

Postembryonic Development of the Redwood Nematode, *Rhizonema sequoiae* (Nemata: Heteroderidae)¹

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Abstract: Second-stage larvae of *Rhizonema sequoiae* Cid del Prado Vera et al. developed into adult females in 6 months or adult males in 3-4 months on roots of *Sequoia sempervirens* maintained in a growth chamber at 16 C with a 12-hour light period. Under these conditions the second-stage larvae increased in diameter, the central cells of the genital primordium increased in size, and their nuclei enlarged. Mesenchymal cells accumulated in the esophageal and tail regions. Second-stage larvae become third-stage males or females 2 months after inoculation of redwood roots. Their sex could be distinguished by the ratio of length to width of the genital primordium, 3.4 for males and 1.6 for females. The stylet in both sexes became slender, the median bulb became robust and almost spherical, and rings of punctation on the cuticle were evident. Fourth-stage females developed in 3 months from the time of inoculation, and fourth-stage males in slightly less time. At this stage the females were more swollen than the males, the rectum was conspicuous, their reproductive system was in the process of elongation, and the annulation of the cuticle was more evident. The ratio of males to females was 2.3. Mature females were completely inside the roots and did not form cysts. The cuticle was entirely annulated, and the first eggs were detected inside the female 4 months after inoculation and started the production of abundant gelatin-like material. The new generation of second-stage larvae hatched inside the female 2 months after she matured, completing the life cycle in 8 months. The redwood nematode also completed its life cycle in 8 months under greenhouse conditions, but the ratio of males to females increased to 7.4. The entire nematode population died out at 25 C after 6 months. In a Marin County, California, forest, where this nematode occurs naturally, the temperature averaged only 9 C over the November to June period of this study, and the redwood nematode reached the fourth stage with a male-to-female ratio of 1.8.

Key words: redwood nematode, life history, temperature.

Rhizonema sequoiae Cid del Prado Vera et al. (2) was found in 1979 parasitizing the abundant surface feeder roots of redwood, *Sequoia sempervirens* (D. Don) Endl., in Marin County, California. This paper describes the development of the nematode on redwood under various conditions.

MATERIALS AND METHODS

Redwood seeds were soaked in 0.5% sodium hypochlorite for 15 minutes, in sterile distilled water for 24 hours, dried with an air current, coated with 75% thiram, sown in vermiculite in sterile petri dishes, moistened with sterile distilled water, and incubated at 21 C until germination (10-20 days). Germinated seedlings were individually transplanted to sand in plastic culture tubes (21 cm long × 4 cm in diameter) with drainage holes. After seedlings had grown 1 year in these tubes the surface sand was removed and an aqueous suspension of 1,000 *R. sequoiae* second-stage larvae was added to each tube around the

seedling redwood roots; surface soil was then replaced.

Second-stage larval inoculum of *R. sequoiae* was obtained from redwood feeder roots collected in Marin County, California. These were incubated in a mist chamber and larvae were collected and used at 24-hour intervals.

Twenty-four inoculated plants were placed in each of three conditions: 1) a growth chamber maintained at 16 C with a light period of 12 hours, 2) a growth chamber maintained at 25 C with a light period of 12 hours, and 3) a greenhouse averaging 18 C (9-23 C). All plants were irrigated with one-half strength Hoagland's solution every 10 days.

At the same time that the controlled environment treatments were established, 24 uninoculated seedlings were planted in the *R. sequoiae*-infested area in Marin County where they became infected naturally. The soil temperature at this location averaged 9 C but fluctuated between 3 and 14 C over the November to June duration of this experiment.

Nematodes from roots of three seedling trees from each condition were examined monthly for 8 months. Roots were washed free of sand or soil and macerated in 200

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ml of water in a blender for 2 minutes. The resulting suspension was passed through sieves with openings of 0.83, 0.25, 0.15, 0.07, and 0.04 mm (20, 60, 100, 200, and 325 meshes per inch, respectively). Nematodes were picked from the backwashings of the four finest sieves using a dissecting microscope. In the case of the inoculated seedlings, the nematodes present in the soil were also extracted using Jenkins (5) method. Nematodes from these extracts were fixed with Seinhorst's (7) fixative and infiltrated with glycerin using a method of DeGrise (3).

Material for SEM was prepared using the technique of Wong and Brummer (8). Unless otherwise indicated, observations of live stages of *R. sequoiae* were made using nematodes taken from plants grown at 16 C.

RESULTS

Growing conditions: The rate of development of *R. sequoiae* differed greatly in the four different conditions under which the plants were grown (Fig. 1). At the constant 16 C or an average 18 C (greenhouse), some females reached maturity 4 months after inoculation (Fig. 1; Table 1), and some males were mature in 3 months. Egg production was first observed at 5 months, and the majority of the nematodes became adults within 6 months. After 8 months, a second generation of second-stage larvae was observed.

At 25 C some males were mature in 3 months, but no females developed. No stage of *R. sequoiae* was found at 6, 7, and 8 months.

The percentage of larvae infecting redwood roots at 16 C or the average 18 C was 4%; at 25 C it was less than 1%. A constant 16 C was the most favorable temperature condition tested for *R. sequoiae*. Unfavorable conditions were indicated by

slower development or predominance of males. During the November to June course of this experiment, the average soil temperature in the Marin County, California, forest was only 9 C and development of *R. sequoiae* was very slow. The maximum stage of development found was the fourth-stage female and male (Fig. 1). Because the plants were exposed continuously to the natural population of *R. sequoiae*, second-stage larvae and advanced second-stage larvae were continually observed during the 8 months in the forest environment.

Life stages—second-stage larva: The infective second-stage larva is shown in Fig. 2A. One month after inoculation, the second-stage larva from redwood roots increased in diameter (Table 2; Fig 2B) and the genital primordium enlarged to 15.8 (10.4–20) μm long and 11.5 (8.2–18.4) μm wide. The nucleus of the two central cells of the primordium is also enlarged. Mesenchymal cells accumulate at the level of the dorsal esophageal gland and the median bulb (Fig. 2B). The first 10 annules in the cephalic region are wider than those over the rest of the body. The anus is inconspicuous and there is accumulation of mesenchymal cells in the tail posterior to the anus.

The molt to the third stage is seen in some larvae 1 month after inoculation, but in most larvae it occurs after 2 months. The anterior extremity of the body shrinks from the cuticle and the anterior portion of the stylet separates from the basal portion that later becomes indistinct. The accumulation of mesenchymal cells in the esophageal area becomes somewhat larger. At molting no change in the number of cells of the genital primordium is noted, but the two internal cells increase in size and the two external ones are reduced, resulting in a more elongate primordium. At molting the annulation, labial disc, and la-

TABLE 1. Environmental effect on development and sex ratio of *Rhizonema sequoiae*.

Environment	Number of months after inoculation	Number of nematodes	% females	% males	Ratio of males/females
Forest; average 9 C	8	11 (4th stage)	36.4	63.6	1.8
Growth chamber; constant 16 C	3–4	467 (adults)	30.6	69.3	2.3
Greenhouse; average 18 C	3–4	556 (adults)	11.8	88.1	7.4
Growth chamber; constant 25 C	3	10 (adults)		100.0	

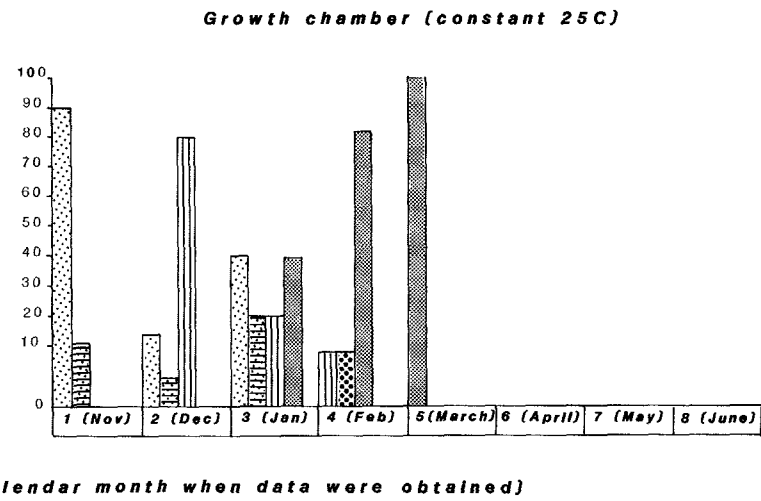
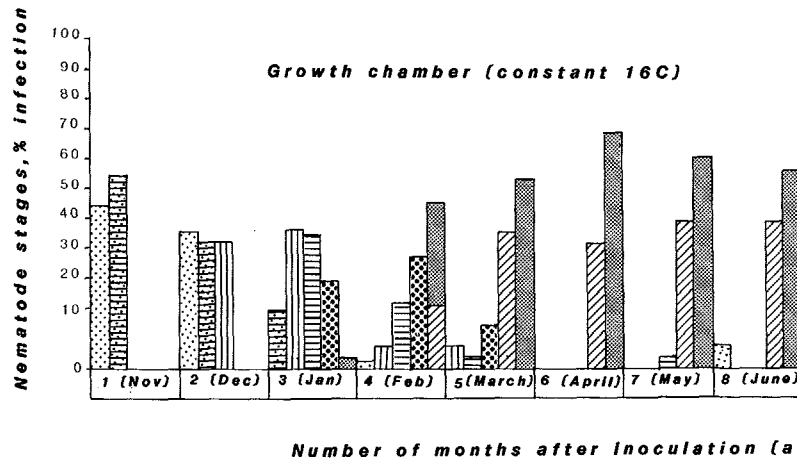
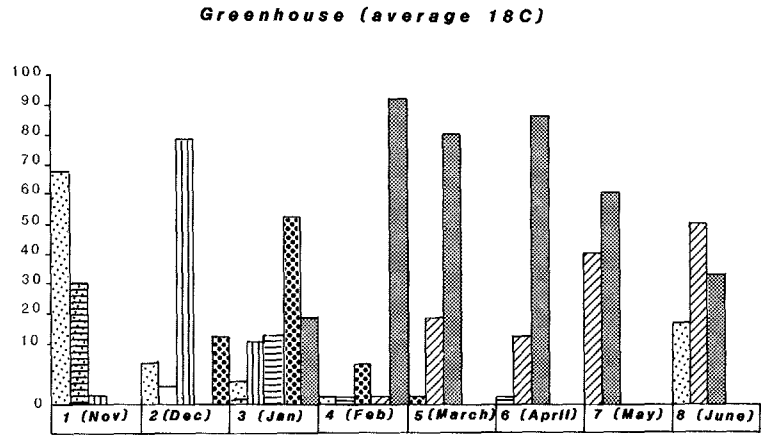
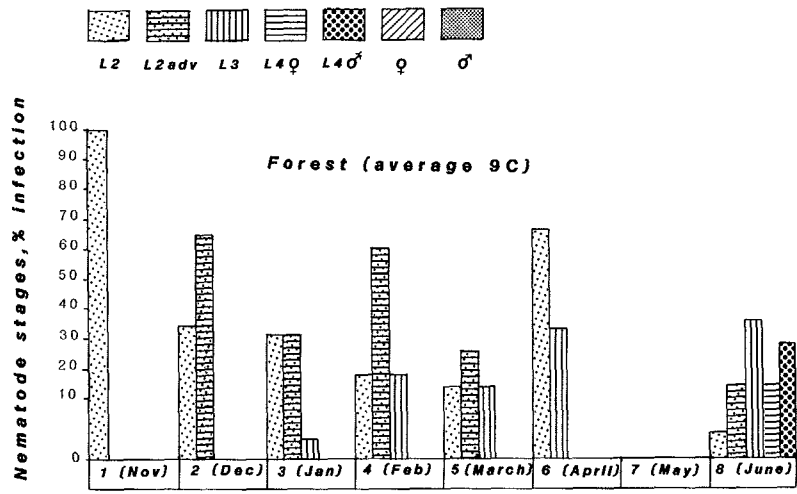


FIG. 1. Monthly percentage of different life stages of *Rhizonema sequoiae* recovered from roots of redwood seedlings grown in four different environments.

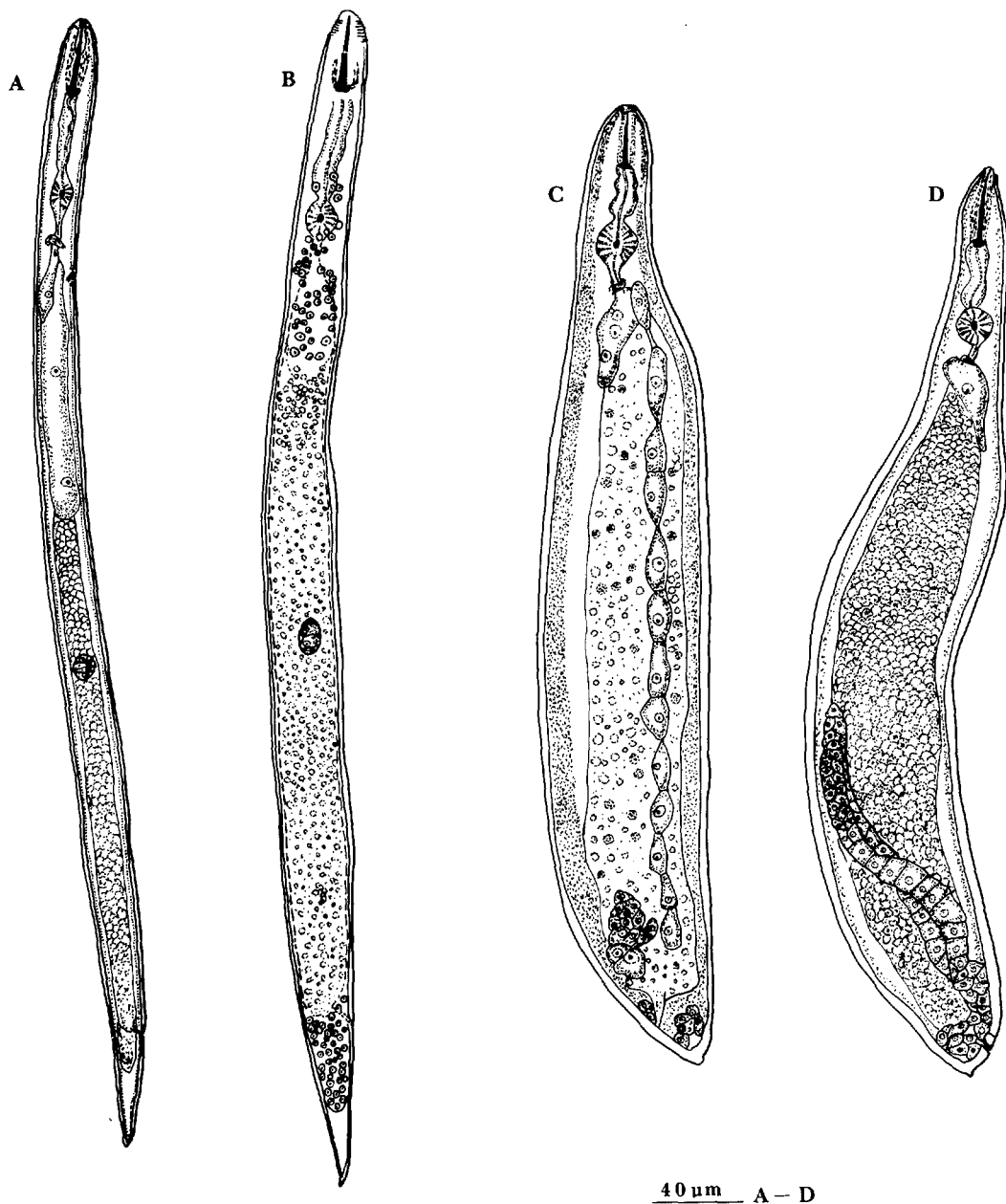


FIG. 2. Stages of *Rhizonema sequoiae*. A) Second-stage larva. B) Advanced second-stage larva molting. C) Third-stage female larva. D) Early fourth-stage female.

bial framework are not yet developed, but the slender stylet is visible. The posterior extremity of the body is formed, and several enlarged cells are present in the terminal area. The number of cells in the genital primordium in the early third-stage larva increases to approximately 10 cells, and the primordium is oval in shape. The

aggregation of mesenchymal cells in the esophageal region remains as described for the second-stage larva but is smaller in size than the aggregation posterior to the anus. At the second molt the tail is formed before the head. The rectum is difficult to see at this time.

Third-stage larval female: The diameter

TABLE 2. Dimensions (μm) of second-stage larvae of *Rhizonema sequoiae*, 1 month after inoculation.

	Range*	Mean	Standard error of the mean	Coefficient of variability (%)
Body length	447-657	534	9.2	8.1
Stylet length	29-37	35	0.5	6.2
Genital primordium: Length	10-20	15.4	0.7	17.9
Width	7.2-18.4	10.4	0.6	24.5
Hyaline tail area	27-39	33	1.2	12.8
Ratios				
a	17-35	22	1.0	20.1
b	2.2-4.3	2.9	0.3	22.4
c	7.1-11.7	9.7	12.0	0.3
Genital primordium: Length/width	0.6-1.9	1.5	21.1	0.1

* Based on measurements of 22 larvae.

of the body increases (Table 3; Fig. 2C), and the lip region is distinguished from the rest of the body by a constriction. The shape of the first lip annule as shown by SEM is rhomboid, like the adult (Fig. 3A). The stylet is slender and curved; its knobs are smaller than those of the second-stage larva. The median bulb is more robust and almost spherical in shape. The oval genital primordium has not yet reached the posterior extremity of the body cavity, ovaries are not yet elongated, and the cuticle is not

clearly annulated (Fig. 3B). In SEM, however, rings of punctations are evident around the body (Fig. 3C). The third-stage larva is fully developed approximately 2 months after inoculation of the roots.

The third molt was detected 3 months after inoculation. At this time the anterior and posterior extremities are contracted; some dense material is found in the anterior and posterior spaces between the new cuticle and the cast cuticle of the third stage. The anterior part of the stylet is molted,

TABLE 3. Dimensions (μm) of third-stage larval female and male *Rhizonema sequoiae* 2 months after inoculation of redwood roots.

	Range*	Mean	Standard error of the mean	Coefficient of variability (%)
Female				
Body: Length	451-537	476.6	9.0	6.2
Width	27.2-76.8	53.6	4.2	25.9
Spear	30.0-32.8	31.7	0.4	4.1
Median bulb: Length	14.4-21.6	19.1	0.9	13.6
Width	12.0-20.8	16.0	1.1	20.0
Genital primordium: Length	17.6-40.0	26.1	2.2	27.9
Width	12.0-20.8	15.6	0.8	17.9
Ratios				
a	5.7-15.8	9.4	0.8	28.5
c	17.9-32.6	24.1	1.3	18.2
Genital primordium: Length/width	1.3-1.9	1.7	0.1	24.8
Male				
Body: Length	418-487	449.7	8.3	5.9
Width	37.6-47.2	42.0	1.1	8.1
Spear	30.4-34.4	32.1	0.7	7.4
Median bulb: Length	16-20	16.6	0.4	7.5
Width	12.8-16.8	15.2	0.5	10.5
Ratios				
a	9.9-12.7	10.7	0.4	10.6
b	22.1-33.7	25.6	1.6	19.1
Genital primordium: Length/width	2.6-4.4	3.4	0.2	22.6

* Based on measurements of 10 larvae.

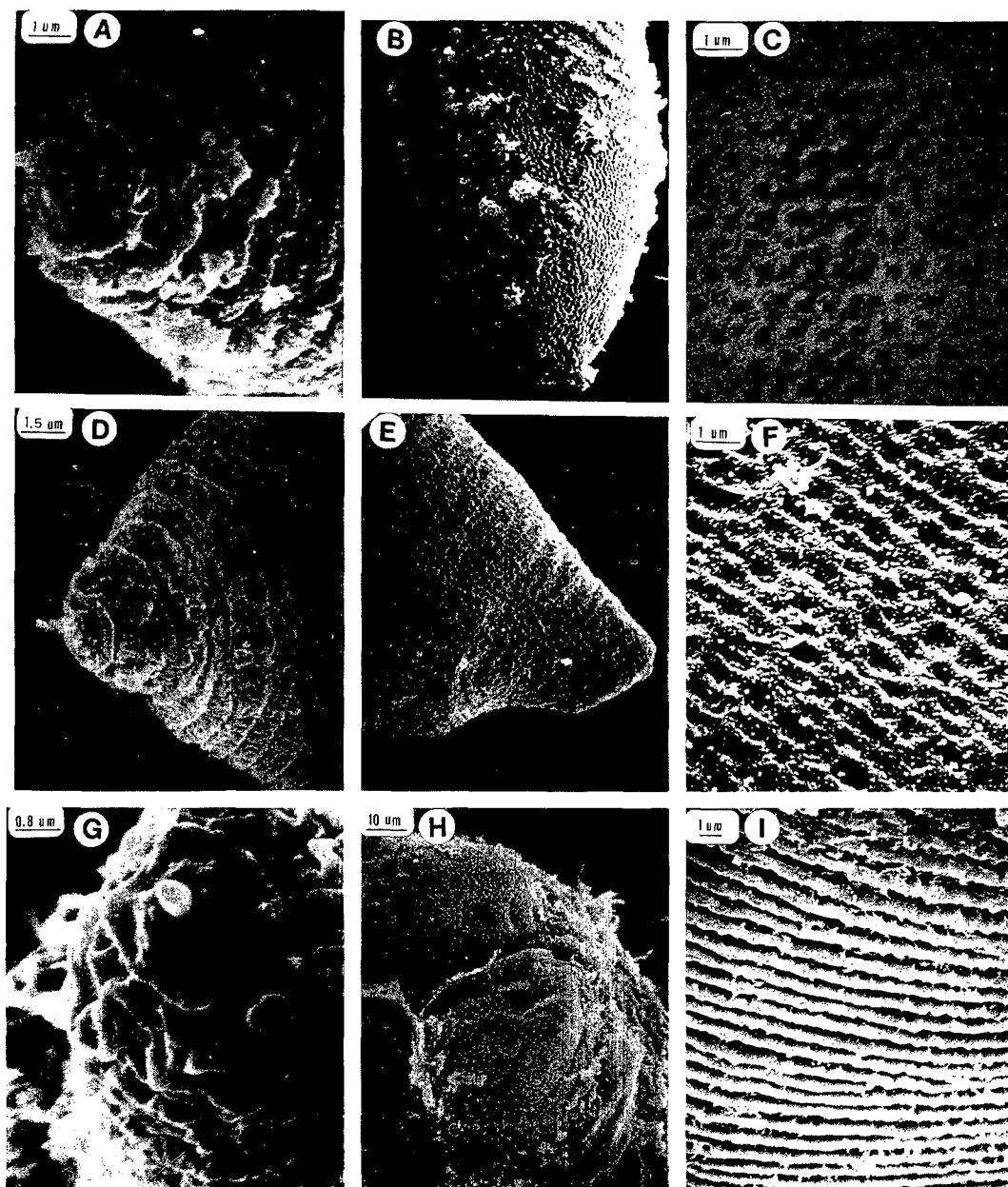


FIG. 3. Scanning electron micrographs of *Rhizonema sequoiae*. A-C) Third-stage female. A) Face view. B) Posterior part. C) Cuticular annules. D-E) Fourth-stage female. D) Face view. E) Posterior part. F) Cuticular annules. G-I) Female. G) Face view. H) Posterior part. I) Cuticular annules.

and a complete new stylet can be seen. No mesenchymal cells are present in the esophageal region. The genital primordium reaches the posterior extremity of the body cavity, and the two germinal zones of the ovaries occupy one-third of the body length. The rectum and future vulva are visible as light spots (Fig. 2D).

Third-stage larval male: The third-stage male is sausage shaped (Fig. 4A). There are approximately 10 distinct annules at the anterior end; there is no distinct body annulation. However, SEM revealed small cuticular punctations around the body (Fig. 3C). The labial region is separated from the rest of the body by a distinct constric-

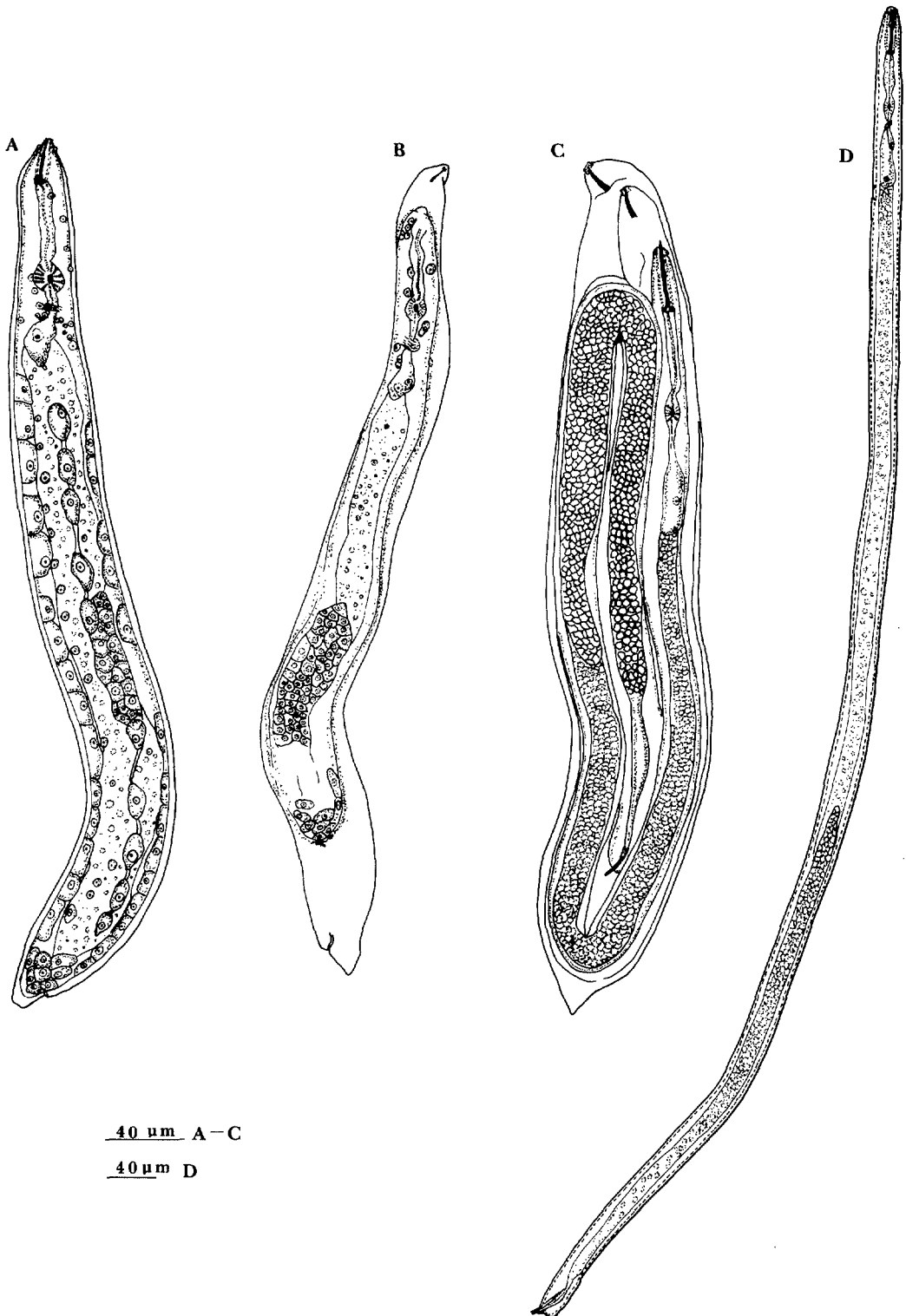


FIG. 4. Stages of *Rhizonema sequoiae*. A) Third-stage male larva. B) Early fourth-stage male. C) Fourth-stage male larva. D) Adult male.

tion. Under SEM the labial disc is circular in shape. The stylet is slender and curved like that of the female, and the median bulb is nearly spherical. The genital primordium is larger than that of the second-stage larva but has not yet reached the rectum. The third-stage male larva completed its development 2 months after inoculation of the redwood roots.

Comparison of the sexes in the third stage: In males the genital primordium is elongate and the ratio of length/width is approximately 3.4; in females the genital primordium is oblong (less elongate) and the ratio approximately 1.6 (Table 3). The rectum in both sexes is well developed. The differences between male and female rectums noted in the third-stage larva of *Globodera rostochiensis* by Chitwood and Buhner (1) were not observed in *R. sequoiae*. There are no differences between sexes in body length or width, esophageal median bulb, or stylet. In the hypodermis of both sexes there are four longitudinal series (two lateral, one dorsal, and one ventral) of conspicuous cells with evident nuclei that extend from the region of the esophageal median bulb to near the anus (Figs. 2C, 4A). Similar cells have been reported in *Heterodera schachtii* by Raski (6) and in *H. glycines* by Ichinohe (4), but only in the third-stage male larvae.

Fourth-stage female larva: At this stage the female became a little more swollen (Figs. 2D, 5A), with clear differentiation of the neck. The lip region is elevated, and the first annule behind the oral disc is rhomboid (Fig. 3D). The stylet is slender, the knobs have slight anterior projections,

and the esophageal bulb is almost spherical (Table 4). The rectum is conspicuous, and the tail is short. The future vulval aperture appears as a light spot, and the ovaries are in the process of elongation. In the advanced fourth stage (Fig. 5A) the presence of the oblong spermatheca is evident. The distance from the vulva to the anus is highly variable (Table 4). In SEM pictures the annulation of the cuticle is more evident than in the third-stage larva, and the punctation around the body continues to be present (Fig. 3E, F). The complete development of the fourth-stage juvenile female required 3 months after inoculation of redwood roots.

Fourth-stage male larva: Three months after inoculation some specimens show the first signs of the third molt; others are in an advanced fourth stage. Some males keep the third larval skin, but a few cast it. Different degrees of elongation are observed at this time. There is an accumulation of cells in the posterior extremity of the body, and a few cells are present in the esophageal area in the early fourth-stage specimens (Fig. 4B). Most fourth-stage males are folded two times, some three, and their heads, stylets, and esophagi are well differentiated. However, spicules and gubernaculum are not yet differentiated. The first indication of the fourth molt is detected 3 months after inoculation. The labial region is fairly well differentiated and the stylet more stout than in earlier stages. The body is folded two or three times; in some specimens the spicules and gubernaculum are still not present and the cloacal tubus is not visible. At 3 months some

TABLE 4. Dimensions (μm) of fourth-stage larval females *Rhizonema sequoiae* 3 months after inoculation of redwood roots.

	Range*	Mean	Standard error of the mean	Coefficient of variability (%)
Body: Length	408-513	464.6	20.5	9.9
Width	56.8-93.6	73.7	6.2	18.7
Stylet length	34-37	34.8	0.7	4.2
Median bulb: Length	16.8-19.2	18.2	0.4	5.5
Width	16-20	18.0	0.9	10.6
Anterior cuticle thickness	1.6-3.2	2.4	0.4	18.6
Posterior cuticle thickness	3.2-8.0	5.76	0.8	31.3
Distance: Embryonic vulva to anus	3.2-21.6	13.7	3.3	53.2
Ratio				
a	5.4-7.1	6.34	0.3	10.7

* Based on measurements of five larvae.

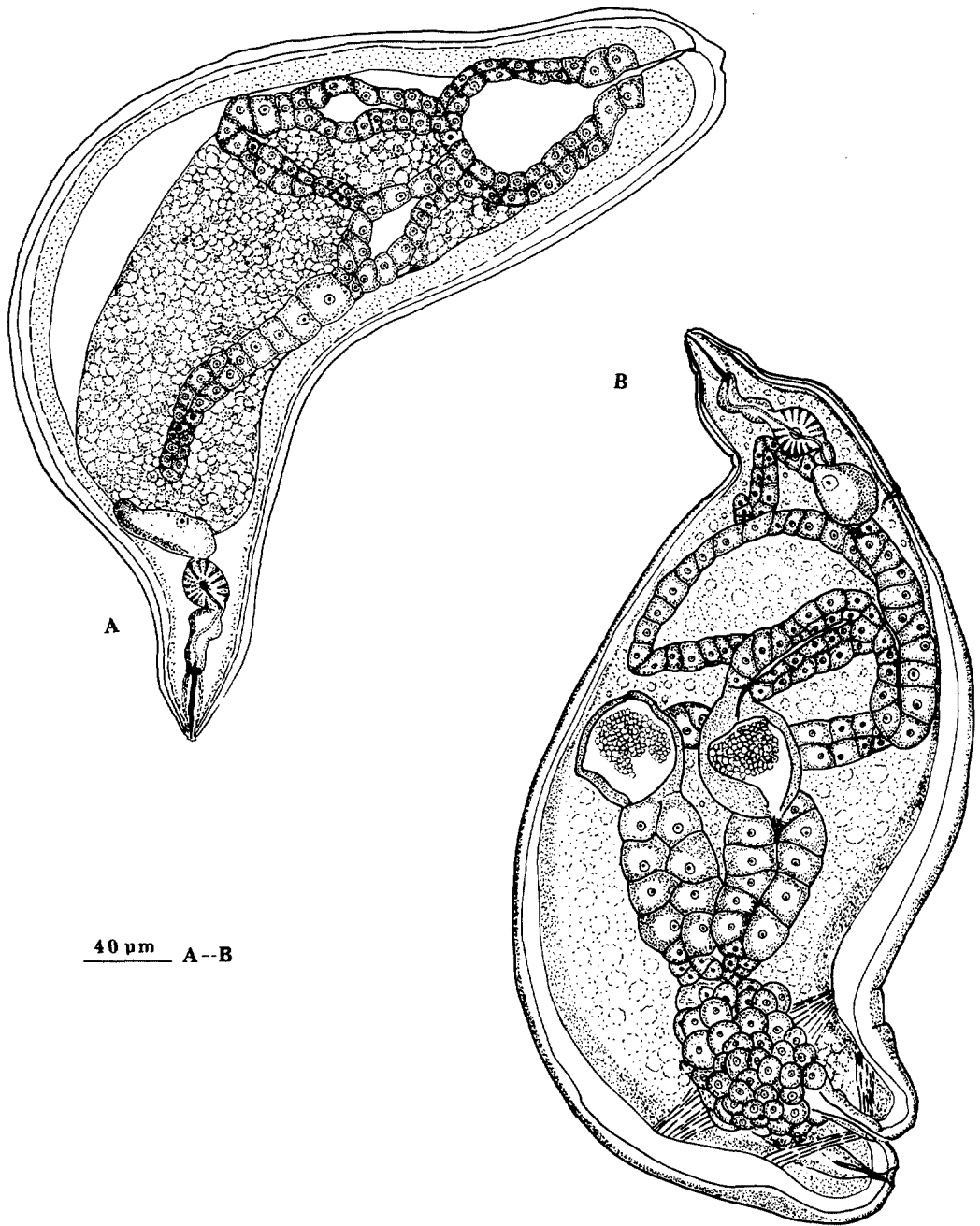


FIG. 5. Stages of *Rhizonema sequoiae*. A) Fourth-stage female. B) Adult female.

fully developed males are detected inside of the fourth-molt cuticle while still retaining the third-stage cuticle (Fig. 4C). When the third-stage male molts to the fourth stage, there is contraction of the posterior, and later the anterior, extremities of the nematode body within the cast skin of the

third-stage larva (Fig. 4B). The anterior part of the stylet is lost, and the genital primordium reaches the posterior extremity. This change occurred in some specimens 3 months after inoculation.

Adult female: Adult females (Fig. 5B) are swollen and sometimes lemon-shaped or

TABLE 5. Dimensions (μm) of adult females (without eggs) and males of *Rhizonema sequoiae* ($n = 10$).

	Range ^a	Mean	Standard error of the mean	Coefficient of variability (%)
Females				
Body: Length	442-603	513.9	15.6	10.0
Width	139-221	182.8	9.1	16.5
Spear	36.0-45.6	42.5	1.0	7.0
Median bulb: Length	16.8-24.8	22.8	0.9	12.7
Width	19.2-23.2	21.0	0.4	6.2
Spermatheca: Length	29.6-52.0	43.3	3.7	21.0
Width	25.6-43.2	34.5	2.3	16.5
Anterior cuticle thickness	1.6-4.8	3.4	0.3	32.4
Posterior cuticle thickness	8.8-15.2	11.0	0.7	20.9
Excretory pore	60-101.6	78.4	10.2	26.0
Neck	76.0-133.6	105.1	7.3	22.1
Distance: Vulva to anus	20.8-41.6	28.0	3.8	30.6
Males				
Body length	629-1,231	791.2	63.4	13.8
Spear	28.8-36.8	32.8	0.8	7.7
Median bulb: Length	8.8-15.2	11.3	0.9	25.7
Width	5.6-8.8	7.2	0.3	15.3
Testis length	218-531	344.1	36.9	33.9
Spicule length	24.8-33.6	29.8	0.9	9.1
Gubernaculum length	7.2-12.0	10.1	0.6	17.8

asymmetrical. Mature females are found completely inside of the roots feeding in the vascular tissue. They cannot be detected without dissection. The entire cuticle is annulated (Fig. 3I), with the exception of the vulval cone which is ornamented with a rugose pattern (Fig. 3H). In addition, there are crescent-shaped depressed areas on both sides of the vulval aperture (Fig. 3H). The neck is short and the labial region is elevated with a distinct rhomboid lip annule (Fig. 3G). The stylet is slender and bent; its knobs possess slight anterior projections. The median bulb is rounded and well developed. The excretory pore is located at the level of the posterior end of the esophageal glandular region. The ovaries have a varying number of flexures and almost reach the esophageal dorsal gland. The spermatheca is distinct and oval shaped (Fig. 5B). The uterus is a nonmuscular sac, connected with the spermatheca by the short columella. The large cells around the uterus, observed at the fourth molt, are still present. The vulva lips are strongly developed (Fig. 3H). The anus is located on the dorsal vulva lip 21-42 μm from the vulva aperture. Eggs are detected in the females 4 months after inoculation of redwood roots; between 4 and 6 months their number increases to 69. Within the same female, first-stage larvae are detected in

the middle part of the body and second-stage larvae can be seen in the posterior region. The first hatching of second-stage larvae inside the females is detected 8 months after inoculation of the roots. This indicates that only one generation per year is produced. Gelatin-like substance is not found with the females extracted with the blender; however, it is observed by dissection of intact roots. Eggs were never detected in this material. When females are placed in water, larvae begin hatching from the eggs and accumulating inside the female.

Adult male: The adult male is elongate and vermiform (Fig. 4D). It can be found in the soil or inside the roots. We do not know whether males are necessary for reproduction, but sperm has been observed in the spermatheca of young females (Fig. 5B). The lip region is set off from the rest of the body by a deep constriction and bears five annules. The labial disc has a low lateral profile and is circular in shape. The cuticle is annulated. There are four longitudinal incisures in the lateral field; this number is reduced to one in the tail region. The median bulb is less developed than that of the females (Table 5). The spicules are slightly arcuate, and the gubernaculum is a simple bar; a cloacal tubus is present.

Additional drawings and photographs of

R. sequoiae are provided with the description of the species (2).

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