Fine Structure of the Cone of *Heterodera schachtii* with Emphasis on Musculature and Fenestration[1]

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Abstract: Fine structure of the posterior cone of monoxenically cultured *Heterodera schachtii* is examined. The cone is not evident at the end of the fourth molt, but as the female matures the cone elongates, vulval lips enlarge, and cuticular patterns on the lips are modified. Body wall cuticle (BW) of the cone includes layers A and B, but C is modified or replaced by a network of fibers which correspond to the semifenestrae. Vaginal lining is continuous with the BW and terminates at the cuticular underbridge near the uterus. Vaginal musculature includes 48 *dilatores vaginae* (DV) as well as a *sphincter vaginae* (SV). The DV include a contractile and noncontractile region with abundant actin and glycogen. A distinct anal depressor muscle is present. In the cyst, only bullae, the underbridge, vagina lining, and traces of the SV muscle persist. Detailed morphology of the cone of *H. schachtii* provides insight into characters which, when compared with other heroderines, will be useful in phylogenetic analysis of Heteroderinae.

Key words: cone, cyst, *dilatores vaginae*, fenestrae, fluorescent phalloidin, *Heterodera schachtii*, monoxenic culture, phylogeny, scanning electron microscopy (SEM), *sphincter vaginae*, transmission electron microscopy (TEM), underbridge, vagina.

The cyst cone became important in the taxonomy of Heteroderinae when Wollenweber in 1923 separated *Heterodera schachtii* Schmidt, 1871 from *Globodera rostochiensis* (Wollenweber, 1923) Behrens, 1975 on the basis of the lemon shape of *H. schachtii* (cone present) versus the spherical shape of *G. rostochiensis* (cone absent). Presently, the principal structures used in cyst identification are within the cone or terminal area, and most of these structures persist after death (17,26–28).

Hypotheses of phylogeny in Heteroderinae suggest either single or repeated evolution of the cone (1,14,38). Fine structural investigation of development of the cone may provide evidence to support or reject hypotheses on the basis of homology of the cone character. Therefore, it is important to know if the cones of different taxa are equivalent in development, structure, and function and if transformation series occur among different types of cones. We suggest that cones could develop as a function of differential growth of the fe-

male, attachment points of vaginal muscles, or cuticle structure of the terminal region.

Recently, we examined the cuticle of *H. schachtii* (8) and demonstrated that a diminutive D layer is present in mature females but that, unlike other Heteroderinae examined (5,31), the D layer does not occur in the cone. A study of the cone and the many additional characters associated with it could explain phylogenetically the diminutive D layer in *Heterodera*, relative to its prominence in other cyst-forming, and many noncyst-forming, genera.

Although a D layer is not present in the cone, an E layer occurs in the cone of aging females and cysts of *H. schachtii* and gives rise to bullae (8). An E layer also has been reported in certain ataloderines which lack cysts and bullae but have a cone (5). An E layer apparently does not occur in round cyst nematodes. The functional and phylogenetic significance of the distribution of the E layer in relation to the cone is not yet understood.

Although comparative developmental morphology of the cone and cone-related structures of cyst nematodes needs to be better understood, one constraint to developmental studies has been rapid deterioration of the mature female and cyst by secondary organisms. Investigation using monoxenic culture would overcome this

limitation. Another constraint is the technical difficulty of preparing the relatively large balloon-like senescent females for transmission electron microscopy (TEM). Rapid senescence of tissues and large body size of Heroderinae have been the principal constraints for detailed studies of *H. schachtii*. Furthermore, the cuticle is relatively impermeable to fixatives.

We report on development of the *H. schachtii* cone using a combination of TEM, scanning electron microscopy (SEM), and light microscopy (LM) in an effort to understand cone-related structures for greater insight into phylogenetic analyses of the cyst and the Heteroderinae.

MATERIALS AND METHODS

Heterodera schachtii was established on its type host *Beta vulgaris* L., cv. USH 11, Holly hybrid large, as previously reported (8). Seedlings established in petri dishes were inoculated with surface-sterilized second-stage juveniles (J2), and infection was monitored with a Nikon inverted microscope equipped with Hoffman interference optics and Garr time-lapse high resolution video recorder. Culture on plantlets allowed detailed investigations of up to four generations of the cone area, including cuticle, fenestrae, the reproductive tract, and musculature. With establishment of feeding site recorded as time zero, development of the J2 was recorded and stopped at particular points for morphological studies. Points included immediately after the fourth molt, 2 and 4 weeks after the final molt, and the new cyst. Both TEM and LM were used to study internal cone structures and SEM was used to study surface pattern development at the posterior end of the nematode.

Specimen preparation for LM and TEM was as previously reported (8). Excised cones were fixed for about 8 hours in a modified Karnovsky's fixative followed by rinsing and additional fixation for 8 hours in a glutaraldehyde-hydrogen peroxide solution (4,22). Postfixation was with osmium tetroxide fumes followed by dehydration in a graduated ethanol series and infiltra-

tion with Spurr's epoxy (8). Specimens were prepared for SEM by glycerine infiltration (32). Specimens were examined on a Hitachi 600 transmission electron microscope at 75 kV or JEOL JSM-35C scanning electron microscope at 15 kV.

To visualize actin, and thus the presence of muscles in the cone, females were treated with fluorescent phalloidin (39).

RESULTS

The cone of *H. schachtii* is not evident in the J3 and J4 (Fig. 1). These stages are oblong with longitudinally oriented ridges, but in J4 the ridges become transversely oriented in the terminal region. The cone is still not evident when the posterior end of the female pulls away from the cuticle of the J4 (Fig. 2), but secretion of a clear gelatinous matrix is apparent as soon as the cuticle ruptures. The matrix increases in optical density after copulation.

As the female matures, the cone elongates, the deep ridges become increasingly shallow, and the vulval lips enlarge and remain so in the cyst (Figs. 3–6). Dorsal and ventral to the lips, two semicircular areas, vulval crescents (described by Green, 1975), are not well delimited in young females (Figs. 4, 5). The crescents include short wavy ridges extending dorso-ventrally in a flabellate arrangement from the vulval lips (Fig. 4). In mature females and cysts, the ridges of the crescents shift from the dorso-ventral flabellate orientation to an interlaced lateral orientation (Figs. 5–7). The vulval crescents in females roughly correspond to the fenestrae in mature cones.

Internal structure of the fenestral region is distinct from the body wall cuticle (BW) of the rest of the cone. In the cone, BW includes layers A, B, and C, but at the terminus, C is modified or replaced by a network of electron-lucent fibers (Figs. 8–10). The fibers generally orient randomly except at 1–3 bridges of condensed laterally-oriented fibers on each crescent (Fig. 10). An electron-dense matrix surrounds the fibers in the mature female, but in the cyst the matrix is lost and only the network of fibers remains (Figs. 10, 11). This fibrous
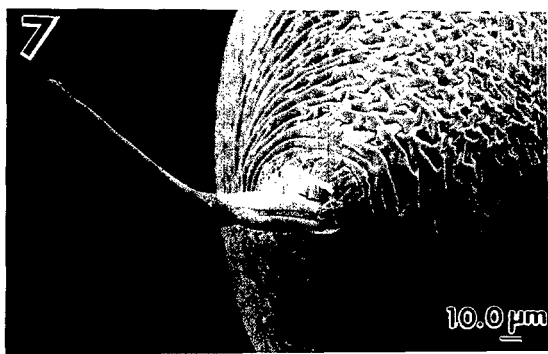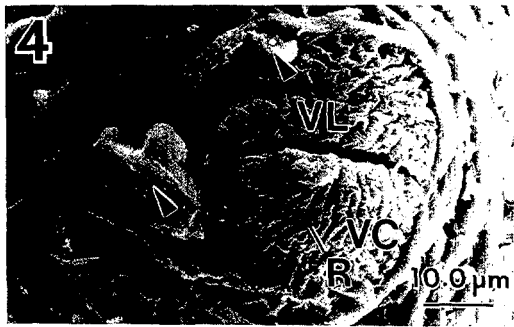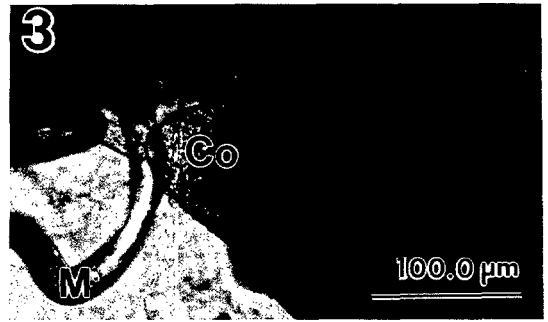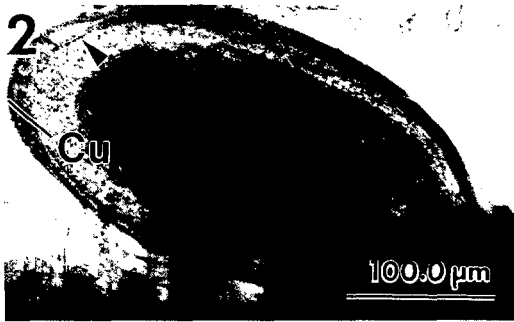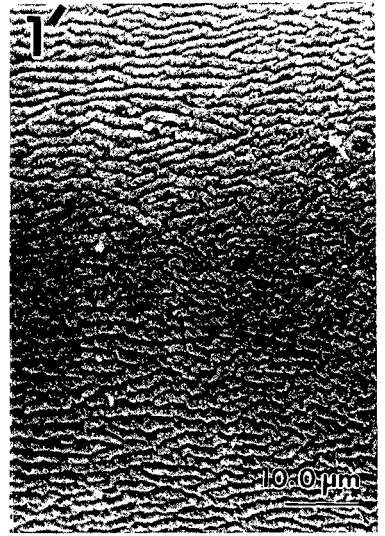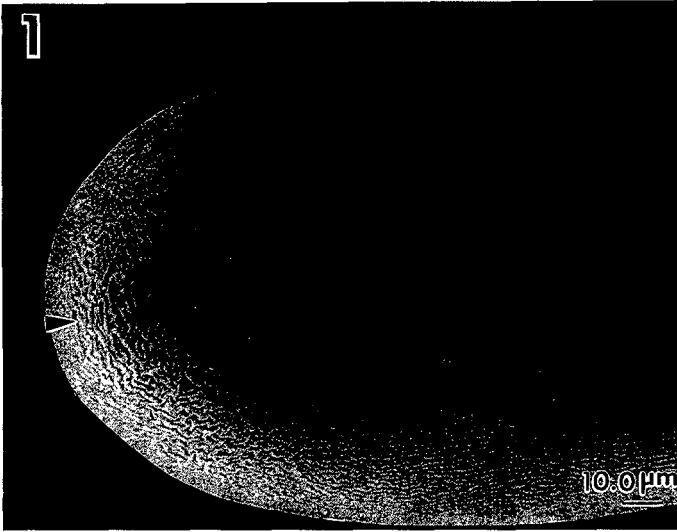
region contrasts to the denser cuticle of the rest of the cyst or adult. Semifenestrae are broken by J2 escaping from the cyst (Fig. 7). Freshly hatched J2 move about the entire cyst but concentrate at the cone, where the stylet is used to pierce the fenestra, particularly in the region closest to the vulval slit. Several J2 contribute to the process of piercing and their collective pressure on the fibrous integument further promotes rupture of the fenestra.

In the cone, a portion of the A layer and C layer outline the irregular sheaf-like vaginal duct (Figs. 12–14). Although C predominates, it is thinner than in the BW and becomes further modified as it approaches the underbridge (Figs. 13B, 14). The modified C layer of the vagina greatly expands in thickness where the vagina joins the uterus (Fig. 12). Close to the uterus, the modified C layer extends on each lateral side forming the underbridge (Figs. 13H, F, 12). The underbridge is a cuticular crosspiece which is not horizontally level. Each arm extends obliquely from the vagina anteriorly to the BW cuticle on each lateral side (Fig. 13H, F). In some specimens the oblique underbridge arms bifurcate close to the BW cuticle. Cuticle of the underbridge is diffuse, and the boundaries are not discrete (Fig. 21).

Distinct vaginal musculature is present in the cone of *H. schachtii* including the *dilatores vaginae* (DV) and the *sphincter vaginae* (SV) (Figs. 13B–D, 14–17, 19, 20, 23). Six prominent DV form a flabellate arrangement on the dorsal side of the vagina, and a corresponding set of six muscles occurs at the ventral side (Fig. 13C, D). The six pairs of DV muscles attach at four different levels along the vaginal length, resulting in 48 muscles (Fig. 13B). The posteriormost six pairs of DV attach

to the vagina lining from the cuticle of the vulval slit to about 5 μm anterior to the cone terminus. Second, third, and fourth pairs attach to the vagina at approximately 13, 22, and 32 μm anterior to the cone terminus. The SV muscle encircles the vagina at about 27 μm anterior to the cone terminus (Figs. 12, 13B, D, 14, 22). Additional vaginal muscles extend from near the uterus to the base of the cone (Fig. 23).

Presence of actin was observed as fine filaments and confirmed by positive staining with fluorescent phalloidin (Figs. 17, 20). The noncontractile region of muscle cells is characterized by a conspicuous large nucleus and patches of glycogen; glycogen also is present between muscle fibers (Figs. 13B, 15, 16). Junctional complexes and hemidesmosomes are associated with attachment of muscle cells to the vaginal cuticle and fibers connect across the hypodermis to the BW (Fig. 19). Muscle mass decreases as females age, although a few filaments persist in the cyst (Figs. 13F, 22).

The hypodermal syncytium in a young female is expanded, lobed, and highly active with dense cytoplasm and large nuclei. Only at the point of muscle attachment does it narrow to less than 1 μm. Where the hypodermis is broad, dense nuclei, Golgi, rough endoplasmic reticulum, and free ribosomes are abundant. In young females, large irregular patches of electron-lucent material nearly fill the hypodermis (Fig. 23), but later in development, when an E layer and bullae appear, they are replaced by clear vacuoles.

An anus is present in all stages of *H. schachtii*. In young females, the rectum is ca. 13 μm long (Figs. 13B, 18); the cuticle lining is very thin compared to that of the vagina. An anal depressor muscle is apparent (Figs. 13B, 18). The intestine is highly

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FIGS. 1–7. 1) SEM of fourth-stage juvenile. Arrowhead indicates posterior end. Inset 1′ is an enlargement of midbody pattern. 2) LM of young female with fourth-stage cuticle (Cu) separated from adult cuticle (arrowhead). 3) LM of adult female cone (Co) 1 week after ecdysis. M = male. 4) SEM of newly emerged cone top with surrounding remnants of fourth-stage cuticle (arrowheads). R = parallel ridges, VL = vulval lip, VC = vulval crescent. 5) SEM of cyst cone. VC = vulva crescent, VL = vulval lip. 6) SEM of dorso-lateral view of posterior region of mature adult female. An = anus. 7) SEM of second-stage juvenile escaping from cyst after rupturing the semifenestra.

8

A
B
C
1.0μm

9

A
F
H
VS
1.0μm

12

VaC
SV
C
U
1.0μm

10

A₂
VS
Br   Br
Br   F
1.0μm

11

1.0μm
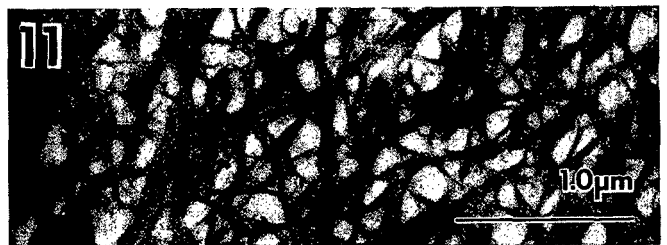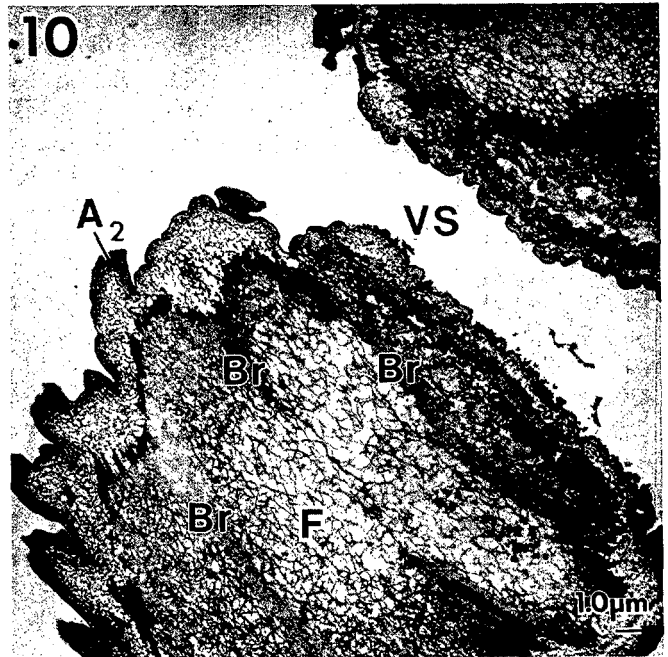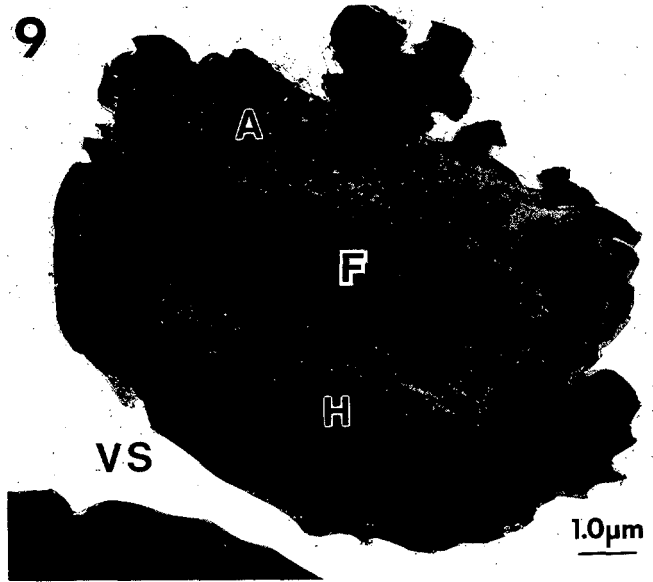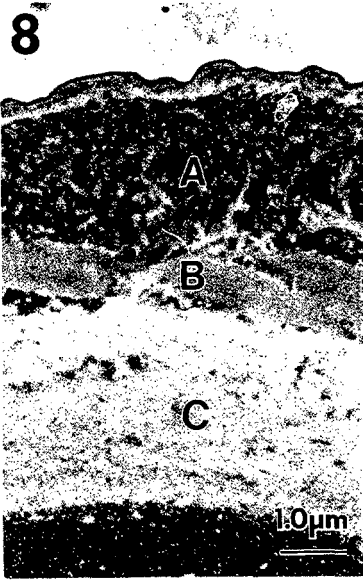
distended, filling the large area not occupied by the reproductive system.

In the cyst, only bullae, the underbridge, vagina lining, and traces of SV persist (Figs. 13F–I, 22). Close to the underbridge many electron-dense globules are evident in the remains of the cuticle lining of the vagina (Fig. 22).

DISCUSSION

The cone of *H. schachtii* develops after the fourth molt and enlarges for about 1 week after ecdysis. Age-dependent variation in size and shape of the cone must be considered in using cone measurements as taxonomic characters. Previous reports document interspecific differences in surface ornamentations at the terminal area of *Heterodera* spp. (17,28). Othman et al. (29) suggested that surface changes with age may account for intraspecific morphological variation and pointed out that the ontogenetic changes need to be identified for reliable phylogenetic interpretation. SEM studies of *H. schachtii* revealed that surface ornamentations vary with development. In the J4, longitudinally oriented ridges at midbody smoothly shift to a transverse orientation at the posterior end. In the adult, the pattern is reticulate at midbody and transverse at the posterior end. This ontogenetic polarity may be similar to a phylogenetic polarity of surface ornamentation in *H. schachtii* with a longitudinal surface pattern ancestral to a reticulate pattern.

Evidence for a transformation series of cuticular patterns can be found in Heteroderinae. The longitudinal pattern, typical of J4 of *H. schachtii* and *H. lespedeza* Golden and Cobb, 1963, is retained in the adult of *Ekphymatodera thomasoni* Baldwin et al., 1989 (2). The adult cuticle of *Atalodera trilineata* Baldwin et al., 1989 may be an intermediate state, since the surface has a longitudinally oriented wavy pattern at midbody which becomes zigzag and transverse posteriorly. The reticulate pattern of the adult *H. schachtii* and *H. lespedeza* may be a terminus in the transformation series.

The surface pattern at the vulval area also changes with development and may vary within Heteroderinae. In young females, dorso-ventral parallel ridges characterize the vulval crescent, whereas in mature females and cysts, these ridges become laterally oriented.

Although the basic surface pattern outside the vulval area in *H. schachtii* does not change in the adult, we noted that the depth and of the ridges decrease with maturity. Young females with deep ridges and compact patterns can become cysts under adverse conditions such as removal from roots (19,21). Thus, compact cuticular ornamentations with deep ridges in rare small cysts might be regarded as atypical variants in a population, if studied independently of age.

Layering of the BW cuticle of the *H. schachtii* cone changes with maturity. Although a D layer was not detected in the cone during development, a thin D layer occurs anterior to the cone of mature females (8). In the cone of mature females and cysts, a fibrous E layer and associated bullae occur next to the hypodermis, increasing the overall thickness of the cone cuticle. Addition to the C, D, and eventually E layers may impart cuticle strength and resistance to extension. Differential rigidity combined with deposit of additional

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FIGS. 8–12. 8) TEM of cross-section of body wall cuticle of young female cone. Letters A, B, C indicate corresponding layers. 9) TEM of cross-section of cone terminus of maturing female. A = corresponding cuticular layer, H = hypodermis, VS = vulval slit, F = network of electron lucent fibers at the area corresponding to the fenestra. 10) TEM of cross-section through cyst fenestral area. Bridges (Br) are of condensed laterally oriented fibers. A_2 = corresponding zone within layer A, F = mesh-like network of fibers in area corresponding to fenestra. VS = vulval slit. 11) TEM of cross-section through fenestral area of cyst showing fibers. 12) TEM of transition region between terminus of vagina and uterus (U). C = corresponding layer of cuticle, SV = *spincter vaginae*, VaC = vaginal cuticle lining.
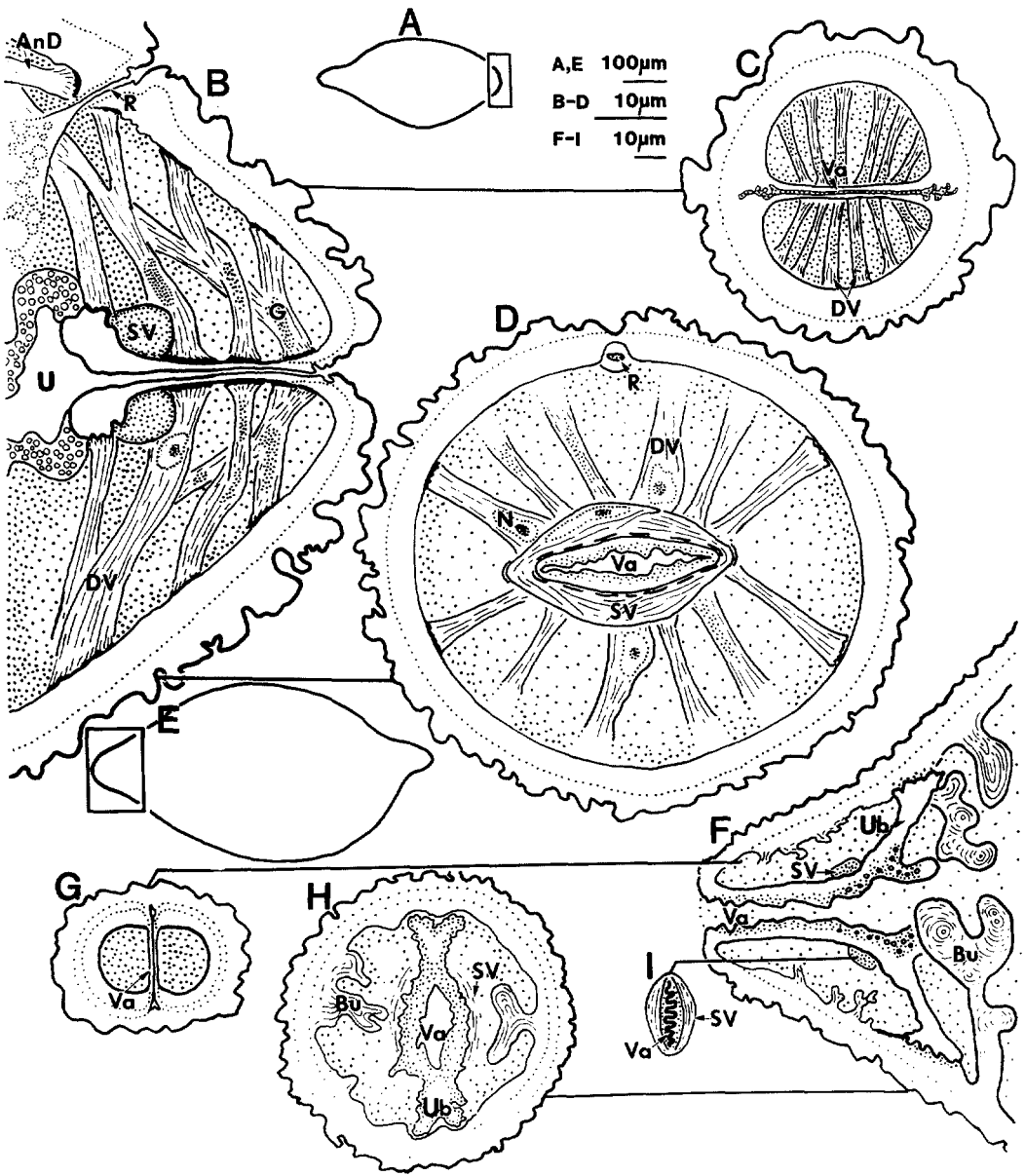
FIG. 13. Schematic representation of cone of newly emerged female and cyst of *Heterodera schachtii.* A) Newly molted female. Box corresponds to area depicted in B. B) Longitudinal view through vagina of newly emerged cone in a lateral orientation. C) Lateral view at cone terminus of young female. D) Lateral view of young female at anus level. E) Cyst. Box corresponds to area depicted in F. F) Longitudinal view through remnant of the vagina in cysts in a dorso-ventral orientation. G) Cross-section of cyst cone terminus. H) Cross-section of cyst close to the underbridge. I) Cross-section of vagina of cyst at the level of the *sphincter vaginae* muscles (SV) remnant. AnD = anus depressor muscle, Bu = bullae, DV = *dilatores vaginae,* G = glycogen, N = nucleus, R = rectum, SV = *sphincter vaginae,* U = uterus, Ub = underbridge, Va = vagina.

material at sites concentrated in the posterior region could result in a cone. Data from other nematode taxa indicate that in general the cuticle is relatively rigid and growth is not necessarily a function of cuticle stretching (3,15,33,36).

Ontogeny of bullae was recently described by Cordero C. and Baldwin (8).

Place, position, size, and number of bullae may have taxonomic value (7,25,26,28). Bullae have been considered as derivatives of tensioned inelastic ligaments between the underbridge and the body wall. They have also been considered points of muscle attachment (15,17); however, muscles are present in the cone before development of bullae. Bullae are projections from the E layer of the cuticle present in cones of mature females (8). These findings confirm and expand on previous speculation that bullae are thickenings of the inner surface of the vulval cone (18,19,34). Mulvey suggests not all bullae are homologous, however, and they may have different origins in different Heteroderinae (26). It is not known if ontogeny of bullae is similar among cyst species and if any structures homologous with bullae are present in noncyst-forming Heteroderinae.

Cuticle lining the vagina persists within the cyst as the "sheaf" described by Taylor (35). Its transition with the BW cuticle forms the vulval bridge, which has been described as a short line of contact between semifenestrae in *Heterodera* sensu lato (7). Layers A and C are present in the vagina and although C predominates, it appears modified as it approaches the underbridge. This modified C layer greatly expands in thickness near the uterus forming extensions on each lateral side. These extensions are the arms of the underbridge. The underbridge may support the vagina by anchoring it to the broad cone base. Contrary to previous reports, the underbridge is not a muscular crosspiece, nor does it control oviposition by contracting the vagina (10,17). In addition, the underbridge in *H. schachtii* does not attach to the inner wall of the cone by muscular bands running parallel to the vulval bridge as suggested previously (28).

Variation in morphology of the underbridge may be expressed as a transformation series within Heteroderinae. For example, the underbridge varies from long and well developed to slender or absent (25,27). The underbridge also varies in distance from the vulva (7,27). Cuticle of the underbridge arms is morphologically distinct and not fully continuous with the BW cuticle on the sides of the cone in *H. schachtii*. Variability in underbridge length, position, and attachment may prove useful characters in phylogenetic analysis of the group.

Musculature of the cone in *H. schachtii* is prominent, with 48 DV and a distinct SV. Attachment of these muscles to the relatively long vagina suggests that the vagina functions as an ovejector. Vaginal muscles are similar to somatic muscles of migratory stages in that they include a contractile and noncontractile region. Fluorescent phal-

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Figs. 14–20.   14) TEM of longitudinal section through vagina of newly emerged cone in a lateral orientation. Letters A and C indicate corresponding layers. Subscripts indicate zones within layers. DV = *dilatores vaginae* muscles, SV = *sphincter vaginae*, VaC = vaginal cuticle lining. 15) TEM of cross-section at cone terminus of young female showing muscles extending from the vagina (Va) to the body wall cuticle. DV = *dilatores vaginae*, G = glycogen. 16) TEM of cross-section showing a *dilatores vaginae* (DV) cell extending from the vagina (Va) to the body wall cuticle. H = hypodermis, N = nucleus (outlined by dots). 17) LM of newly emerged cone incubated in FITC labeled phalloidin. Dorso-ventral view with positive fluorescent staining indicating presence of actin. DV = *dilatores vaginae*. 18) TEM of longitudinal section through rectum (R) of newly emerged cone in a lateral orientation. Arrowheads indicate anal depressor muscle. An = anus. 19) TEM of cross-section of female showing attachment of *dilatores vaginae* (DV) to cuticle of vaginal lining (VaC). He = hemidesmosomes. 20) TEM of cross-section of female through *sphincter vaginae* (SV) showing an enlargement of fibers. VaC = vaginal cuticle lining.

Figs. 21–23.   21) TEM of cross-section of underbridge (Ub) in young female. BW = body wall cavity, Va = vagina. 22) TEM of cross-section of the vagina in a cyst. ED = electron-dense globules in vaginal cuticle; SV = *sphincter vaginae* remnant. 23) TEM of longitudinal section through the hypodermal region of the cone including one of the the posteriormost *dilatores vaginae* (DV) muscles. BW = body wall cuticle, EL = electron lucent matrix, N = hypodermal nucleus, U = uterus wall, Va = vaginal lining, Vc = Vacuole.
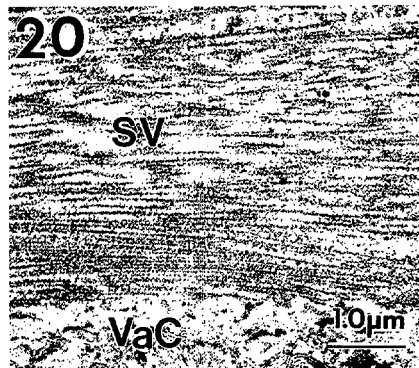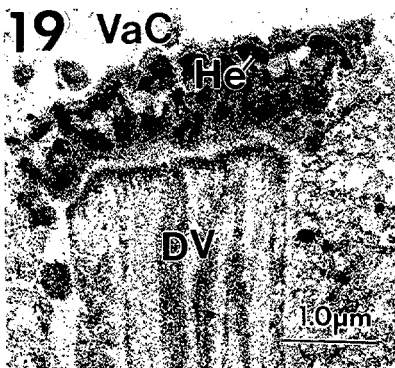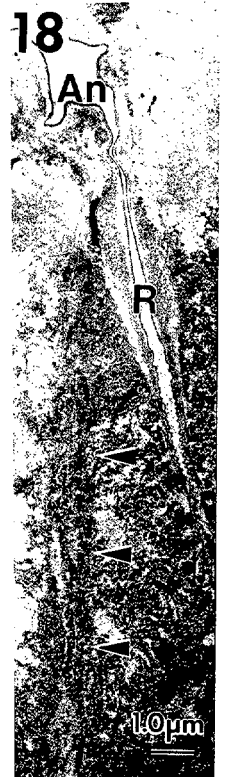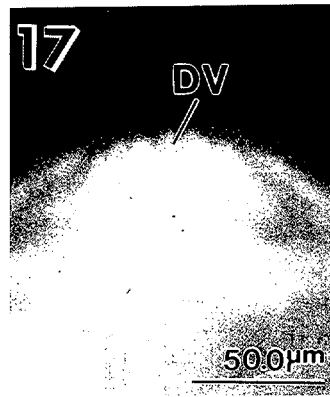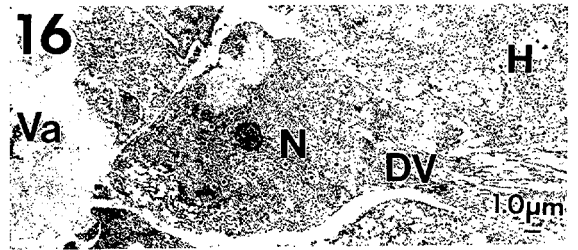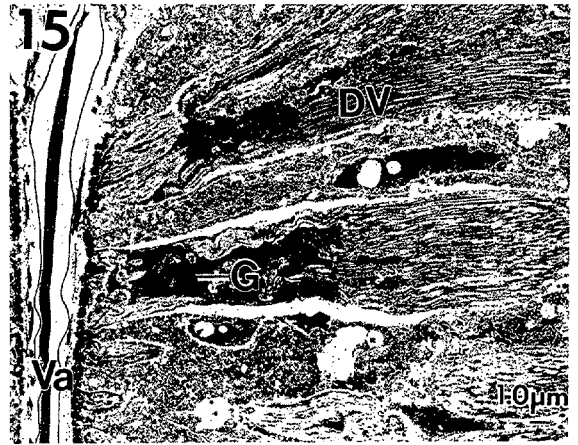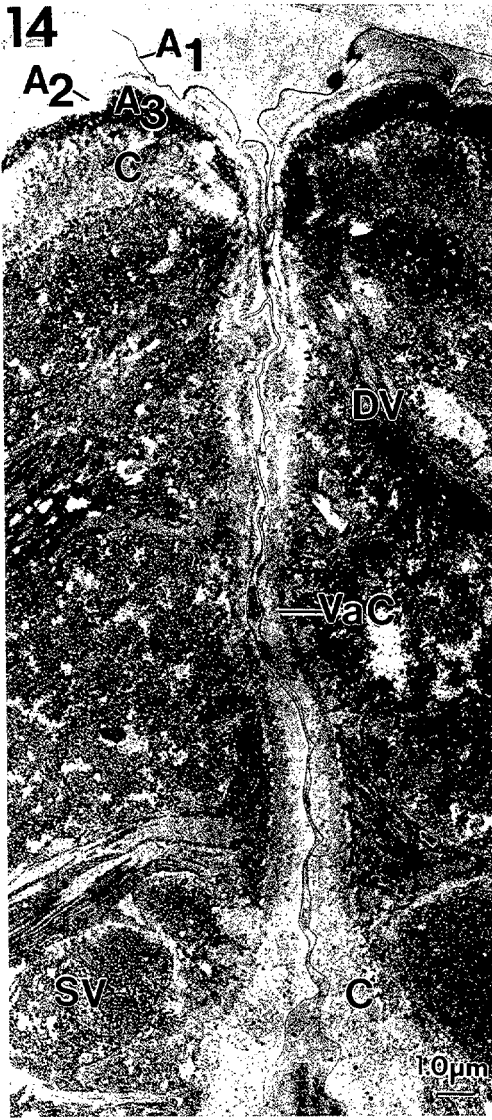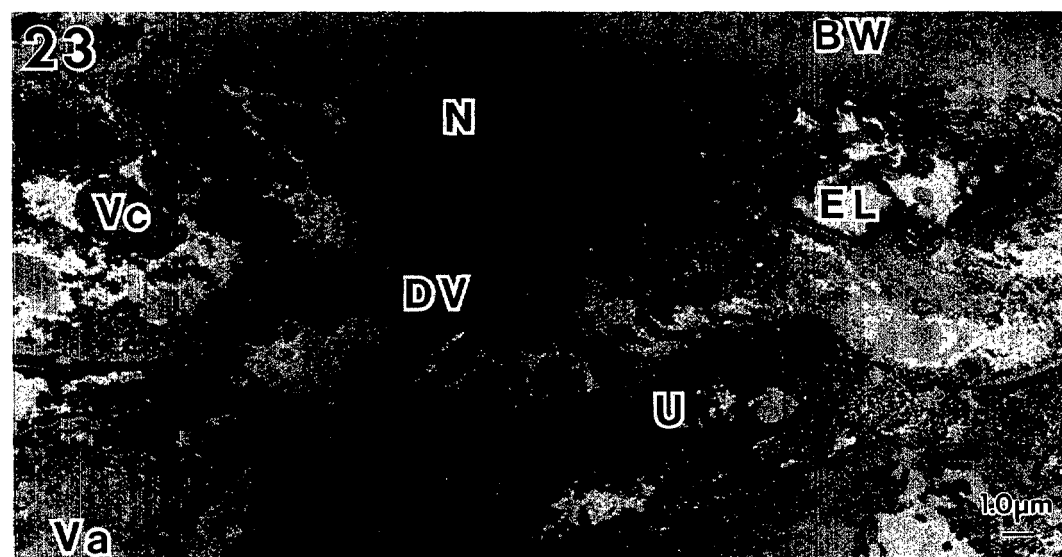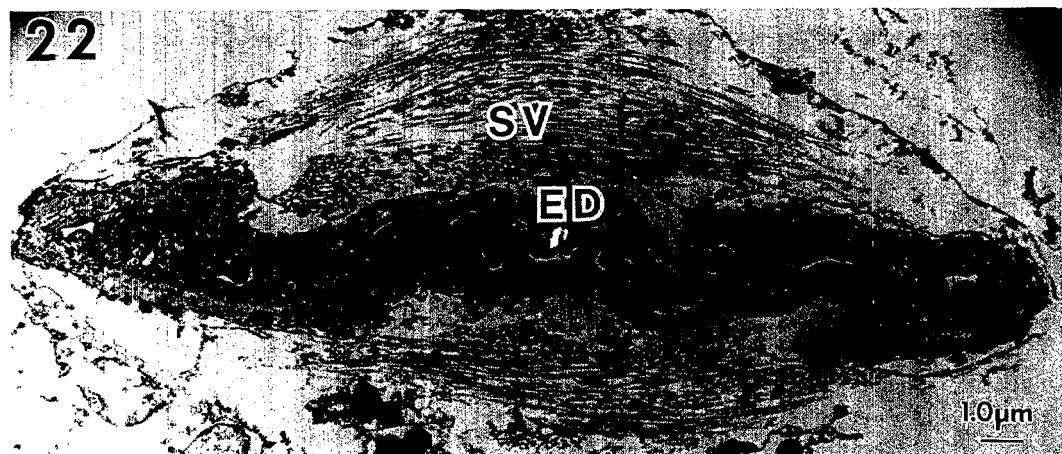
loidin verified presence of actin in areas corresponding to the location of the contractile region of vaginal muscles, and large amounts of glycogen present in muscles of young females provide an energy source.

Flabellate arrangement of DV and presence of a SV apparently occur throughout Nemata (6,12,23,30), but the number and position of muscles is highly variable. Although it is not known if DV and SV are generally present in Heteroderinae species that do not lay eggs, in *Cactodera cacti* Krall and Krall, 1978, which retains eggs, vaginal musculature is greatly reduced (12). A transformation series with vaginal musculature may occur throughout Heteroderinae and may prove to be a reliable character for use in phylogenetic analysis.

Fenestrae have a unique fibrous structure distinct from other BW cuticle. The optically and electron-dense matrix which infuses the network of fibers in females is similar in appearance to, and may be homologous with, a dense material in zone A3 of the BW cuticle. The dense matrix may interfere with LM visualization of the fenestral region in females. Subsequent loss of the matrix apparently allows the unruptured fenestral area to be easily seen in young cysts. Rupture of the fenestrae was previously widely considered to be by decay (10,16,20,34), but our observations confirm that fenestrae are broken by J2, as proposed by Cooper (7).

We suggest that semifenestrae occur in Heteroderinae where the vagina lining is differentially stronger than the cuticle of the perivulval region. In *H. schachtii*, layers A and C line the vagina and underbridge providing a supporting framework. This framework prevents the vulval bridge from breaking away during fenestration. Perhaps fenestrae, including semifenestrae, allow external chemical stimuli to penetrate the otherwise relatively impermeable cyst. Such stimuli also may provide a path for J2 to follow during their escape from the cyst.

Aging of the female and development of the cyst includes occurrence of the E layer and bullae in the cone. When the E layer develops, multilamellar vesicles are prominent in the hypodermis and body cavity. In addition, the hypodermis and muscles become less organized and vacuolated and muscle mass decreases. Similar changes characterize senescent tissues in other nematodes (13,24,40).

The phylogenetic value of cone characters of *H. schachtii* can be interpreted only by fine structure of development of the terminal region of additional species and genera of Heteroderinae. Of particular interest is *Cactodera* which has a cone but differs from *H. schachtii* by the presence of a pronounced D layer in the cone, by retention of eggs, and by formation of a circumfenestrae. These additional comparative investigations will provide a basis for reliable coding to include cone characters in phylogenetic analysis of Heteroderinae.

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