[Jpn. J. Parasitol., Vol. 34, No. 6, 479-491, December, 1985]

Scanning Electron Microscopic Study on Developing Larvae of *Brugia pahangi* in the Vector Mosquito

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(Received for publication; April 17, 1985)

key words: scanning electron microscopy, ultrastructure, *Brugia pahangi*, larval stages, cuticle

Introduction

The detailed morphologies of larval stages of *B. pahangi* in the mosquitoes were described by Schacher (1962). Recently ultrastructural studies on the developing filarial larvae have been reported by many parasitologists including Rogers *et al.* (1974), Aoki and Katamine (1975), Aoki (1976), Lehane (1978), Tongu *et al.* (1978), Suguri *et al.* (1978), Vincent *et al.* (1978, 1979), Aoki *et al.* (1980), Franz and Zielke (1980) and Franz and Schulz-Key (1981). The surface architecture of *B. pahangi* however has not been viewed. The present study deals with the surface structure of the developing larvae of the following stages examined by SEM; the early first, the sausage or the late first, the second and the third stages.

Materials and Methods

Larvae of *B. pahangi* were obtained from experimentally infected mosquitoes, *Aedes aegypti* and *Armigeres subalbatus* which had fed on jirds, *Meriones unguiculatus*.

The mosquitoes were reared at 27°C and then dissected at 10 hr and subsequently every day from the second day to the twelfth day after the infection in order to obtain developing larvae. The dissections were done in Earle's balanced salt solution (Ash and Schacher, 1971) and larvae were fixed in 2% glutaraldehyde (phosphate buffered at pH 7.4) chilled with ice for 1.5 hr, washed with the phosphate buffer and then post-fixed with 1% osmium tetroxide (phosphate buffered at pH 7.4) for 2 hr, at 4°C. The fixed materials were dehydrated through a series of graded ethanol solution, transferred into isoamyl acetate and finally dried in a critical point dryer using liquid carbon dioxide. The dried specimens were mounted on stubs, sputtered with gold, and examined with a Hitachi S-550 scanning electron microscope.

Results

First-stage larva

The microfilaria taken into the vector with the blood meal loses its sheath and migrates to the thoracic muscles. During the first 48 hr the larva becomes shorter and thicker until it reaches a minimal length of 131 μm on the average. The basic structure of the larva is similar to that of the microfilaria by the time larvae attain to the minimal length; at this time it has assumed the so-called sausage shape (Fig. 2). By the third day, the sausage-shaped larva has grown in length and by the fourth day it has become 199 μm long (Table 1). The first molt takes place five days after the infection.

A cephalic disk is situated on the anterior end and its size, varying between 1.5 and

This study was supported by a Grant-in-Aid for the Japan-United States Cooperative Medical Science Program.

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Table 1 Development of *Brugia pahangi* in *Armigeres subalbatus*

Time after infection	Stage of development	Length (μm)	Diameter (μm)
10 hours	early first stage	194.5 ± 5.5	3.6 ± 0.1
24 hours	early first stage	140.0 ± 22.0	6.4 ± 0.3
2 days	early first stage	134.5 ± 13.8	10.1 ± 1.0
	sausage stage	130.9 ± 10.2	15.6 ± 1.2
3 days	sausage stage	185.1 ± 44.1	19.6 ± 2.9
4 days	sausage stage	199.1 ± 11.3	18.3 ± 1.9
6 days	2nd stage	671.2 ± 163.5	21.6 ± 2.0
8 days	3 rd stage	1118.2 ± 215.0	20.0 ± 1.5
12 days	3 rd stage	1260.3 ± 36.6	17.5 ± 2.3

(Measurement of 10 larvae each, expressed as the average±SD)

2.0 μm in diameter, does not change much during the first-stage. The cephalic disk consists of three segments (Fig. 3). The anterior segment has a hook on its left side and two openings on the front surface. The length of the hook ranged between 0.79 and 0.97 μm. The right opening is one of the amphidial openings and the left one is the mouth. The shape of the former structure is elliptical (0.35 by 0.2 μm) and the latter one is round (about 0.16 μm in diameter). The middle segment has no special structures on its surface. The posterior segment has three spines on the right side and their length varied from 0.4 to 0.5 μm (Fig. 4).

The excretory pore of the early first-stage larvae was not seen in the present study, but that of the sausage-stage larva was situated at anterior 1/4 of the worm and its shape is round or elliptical (about 1.5 μm in diameter) (Fig. 5).

The cuticular annulations of the early first-stage larvae are clear. The breadth of the annulations at the midbody was 0.4 (±0.035) μm in a 10-hour larva and they are reduced to 0.29 (±0.07) μm in a 24-hour larva (Figs. 6 and 7). In the sausage-stage larvae, the annulations can't be observed except on the tail surface of the worm, while cuticular wrinkles are observed on the anterior or posterior parts of the body.

The anal pore is oval shaped in the early first-stage larva (about 0.1 by 0.2 μm in a 10-hour larva). The rectal plug appears when the larva develops to the sausage-stage and it shows oval shape (about 8.3 by 11.1 μm) (Fig. 8). The size of the rectal plug does not change very much while the larva stay as the sausage stage but its thickness increases with the development. On the surface of the rectal plug there are many cuticular wrinkles running transversely.

The tail is tapered and has a terminal appendage which is characteristic of the genus *Brugia* when larvae are in the first stage. The appendage has a club-shaped appearance. The terminal appendage decreases in length, from 21 to 12 μm, and increases in width at its basal part, from 1.2 to 2.3 μm, during the first-stage larval development. The annulations of the cuticle on the terminal appendage also become deeper and more prominent as the development proceeds. The number of annulations on the broad basal part of the terminal appendage is 20 to 28 and that on the slender apical part is 16 to 18 (Fig. 9).

Second-stage larva

Variations are marked in the second-stage larvae ; the length of 6-day old larvae ranged from 438 to 998 μm (671.2±163.5 μm). The second molt took place on the sixth or seventh day after infection. The second-stage larva is long and slender (Fig. 10). The second-stage larva increases in length, but changeless in width (Table 1).

The cephalic disk is lost at the beginning of the first molt. The second-stage larva has an oral opening on the anterior end and its

shape is oval or round (1.1 to 1.3 μm by 0.9 to 1.1 μm) (Fig. 11). Two papillary protuberances exist on the lateral sides of the mouth and slant foward the mouth opening. The size of each protuberance is 0.65 μm in height and 0.6 μm in diameter.

The annulations of the cuticle and the lateral line in the second-stage larvae were invisible.

The rectal plug still exists and its size (9.9 by 11 μm) and the appearance is similar to that of the first-stage larva (Fig. 13).

The tail is short and ends in spikelike tip (Fig. 14). The tip length (about 4.3 μm) is shorter than the terminal appendage of the first-stage larva.

The second molt takes place on six or seven days of the infection. At the end of the molt, the esophageal cuticle of the second-stage larva is shed (Fig. 16).

Third-stage larva

The length of the larva at this stage increases continuously after the second molt, while the thickness of the larva is rather changeless (Table 1).

The mouth opening is flattened dorso-ventrally and the length is about 1.6 μm (Fig. 17). The buccal area surrounding the mouth opening is rather flat and smooth. Four inner and 4 outer papillae are present on the anterior end. The inner papillae are situated around the buccal area at dorso-lateral and ventro-lateral positions, 2.5 μm from the tip of the anterior end. The inner papilla is composed of a basal part (0.52 by 1.2 μm) and a nipple-like protuberance (0.5 μm in diameter). The outer papillae, 1.3 μm in diameter and 0.6 μm in height, are also situated at dorso-lateral and dorso-ventral positions, 2.5 μm posterior from each inner papilla. The outer papilla is surrounded by a groove at its base. When the larvae developed to the late second-stage larvae, the cephalic papillae of the third-stage larva covered by old cuticle were observed (Fig. 12). An amphid opens at each lateral side, and they are located slightly posterior to the inner papillar level. The opening is elongated dorso-ventrally (1.35 by 0.06 μm).

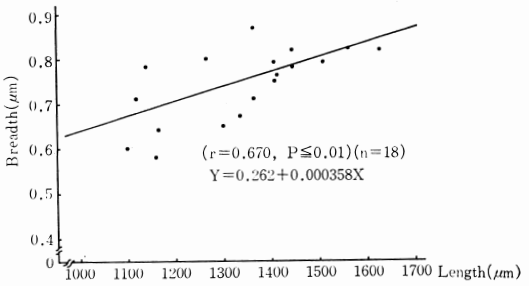

Fig. 1 Correlation between worm length and the breadth of annulations measured at the anterior 1/3 point.

The annulations of the cuticle are very prominent. The breadth of the annuli and the depth of grooves increases with the development (Figs. 18 and 19). There is a correlation between worm length and the breadth of annuli at a position 1/3 the distance from the anterior end ($r=0.670$, $p \leqq 0.01$) (Fig. 1), but no correlation between the length and the breadth at a position 1/3 the distance from the tail end ($r=0.220$, $p \geqq 0.005$). The breadth decreases as one nears the anterior end or the tip of the tail.

The lateral line is also prominent in this stage (about 3.0 μm in width). It is recognized as the interruption of the transverse annulations of the cuticle on the lateral sides of the worm (Figs. 18 and 19). Longitudinal folds, about 0.29 μm in width are observed all over the worm surface (Fig. 21).

The anus is situated at about 37 μm from the posterior end in the 8-day-old larva, and this distance increases with larval development and reaches approximately to 51 μm in a 12-day-old larva. The anus is crescent-shaped and it has a prominent anterior border (0.7 to 0.8 μm in thickness) (Fig. 20). The anal opening is about 5.5 μm in length and 0.8 μm in width on the average and its caudal side is connected to the anal field which is about 5 μm by 7.5 μm in size and has many longitudinal folds directed to the anal opening.

A papilla (Pa) is seen only on the left lateral side of the worm (Fig. 21). The papilla is about 1.3 μm in diameter and has an encirclement of about 0.67 μm in width. It is sit-

uated at a position from the level of the anal pore to about 20 μm posterior to it.

On the tail end, three caudal papilliform processes can be seen (Fig. 21). These processes are surrounded by a groove at their base and the diameter of these processes is about 2.8 μm. The shape of the lateral processes is hemispherical and that of the terminal one is conical. In almost all the larvae, the terminal process is a little off-center. The caudal papillae of the third-stage larva are already visible under the old cuticle of the late second-stage larva (Fig. 15). The phasmids open in the caudal region immediately anterior to the lateral papillae. The opening is lined by the elevation of cuticle which is elongated (1.8 μm in length) dorso-ventrally.

Discussion

The development of larvae in the vector mosquito is roughly synchronized in the first stage but differs considerably between individuals in the second and the third stages. Larvae with various lengths were observed simultaneously in the same mosquitoes after the fifth day of the infection. In the present study the first molt occurred on day 5 and the second one, on days 6 or 7 of infection in *Armigeres subalbatus*. Schacher (1962) reported that the first molt of this species occurred between 4 and 5 days and the second molt at about 8 days in *Aedes aegypti*. The discrepancy may be attributed to the differences in the vector mosquitoes used and the rearing temperatures of the mosquitoes. The diameter of larvae was the biggest in the second stage and it decreased slightly with larval development to the third stage. This phenomenon was reported by Kobayashi (1940) in *W. bancrofti* and Schacher (1962) in *B. pahangi*.

The structure of the cephalic disk in the first-stage larvae was basically the same as seen in *W. bancrofti* (Franz and Zielke, 1980) and in the microfilariae of *B. malayi*, *B. pahangi* (Aoki and Katamine, 1975) and *D. immitis* (Aoki *et al.*, 1976). In the first-stage larva of *W. bancrofti*, the right amphidial opening is situated at the front surface of the cephalic disk and the opening is surrounded by a prominent border. In our study on *B. pahangi*, this border was not observed. The location of the other amphidial opening is at the posterior base of the hook in the microfilariae of *B. pahangi* (Suguri, 1977), *B. malayi* (Tongu, 1974) and other species (McLaren, 1972) as seen by TEM observation.

The length of the hook in microfilariae of *B. malayi* and *B. pahangi* was 0.7 to 1.1 μm (Aoki *et al.*, 1976) and that of *W. bancrofti* was 0.5 μm. In the present study it was 0.79 to 0.97 μm in the first-stage larva, so that the size of hook did not differ between microfilaria and the first-stage larva. The size of spines in the microfilaria of *B. malayi* and *B. pahangi* (Aoki *et al.*, 1976) was 0.6 to 0.7 μm and 0.4 μm in the first-stage larvae of *W. bancrofti* (Franz and Zielke, 1980). In the present study the size of spines of the first-stage larva was 0.4 to 0.5 μm. From these results it appears that the sizes of the cephalic structures during early larval development do not show remarkable change in comparison with other portions of the body. Laurence and Simpson (1968) found three spines in *B. pahangi* four in *W. bancrofti* and up to eight in *Loa loa* by light microscopy. In the present study only one first-stage larva demonstrated on the fourth segument of the cephalic disk a single spine ; all the other larvae lacked this spine.

In the second-stage larva the oral opening becomes large (0.9 to 1.3 μm in diameter) and it is almost the same size as that of *W. bancrofti* (0.8 to 1.5 μm, Franz and Zielke, 1980). Two papillary protuberances existing at the lateral sides of the oral opening were also reported in the second-stage larva of *W. bancrofti* (Franz and Zielke, 1980) but not in *O. volvulus* (Franz and Schulz-Key, 1981). This structure is thought to be the characteristic of the second-stage larva of genera of *Brugia* and *Wuchereria*. Tongu *et al.* (1978) reported the existence of an amphid in a five-day larva (the second-stage) by TEM but in the present study and that of Franz and Zielke (1980) by SEM the amphidial openings could not be detected.

In third-stage larvae the mouth was slitlike

and the 4-2-4 configuration, 4 inner papillae, 2 amphidial openings and 4 outer papillae, was seen as well as those reported in *D. immitis* (Hendrix *et al.*, 1984) and *W. bancrofti* (Franz and Zielke, 1980). However, the shape of the nipple-like protuberance of the inner papilla in *B. pahangi* was more pointed than that in *D. immitis* and less pointed than that in *W. bancrofti*. Hendrix *et al.* (1984) previously observed variations in the total number of inner and mid-position papillae surrounding the oral opening, ranging from three to six papillae, but in the present study the number was constant.

The excretory pore of the sausage-stage larva observed in the present study (about 1.5 μm in diameter) was much larger than that in the microfilaria of *D. immitis* (about 0.2 μm in diameter) and it was situated at nearly the 83rd annulus from the anterior end (Aoki and Katamine, 1975). The excretory pores in the microfilariae of *B. pahangi* and *B. malayi* (about 0.3 μm in diameter) are situated around the 150 th annulus from the anterior end (Aoki *et al.*, 1976). The excretory pore in the larvae of other stages was not be observed in the present study.

The cuticular annulations of the early first-stage larva and those of the third-stage larva were prominent. The breadth of the former was reduced as larval development proceeded and that of the latter enlarged. A correlation between the worm length and the breadth of annuli at a position 1/3 the length of the worm from the anterior end is thought to reflect the constant growth at that position. Franz and Schulz-Key (1981) and Franz and Zielke (1980) observed the cuticular annulations of the second-stage larvae of *O. volvulus* and *W. bancrofti* respectively but the annulations are flatter and smaller. In the present study clear cuticular annulations could not be observed. Tongu *et al.* (1978) and Lehane (1978) also could not detect by TEM cuticular annulations in the sausage stage or the second stage.

The cuticle of the microfilariae of *B. malayi* (Tongu, 1974) and *B. pahangi* (Suguri, 1977) consists of three layers but the layering of the cuticle is poor during larval morphogenesis. Between 2 and 5 days the cuticle consists of only a thin fibrous layer and the 7-day-old larva is covered only by a membrane-like structure (Tongu *et al.*, 1978 and Lehane, 1978). In the third stage, the cuticle is composed of three layers *i. e.* cortical, matrix, and basal layers (Rogers *et al.*, 1974). The longitudinal folds, as observed in *W. bancrofti* by Franz and Zielke (1980) which have been thought to be characteristic of the infective larvae, appeared in old third-stage larvae in the present study.

The anal pore of early first-stage larvae was very small compared to that seen in microfilariae of *D. immitis* (0.4 to 0.5 μm) by Aoki and Katamine (1975). The rectal plug exists in the sausage-shaped and the second-stage larvae. The anus of the third-stage larva is similar to that in *W. bancrofti* in shape and size (Franz and Zielke, 1980).

A papilla situated at the level of the anus or posterior to that was observed only on the left side of the third-stage larva. This papilla was not reported in *W. bancrofti* by Franz and Zielke (1980) but was present in our observation on the same species (unpublished). In *D. immitis*, however, Hendrix *et al.* (1984) could not observe it.

In the first-stage the tail has an appendage which is characteristic of the genus *Brugia*. In the second-stage, the spinelike tip is small and it is shorter than the terminal appendage in the first-stage larva. In the third-stage, there are three papilliform processes. Their sizes are almost the same as those of *W. bancrofti* (2.5 μm in diameter) but the shape of the lateral processes is hemispherical and the terminal one is conical in the present study whereas the caudal papillae in *W. bancrofti* are bubble-like (Franz and Zielke, 1980 and Yen *et al.* 1982). The phasmids open immediately anterior to the lateral papillae. This has been confirmed by TEM observation (Suguri *et al.*, 1978).

Summary

The surface structures of *B. pahangi* larvae in the first, second and third stages in the

vector were observed with a scanning electron microscope.

The cephalic disk is situated on the anterior end of the first-stage larva and consists of three segments. The anterior segment has a hook on its left side, the right amphidial opening and the oral opening on its front surface. The posterior segment has three spines on its right side. The second-stage larva has a rounded mouth opening and two papillary protuberances at the lateral sides of the mouth. On the third-stage larva, around the dorso-ventrally flattened mouth opening, the four inner papillae, the two amphidial openings and the four outer papillae are arranged in a 4-2-4 configuration. The excretory pore of the sausage-shaped larva is 1.5 μm in diameter. The cuticular annulations are clear in the early first-stage but clear annulations of the cuticle could not be observed in the sausage and the second stages except on the tail surface. The third-stage larva has prominent cuticular annulations. There was a correlation between the third-stage larval length and the breadth of annuli at a position 1/3 the distance from the anterior end. The lateral line is observed as an interruption of the cuticular annulations. Longitudinal folds are observed on the surface of all over the third-stage larvae. The anal pore is observed in the early first-stage and the rectal plug in the sausage and the second stages. The anal opening of the third-stage larva is crescent-shaped. A papilla is seen only on the left lateral side at the level of the anus or posterior to it. On the tail end, a terminal appendage exists in the first-stage larvae, a spine-like tip in the second-stage larvae and two hemispherical lateral processes and one conical terminal process are observed in the third-stage larva. The phasmids open adjacent anteriorly to the lateral papillae.

Acknowledgements

The authors wish to thank Professors Hisashi Yamamoto, School of Medicine of Dokkyo University, and Yoshiki Aoki, Institute for Tropical Medicine, Nagasaki University, for offering us the jirds infected with *Brugia pahangi*. The authors also wish to thank Messrs Kazuo Itano and Max Lent for technical assistance.

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蚊体内における *Brugia pahangi* 幼虫期の走査電子顕微鏡による観察

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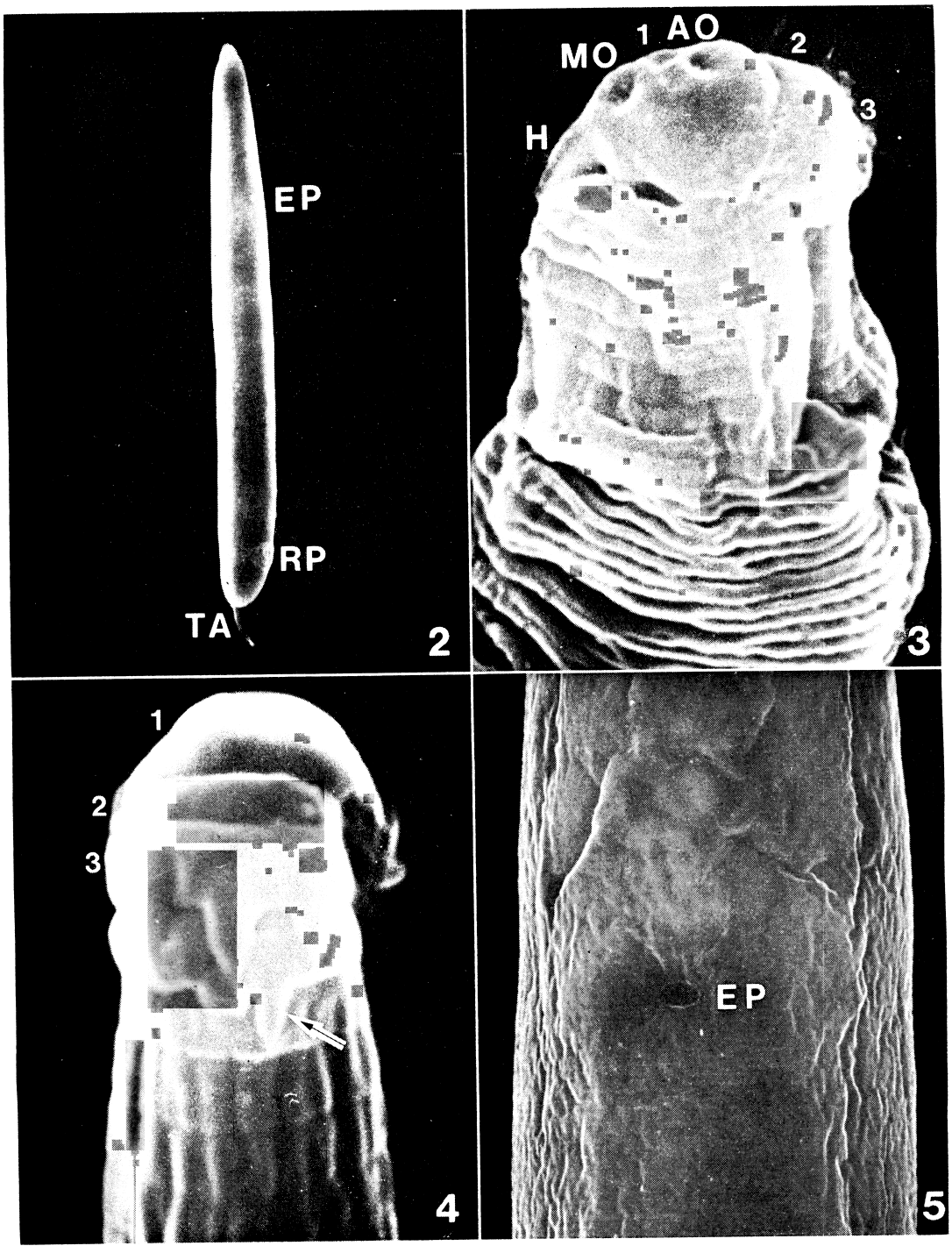
Brugia pahangi の蚊体内における第1期，2期，3期幼虫の表面構造を走査電子顕微鏡にて観察した．

Cephalic disk は1期幼虫の前端にあり，3つの segment より成っている．前部の segment はその左端に1本の hook と前面に右側 amphid の開口部と口を有しており，後部の segment は3本の spine をその右端に有している．第2期幼虫は円形の口とその両側に乳頭状の突起を有している．また第3期幼虫では口は背腹方向に延びており，その口の周囲に4個内側乳頭と2つの amphid の開口部，4個の外側乳頭が 4-2-4 の配列をなしている．ソーセージ期幼虫の排泄口は直径 1.5 μm であった．角皮の輪状溝は早期の第1期幼虫でははっきりとしているが，ソーセージ期及び第2期幼虫においては尾部の表面を除いては観察されなく，第3期幼虫では再びはっきりとした輪状溝が見られる．第3期幼虫において虫体の長さと虫体の前端より体長の 1/3 の部位の輪状溝の間の幅との間に相関関係がみられた．第3期幼虫において輪状溝は側線の部分では途切れていた，虫体の長軸方向に走るひだが第3期幼虫の全表面に観察される．肛門口は早期の第1期幼虫に見られ，anal plug はソーセージ期及び第2期幼虫期において見られる．第3期幼虫の肛門口は三日月形をしており，肛門付近又はそれよりやや後部の左体側に位置する乳頭が存在する．第1期幼虫の尾部は二段構造になっているが，第2期幼虫のそれは小さく特別な構造がない．第3期幼虫の尾端には2つの半球形の突起が両体側部に又，1つの円錐形の突起が後端に見られる．phasmid はこの両側部突起の前端側の付け根に開口する．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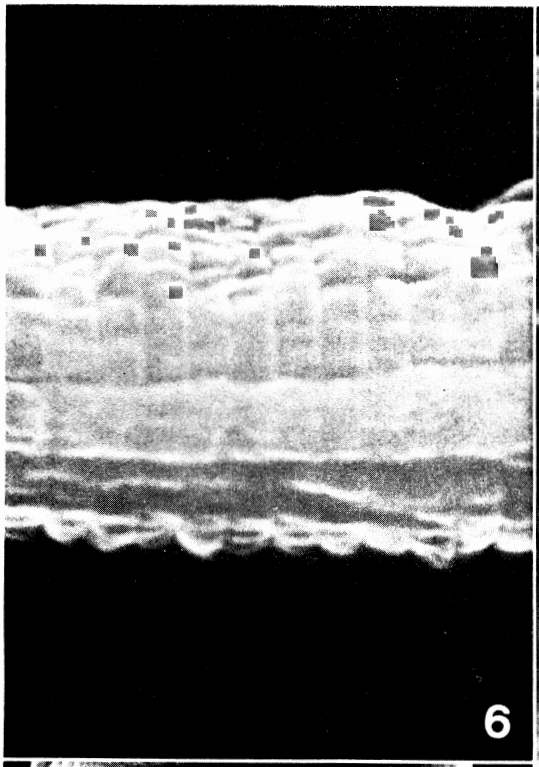
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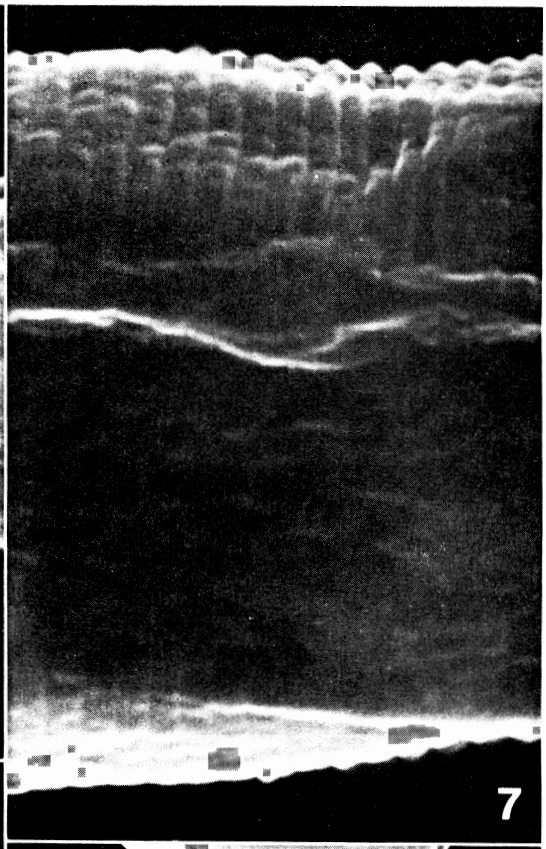
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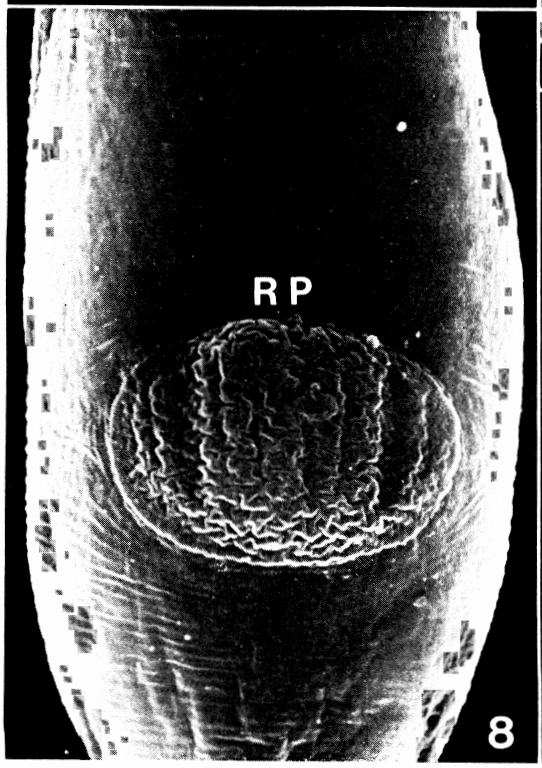
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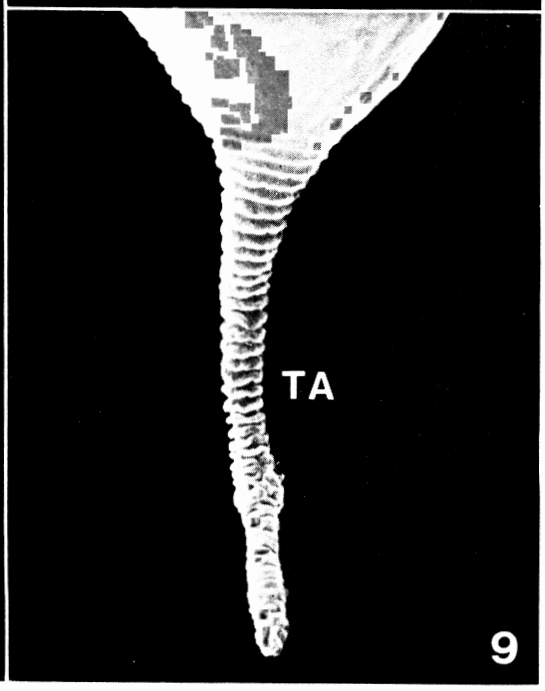
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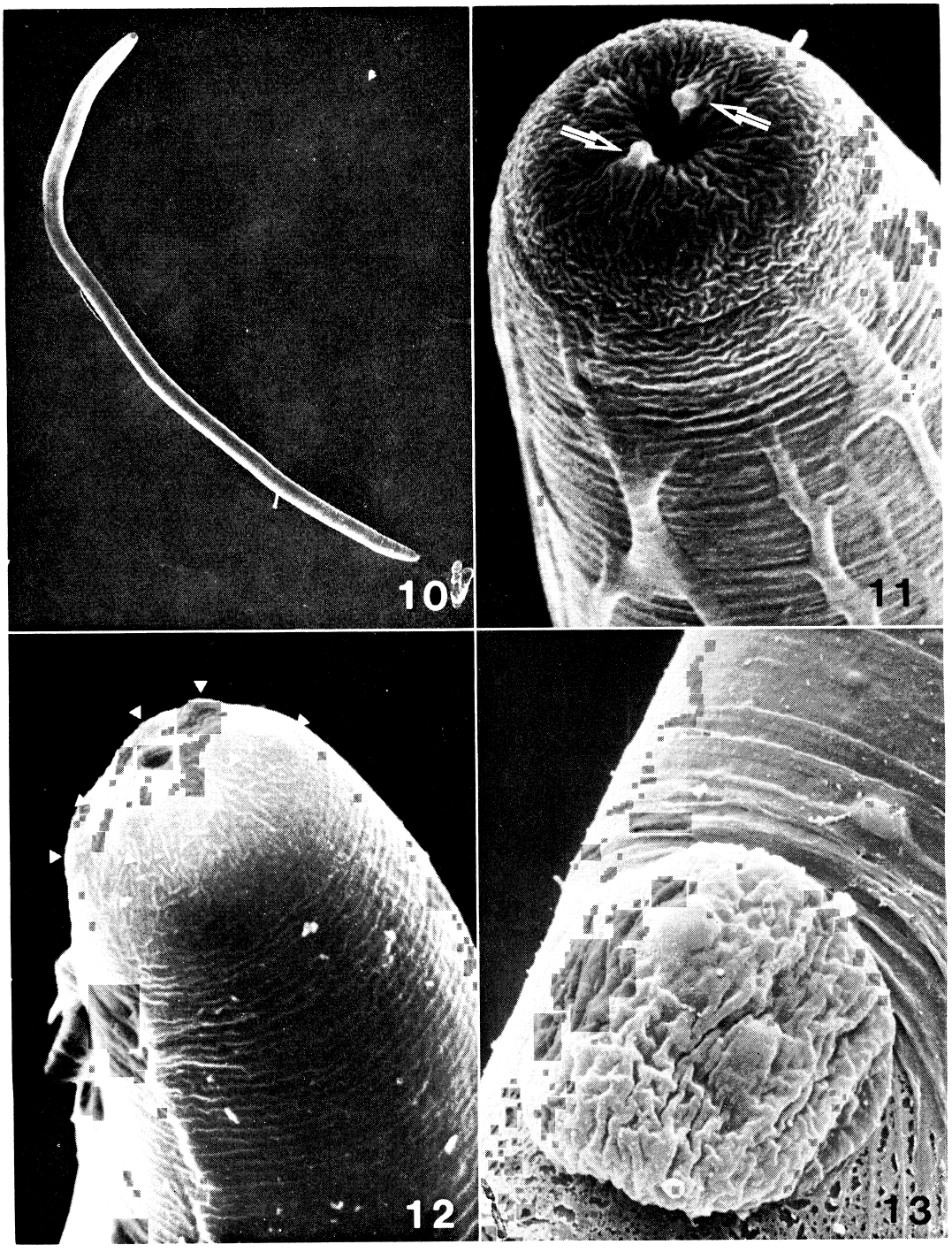
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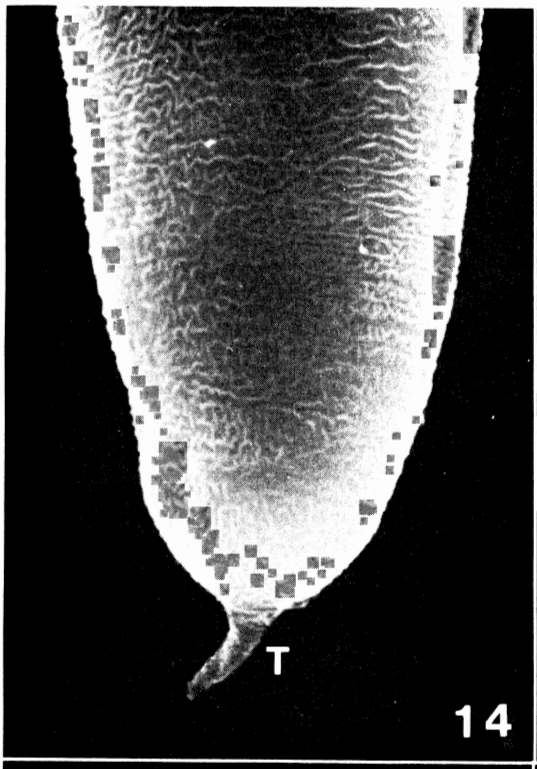
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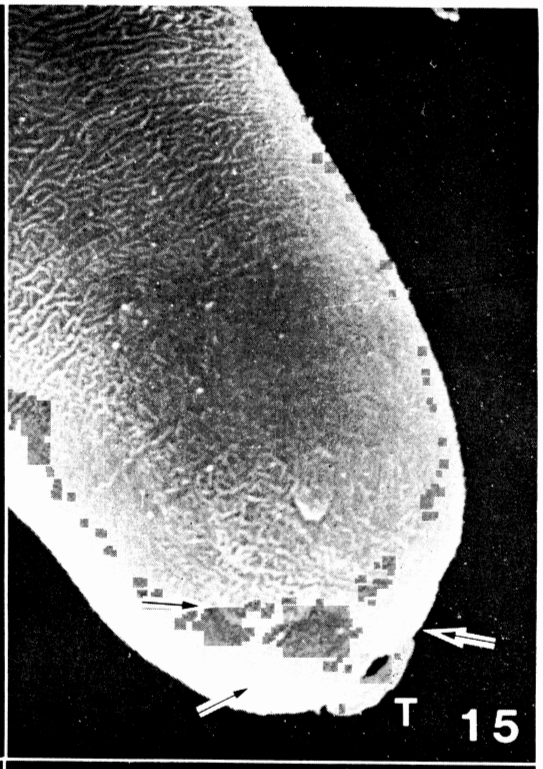
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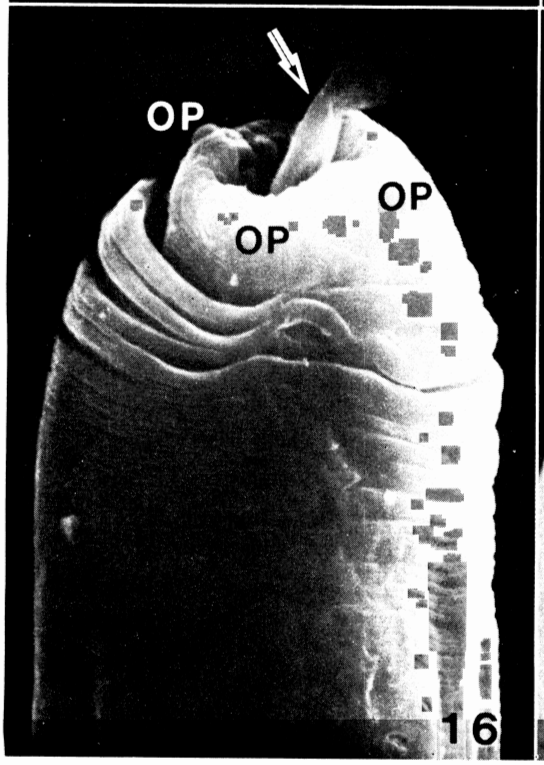
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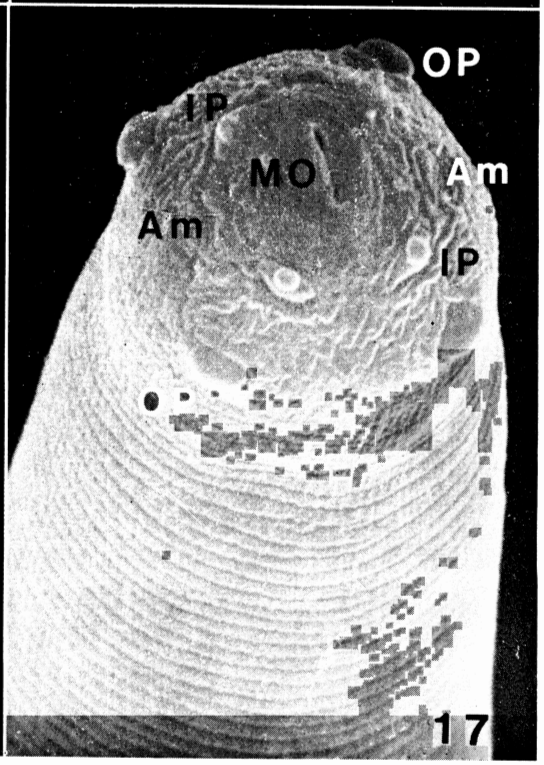
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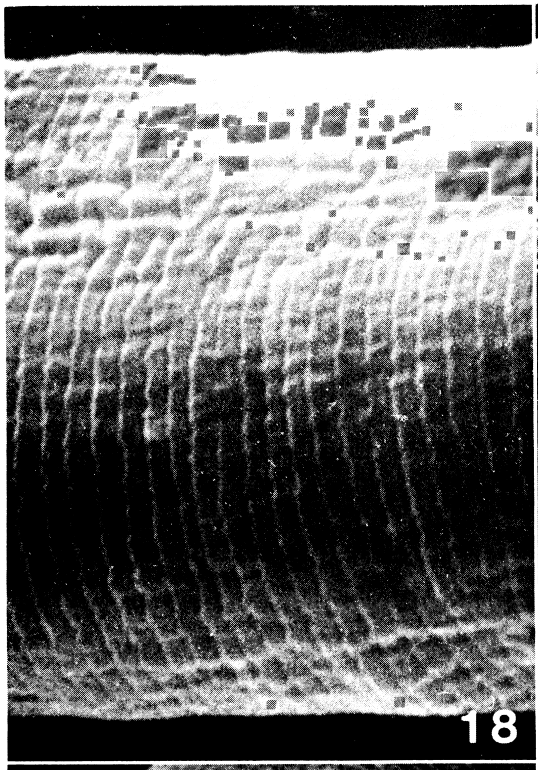
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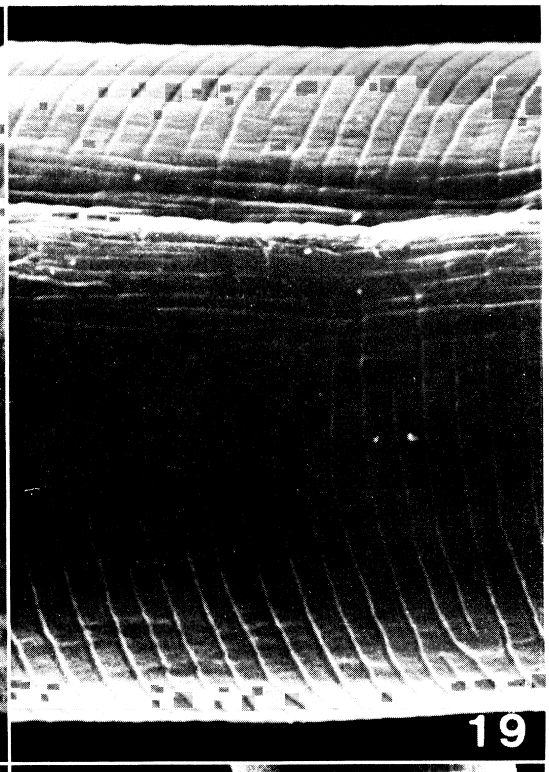
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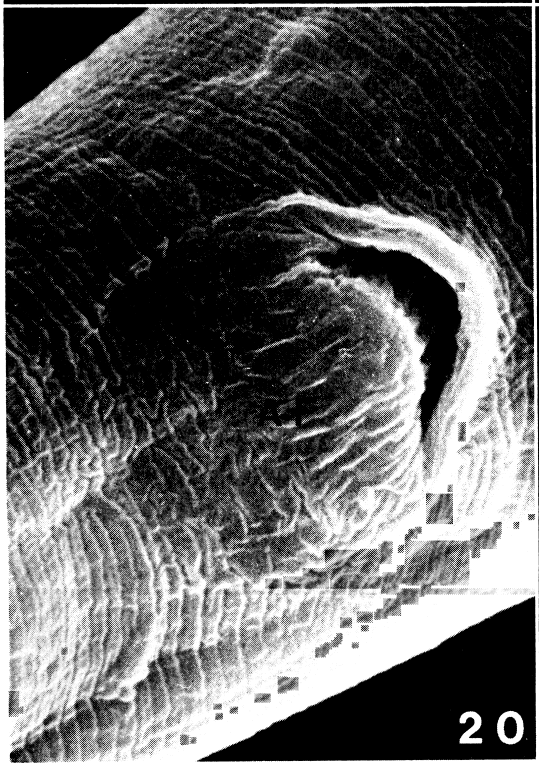
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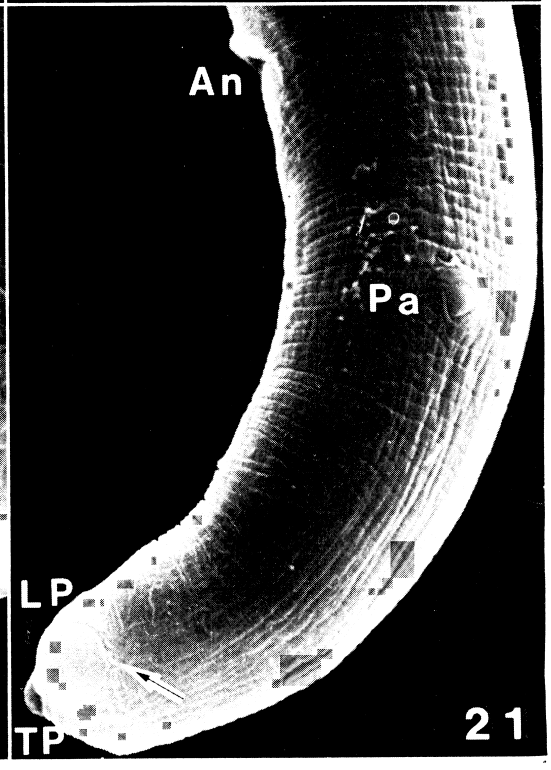
An
Pa
LP
TP
21

Fig. 2 Four-day-old sausage-shaped larva. Note the terminal appendage (TA), the rectal plug (RP) and the excretory pore (EP). ×480.

Fig. 3 The anterior end of a 24-hour-old larva. The cephalic disc is composed of three segments. anterior (1), middle (2) and posterior segments (3). The anterior segment bears a hook (H), the amphidial opening (AO) and the mouth opening (MO). ×15,700.

Fig. 4 Right lateral veiw of the anterior end of 10-hour-old larva. The middle segment (2) of the cephalic disk shows a smooth surface lacking any structures. The posterior segment shows three spines. This particular larva had an unusual single spine (arrow) on the fourth segment. ×2,160.

Fig. 5 The excretory pore (EP) of 4-day-old larva. The shape is elliptical and its size in this figure is 1.4 by 1.0 μm. ×3,600.

Fig. 6 Cuticular surface of 10-hour-old larva. The breadth of annuli is 0.35 μm. ×15,600.

Fig. 7 Cuticular surface of 24-hour-old larva. The breadth of annuli is 0.32 μm. ×15,600.

Fig. 8 The rectal plug (RP) of 4-day-old larva. The shape is elliptical and many wrinkels of the cuticle run longitudinally on its surface. ×3,700.

Fig. 9 Terminal appendage (TA) of 4-day-old larva. The annulations are prominent on the appendage of the tail. ×5,280.

Fig. 10 Seven-day-old larva. The shape is long, about 700 μm, and slender. ×150.

Fig. 11 Anterior end of 7-day-old larva. The shape of the mouth is round and two papillary protuberances (arrows) are seen on both sides of the mouth. ×6,750.

Fig. 12 Anterior end of 7-day-old larva. The cephalic papillae (four inner and four outer papillae) of the third-stage larva are visible under the old cuticle (arrowheads). ×4,720.

Fig. 13 Rectal plug of 7-day-old larva. The size is 9.9 by 11 μm and the extensive wrinkling of the cuticle is visible. ×4,500.

Fig. 14 Posterior end of 6-day-old larva. The spinelike tip (T) of the second-stage larva is small and the length is about 4.3 μm. ×3,800.

Fig. 15 Posterior end of 7-day-old larva. The caudal papillae of the third-stage larva are visible under the old cuticle (arrows). T: spinelike tip. ×4,600.

Fig. 16 Anterior end of 8-day-old larva. The old esophageal cuticle of the second-stage larva is shedding off (arrow). OP: outer papilla. ×4,480.

Fig. 17 Anterior end of 12-day-old larva. The mouth opening (MO) is dorso-ventrally flattened. The amphid (Am) opens on the lateral side at the level slightly posterior to the inner papillae. IP: inner papilla, OP: outer papilla. ×6,340.

Fig. 18 Cuticular surface of 7-day-old larva. The breadth of annuli is 0.6 μm. The lateral line is observable as the interruption of the transeverse annulations. ×5,960.

Fig. 19 Cuticular surface of 9-day-old larva. The breadth of annuli is 0.72 μm and the annulations are distinct. The lateral line is observable as the mass of cuticular folds running longitudinally. ×5,960.

Fig. 20 Anal field (AF) of 12-day-old larva. The anus is crescent-shaped and the anal field has many longitudinal cuticular folds of the cuticle. ×5,760.

Fig. 21 Posterior end of 12-day-old larva. One of the two lateral papilliform processes (LP) and the terminal process (TP) are seen in this figure. The phasmid (arrow) opens adjacent anteriorly to the lateral papilla. A papilla (Pa) is observed only on the left lateral side. Longitudinal folds run over the entire surface. An: anus. ×3,540.