4% of the larvae recovered from roots of pine seedlings were developing as males. It was also observed that all these males were associated with the outer layers of the root cortex and, apparently, had not established a feeding relationship with the pine roots. Body size similarly indicated that they had not grown beyond the size of second-stage larvae, suggesting development without feeding. It can, therefore, be assumed that larvae which start feeding on pine roots develop into females, whereas larvae that do not feed develop into males.

To explain the developmental behavior of *M. floridensis*, particularly with regard to sex expression, one should consider that this species is a triploid and reproduces by mitotic parthenogenesis (11). Normally, it should produce one type of eggs with the capacity to develop as females. It must be a thelytokous parthenogenetic species. The production of large numbers of males in our tests indicates that second-stage larvae also have the genetic capacity to develop as males.

The direction of sexual differentiation of second-stage larvae appears to depend largely on the environmental conditions under which they live. Larvae entering the roots of a host plant, and successfully establishing a feeding relationship with that plant, grow and develop as females. Larvae that enter the roots but fail to feed on them do not grow, and develop as males. Larvae that remain in the soil usually exhaust their food supply and die, although a small percentage of them may develop into males. Finally, larvae kept in tap water without feeding preserve their food supply by entering a state of low metabolic activity, during which body movement is reduced to a minimum or ceases completely. In this metabolic state, development toward the male direction appears to be enhanced, and the majority of the larvae start developing and become adult males. The biochemical mechanism that triggers initiation of development of the second-stage larvae is not known. It can be assumed that feeding initiates development toward the female direction; whereas, prolonged starvation triggers initiation of development toward the male direction. A second requirement for the development in the male direction appears to be the presence of sufficient stored energy in the intestine of the larvae. This requirement is met when larvae enter a state of pseudoquiescence, as when kept in tap water.

The relatively simple system involved in the development of *M. floridensis* males, without the need of a host plant, may provide a useful tool in the study of the developmental aspects of sex differentiation from a physiological and biochemical point of view. Undoubtedly this pattern of development is closely controlled by a hormonal system which is genetically regulated, but which can easily be modified by various environmental factors such as feeding or starvation.

Similar patterns of sex differentiation have been described in the genus *Meloidogyne* and *Heterodera* (10), but they are more complex,

because they involve continuous or partial feeding of the nematodes on a host plant.

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