Ultrastructure of the Head Region of Molting Second-Stage Juveniles of *Heterodera glycines* with Emphasis on Stylet Formation

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Abstract: The morphology and alterations of infective juvenile (J2) body components with emphasis on the body wall, stomatal wall, stylet, and sensilla of *Heterodera glycines* were observed. During the molt of J2 to J3, the J2 hypodermis separates from the J2 cuticle and forms an extracellular space, continuous with an invagination at the anterior, center of the J3. The space between the J2 cuticle and the enlarged J3 hypodermal cells is filled with electron-dense material resembling a fluid observed in insects during molt. Regeneration of the J2 during molt was traced in a series of ultrathin sections. The site of stylet regeneration is in the hypodermal and myoepithelial tissues of the invaginated anterior, center of the J3. Four arcade-like cells are related to specific components of the stomatal wall, the stylet cone, and the stylet shaft of the J3. The first and second arcade-like cells are primarily related to stomatal wall development, whereas the third and fourth arcade-like cells are related to stylet cone and shaft development. Spherical, electron-translucent vacuoles that occur in myoepithelial cells just posterior to the arcade-like cells appear to be progenitors of the stylet knobs. Early stages of protractor muscle attachment to the vacuolar membrane were observed.
Key words: stylet development, Tylenchida, *Glycine max*.

Soybean cyst nematodes (*Heterodera glycines* Ichinohe, 1952) penetrate soybean roots as second-stage juveniles (J2) and undergo three molts before maturing. Regeneration of a functional stylet, lost early in molt, is critical for survival and is characteristic of the ontogeny of the Tylenchida. Light microscope studies have elucidated the molting process in tylenchid nematodes (1,12,13). Stomatal wall and stylet components disappeared or remained attached to the cuticle, and tissues from which new stomatal wall and stylet components arose were identified. The parts of the stylet of adult tylenchid nematodes were named, and attempts were made to homologize the parts with those of the cylindrical stomata of rhabditids (2,4,10,15).

Recently, the relationship of the molting cuticular lining of the stoma with subjacent cells in the rhabditid *Caenorhabditis elegans* was established (17). In contrast to the cell-to-cell reassembling of stomatal parts as described in these nematodes, the tylenchid species have a stylet initiating zone at an invaginated center anterior of the J3.

This study focuses on the cells in the anterior region of J2 of *H. glycines* at the second molt emphasizing the initial stages of stylet development in the J3.

MATERIALS AND METHODS

Juvenile stages of *Heterodera glycines* in infected soybean roots were prepared for electron microscopy by previously described procedures (6,16). Nematode-infected root segments were fixed in buffered 3% glutaraldehyde (0.05 M phosphate buffer, pH 6.8) at 22 C for 1.5 hours. Rinsing and postfixation in osmium tetroxide were with the same buffer. Fixation was followed by washing for 1 hour in six changes of buffer. The roots were then postfixed in 2% osmium tetroxide for 2 hours, dehydrated in an acetone series, and infiltrated with a low viscosity medium (14). Silver-gray sections of selected nematodes were cut on a Sorvall MT-2 or MT-5000 ultramicrotome with a diamond knife and mounted on uncoated 75 × 300-mesh copper grids. The sections were stained with 2% aqueous uranyl acetate for 10 minutes, then with lead citrate for 5 minutes. Thin sections were viewed in a Philips 301 or 400 electron microscope operating at 60 kV with 20-μm objective aperture.

RESULTS

Infective J2 of the soybean cyst nematode enter soybean roots, migrate intracellularly, and become established at feeding sites where host cells are stimulated to form syncytia. In the course of feeding on the syncytium, the nematodes undergo three molts developing to sedentary pyriform females or mobile vermiform males.

Changes during juvenile molt include enlargement of the hypodermis and the subsequent separation of body cuticle, cephalic framework, stomatal wall, and stylet cone from the hypodermis (Figs. 1, 4, 6). One of the first signs of molting is accumulation of electron-dense granules between the outer surface of the hypodermis and the separating cuticle (Fig. 1). The electron-dense granular accumulations (EDD) appear to merge with the less dense accumulations (EDM) within the cephalic framework and under the cuticle of the molting nematode (Fig. 1). The electron-dense granules extend between the retracted hypodermis and the stomatal components (Fig. 2). Subsequently, the granular region between the hypodermis and cuticle extends along the body wall and is continuous with the invaginated central anterior region of the nematode (Figs. 3, 5). Within the invaginated region are the cephalic framework, stomatal wall, and the stylet cone with attached cell membranes and stylet shaft residue (Fig. 5). The electron-dense granular region also contains remnants of cilia and cuticular walls of the J2 inner labial and amphidial sensilla (Fig. 7). These cilia occur near their original sites in the J2 prior to molt. Rudimentary components of the J3 stomatal and sensory organs lie within the hypodermal cells that partially enclose the invaginated anterior region (Figs. 5, 7). Rudimentary amphidial canals of the J3 are delineated by cilia that occur in the J2 amphidial canals (Figs. 5, 7). Junctional complexes and secretion-laden cells surround these sites of rudimentary amphidial canals.

Similarly, at the base of the invaginated region, junctional complexes, secretion-laden cells, and cilia that emerge from the inner labial sensory organs indicate early stages of J3 lip region development (Fig. 7). Cilia of the J2 can be traced to the basal body regions of either the amphidial or inner labial sensilla or sense organs where new cilia would be expected to develop (Figs. 5, 7).

Stomatal wall components of developing stylet: Sections posterior to the base of the invaginated region show secretion-laden cells and cell junctions with direct connections to the developing stomatal wall (Fig. 8a) and components of the stylet (Figs. 5, 7).

Submedial longitudinal sections (Figs. 5, 6) reveal the junctional complexes and cells that develop into inner labial sensilla. In medio-longitudinal sections (Figs. 7, 7a), there is continuity between the hypodermis and the surrounding inner labial sensilla apertures, and the stomatal aperture with cellular and structural components of submedial section.

Submedial longitudinal sections show three linearly arranged cell junctions that join four layers of cells with high secretory activity (Figs. 5, 6). In medio-longitudinal sections (Figs. 7, 7a), these junctions appear as distinct narrow bands between adjacent cells on either side of the centrally located stomatal wall and stylet components. Thus, the cell junctions can be visualized as circular bands in cross section through the nematode.

The first arcade-like cell below the apertures of the inner labial sensilla is bordered anteriorly with a belt-like junctional complex (Figs. 7, 7a). This arcade-like cell contains granules that appear to secrete material for stomatal wall development. A second arcade-like cell adjacent and posterior to the first cell contains secretory granules that are related to the flexed arm portions of the stomatal wall. The two layers of arcade cells appear to be part of the hypodermis.

The arcade-like cell that supports the stylet cone lies posterior to the cells that support the stomatal wall (Figs. 7, 7a). The adjacent cell membrane is joined by a third junctional complex. A submedial section (Fig. 6) reveals a continuous layer of secretion-laden, granular cytoplasm that is bounded anteriorly with the stomatal wall-supporting cell and bounded posteriorly with the shaft-supporting cells. A median section of this nematode (Figs. 7, 7a) shows the early stages of stylet cone development. Secretory granules interact with the developing stylet, and deposition of products
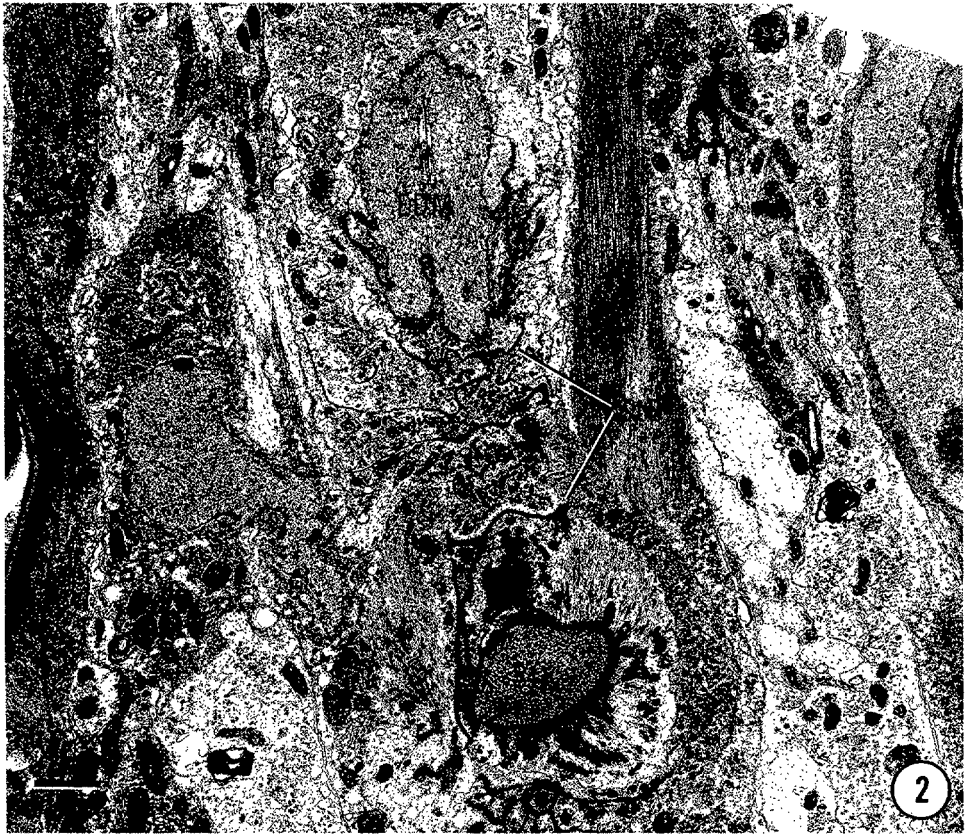
FIG. 1.   Submedial longitudinal section through the lip region of *Heterodera glycines* 4 days after inoculation of 'Lee' soybean roots. The nematode is in molt between the second and third stage. During molt, the J2 hypodermis swells to become the J3 hypodermis (J3H). It is separated from the J2 cuticle by electron-dense granular material (EDM). These deposits extend throughout the cephalic framework. Extremely electron-dense deposits (EDD), present in the cephalic region and observed as deposits in the infective J2, appear to disintegrate during the molt to combine with the larger mass of granular material. Feeding plug (FP) in the host cell wall forms during the initial feeding stages of J2.

FIG. 2.   Same nematode in Figure 1 viewed near the site of the deteriorated J2 stylet shaft. Electron-dense

FIG. 3. Submedial section of *Heterodera glycines* in 'Lee' soybean roots 5 days after inoculation. Electron-dense granular material (EDM) occurs between the J2 cuticle and the J3 body. Remnants of ciliary membranes (CM) and ducts of J2 sensory organs are present throughout the granular matrix. The feeding plug (FP) deposited by the J2 shows the feeding site and the adjacent host syncytium (Syn). CW = cell wall.

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material (EDM) fills the invaginated center anterior region of the J3. Posterior to this region are arcade-like cells that contribute to development of the stoma wall and stylet. The secretion laden arcade-like cells have integrating cell junctions. StK = stylet knob of J2. StP = stylet initials of J3.

FIG. 4. Tangential longitudinal section of a *Heterodera glycines* juvenile 4 days after inoculation of 'Lee' soybeans. Hypodermal cells (HC) of the J3 contain microtubules (Mt), mitochondria (Mc), and secretion granules (SG). Secretion granules appear to be deposited by microectocytosis into the surrounding electron-dense material (EDM). The anteriormost cell is sharply defined by cell junctions (CJ) that separate it from the adjacent posteriad hypodermal cells. A portion of the hypodermis extends between two protractor muscles (PM), which in turn are bordered by somatic muscles (SM). Cu = J2 cuticle.

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FIG. 5. Submedial longitudinal section (LS) through a molting J2 nematode of *Heterodera glycines* 4 days after inoculation of 'Lee' soybeans. The invaginated central anterior region of the J3 contains a granular matrix resembling molting fluid. The matrix is bounded by the body cuticle (Cu) and J3 hypodermis and contains the stomatal wall (SW), stylet cone (StC), and stylet shaft remnants (StShR). This dorso-ventral

longitudinal section shows portions of the amphidial cuticular channels (ACC) that enclose ciliary (Ci) processes. Two of six inner labial receptors (ILR3) of J3 are shown at the base of the invaginated region filled with electron-dense material (EDM). Remnants of the J2 inner labial receptors (ILR2) lie adjacent to the stomatal wall. Junctional complexes (JC) unite arcade-like cells that contribute to stomatal wall and stylet cone and shaft development. The electron-translucent spherical vacuole (ETS) represents the early stage of the dorsal part of the tripartite stylet knob. PM = stylet protractor muscle.

FIG. 5a. Section through the vacuole (ETS) of Figure 5 at a different plane shows myofilaments of protractor muscles (PM) that are attached (→) to the membrane.

FIG. 6. Submedial section of the stylet initiation zone of J3. Arcade-like cells designated as A1, A2, A3, and A4 represent the cells that contribute to stomatal wall and stylet components. Cell A1 is related to the stomatal wall development, A2 to the flexible arm portion of the stomatal wall, A3 to the stylet cone, and A4 to the stylet shaft. These arcade-like cells can be compared to the stomatal wall and stylet components in Figures 7 and 7a. Numerous secretory granules (SG) occur in the arcade-like cells. Portions of electron-translucent vacuoles (ETS) within myoepithelial cells are separated from cell A4 by a thickened membrane (TCM). ETS = electron-translucent vacuole. PM = stylet protractor muscle. StShR = stylet shaft residue.

FIG. 7. Median longitudinal section of the anterior of a molting J2 showing stomatal wall and stylet initials of J3 of *Heterodera glycines*. Arcade-like cells A1–A4 shown in tangential sections (Fig. 6) are now shown in direct relation to the developing stages of the stomatal wall (SW), stylet cone (StC), and stylet shaft (StSh). Arrows indicate narrow membrane junctions that join the arcade cells. Just posterior to the shaft supporting cell (A4) are three myoepithelial cells, two with vacuoles (ETS) that will form the J3 stylet knobs. A third vacuole of this same nematode was shown in Figure 5a.

FIG. 7a. Stylet initial zone at a slightly different level and magnification than in Figure 7. Secretion granules, especially adjacent to the stylet cone (StC), show the direct relationship between a developing stylet and the arcade-like cell (A3). AmpCi = amphid cilia. EDM = electron-dense material. HC = hypodermal cell. ILR = inner labial receptor. ILRCi = inner labial receptor cilia. MJ = membrane junctions of amphids. ShC = shaft cell. SW = stomatal wall.
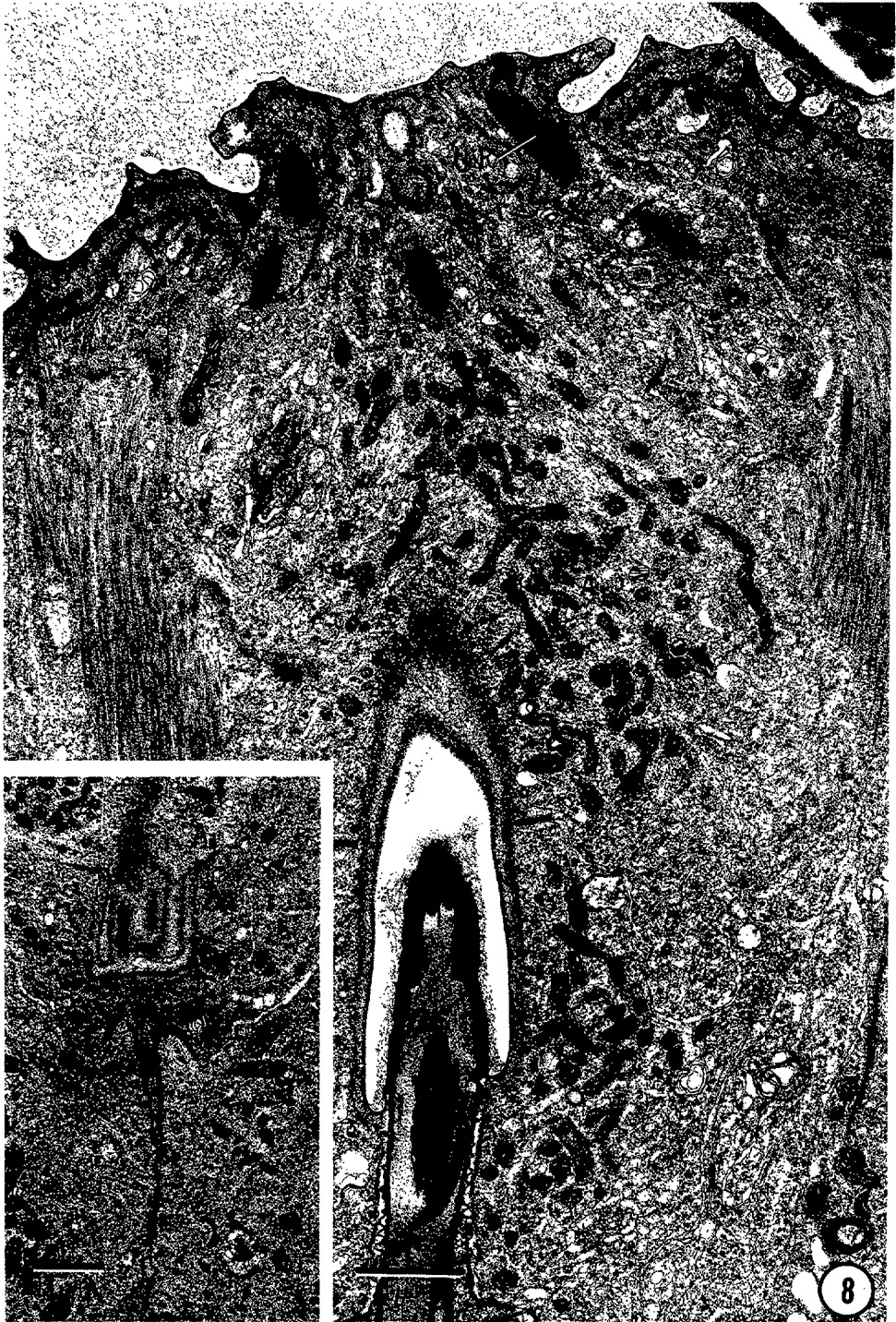
FIG. 8.   Longitudinal oblique section through the anterior of the head region of a J3 *Heterodera glycines,* 6 days after inoculation. Cells A1 and A2 are comparable to the arcade-like cells of A1 and A2 in Figures 7 and 7a. Enlarged stomatal wall, stylet components, and sensory organs are comparable to the stylet initial region and inner labial receptor zones of a molting J2 (Fig. 7). Former base of the invaginated J2 region is now the rounded anterior head region of the J3.

FIG. 8a.   Early stage of stomatal wall and stylet development of a J4. Arcade cells A1–A4 contain numerous secretion granules that are part of the development of stomatal wall and stylet components. Cu = cuticle. ILR = inner labial receptor.

appears to be by micropinocytosis. The point of the stylet cone invaginates the arcade-like cell wall that initiates the stomatal wall and forms a stirrup-like cuticular structure when viewed in medio-longitudinal section.

The cells that support and develop the stylet shaft are joined to the cone-supporting cells by a third circular junctional complex. The arcade-like cell delineated by junctional complexes is also laden with secretion granules (Fig. 6). In median sections (Figs. 7, 7a), these secretion granules appear to have been deposited on the developing stylet shaft. Stylet cone, shaft, and knobs appear to be derived from myoepithelial cells of the esophagus. Three large myoepithelial cells are located immediately posterior to the shaft-supporting cells. They are partially bounded anteriorly with thickened cell membranes. The myoepithelial cells are situated dorsally (Figs. 5, 5a) and subventrally (Fig. 7) in the esophagus; each contains an enlarged electron-translucent spherical vacuole that appears to be a progenitor of a stylet knob. At this early stage of stylet knob development, myofilaments of the protractor muscle elements occur at the anterior and lateral membrane surfaces of the electron-translucent vacuoles (Figs. 5a, 7).

During later stages of the second molt, the newly formed stomatal and sensory apertures extend anteriorly to replace the granular invaginated anterior region (Fig. 8). Subsequently, the J3 lip region contacts the surface of the host plant syncytium for the nematode to resume feeding.

DISCUSSION

The ultrastructure of the cells that contribute to stylet development of *Heterodera glycines* can be related to morphological studies of the anterior region of molting specimens of *Pratylenchus scribneri* and *P. brachyurus* (13).

In studies of *P. scribneri* and *P. brachyurus*, Roman and Hirschmann (13) indicated that the head of the J3 retracts slowly from the second molt cuticle. At this stage the outline of the developing head region became distinct. Ultrastructurally, using *H. glycines* as an example, this accentuation of the head region could be brought about by the invagination of the anterior center of the J3 and the subsequent filling of the

void between the J3 hypodermis surface and the J2 cuticle with a granular electron-dense material. The nature and source of the material is not known, but it could be comparable to a fluid that occurs in insects during the molting process. When the insect cuticle separates from the epidermis, molting fluid is secreted into that space (3). Although the source of such a fluid in nematodes is not clear, the enlarged hypodermal cells, especially at the anterior of the nematode, appear to secrete granules via microectocytosis. Furthermore, an intense electron-dense granular material, often observed in the anterior of infective J2 (7), appears to merge with the fluid.

In *P. scribneri* and *P. brachyurus*, three distinct cutinized rings appeared around the middle of the shaft at the base of a cup-shaped region after the stylet shaft and knobs had disappeared (13). In *Ditylenchus destructor*, these cutinized rings were characterized as part of a primordium for stylet formation (1). The spear or cone developed progressively from the upper ring where the shaft was formed initially within the lower rings. The rings were believed to represent regions where materials accumulated to form the spear and shaft. Observations of the ultrastructure of *H. glycines* agree in part with the light microscope observations by Anderson and Darling (1). The developing head regions of *D. destructor* and *H. glycines* are comparable. The direct contribution of secretion granules to the development of the stomatal wall and stylet is evident (Fig. 7). Furthermore, the cutinized rings observed with the light microscope in *D. destructor* were probably arcade-like cells accentuated by junctional complexes. Tangential views of junctional complexes of arcade-like cells assisted in delineating these cells as contributors to the early stages of stylet and stomatal wall development. The upper ring or junctional complex of *H. glycines* is the anterior surface of the arcade-like cell (A2) that gives rise to the flexed arm of the stomatal wall and not the stylet cone as indicated in the study of *D. destructor* (1). In fact, it is the more posterior cell designated as A3 that contributes to stylet cone development in *H. glycines* (Figs. 7, 7a). The third ring or junctional complex is the anterior boundary of the arcade-like cell (A4) that supports stylet shaft development. Each ar-

cade-like cell has been referred to as a single cell unit. However, each unit may consist of several cells that are not readily delineated.

Although all facets of stylet development during molt have not been documented, the cells joined by the third junctional complex and the slightly obscure fourth junctional complex seem to be related to stylet shaft development. Apparently as the shaft develops, union occurs with knob components within the myoepithelial cells.

Similar junctional complexes were described for the rhabditoid nematode *Caenorhabditis elegans* where the cylindroid cuticular stoma is underlain by thin hypodermal cytoplasm (17). This hypodermis is separated into distinct arcade cells that are attached to each other through a junctional complex (belt junction). The cells form a layer of cytoplasm that encircles the buccal capsule.

The junctional complexes of *H. glycines* differ from the junctional complex reported for *C. elegans* (17) because they are not initially arranged along the periphery of a cylindroid stomatal wall as in *C. elegans*, but are part of the stylet initiating zone at the anterior, center of the invaginated head region of the J3. Although the junctional complexes of the stylet initials of *H. glycines* are confined and closely arranged, they are identifiable as distinct belt junctions alongside the fully developed tylenchid stylet (8).

Although cell changes that contribute to the development and union of the various parts of the stylet are incompletely described, the hypodermis is obviously an integral part of the anteriormost arcade-like cells related to the stomatal wall formation. Furthermore, esophageal cells are primary contributors to stylet cone, shaft, and knob development.

The homology between the stomatostyle of the Tylenchida and the stoma of Rhabditida was discussed and compared (4) using the terminology of nematode stoma parts introduced by Steiner (15). While homology apparently exists among various stomatal sectors in rhabditid species compared with Dorylaimida (5,9,11) and Tylenchida (10), the homology with tylenchid species is obscure during early developmental stages. In rhabditid species, such as *Caenorhabditis elegans*, the new stomatal structures are laid down by secretory deposits adjacent to existing stomatal wall sectors (rhabdions) of the previous molt (17). In contrast, deposits for new stylets of tylenchids are made initially in a localized zone; only through elongation and general increase in size, does a replacement stomatostyle form. Thus, the homology between parts of the stoma in Rhabditida and Tylenchida is more apparent at the completion of stylet development rather than in the initial stages.

Future investigations should be directed toward elucidation of the intermediate stages of stylet development as well as the source and nature of the molting fluid. A broader understanding of the mechanisms that influence molting may provide targets for disruption and may lead to improved nematode control strategies.

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