

## Effect of Age on Body Wall Cuticle Morphology of *Heterodera schachtii* Schmidt females<sup>1</sup>

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**Abstract:** Fine structure of the body wall cuticle of *Heterodera schachtii* is compared with respect to age and body region of the female. The cuticle is more complex than previously reported. In newly molted females only layers A, B, and C are present, but 4 weeks after the final molt a thin D layer is present between the midbody and base of the cone. This D layer is absent in the cone of *H. schachtii*, regardless of age. As females age, an additional layer E is produced and includes zones E<sub>1</sub> and E<sub>2</sub>. Zone E<sub>1</sub> apparently is unique to *H. schachtii*, whereas E<sub>2</sub> is likely to be homologous with a similar layer in *Atalodera*. In the cone of old females (ca. 8 weeks after the final molt) of *H. schachtii*, the two zones become irregular in shape and comprise bullae. The presence of a thin D layer in *Heterodera* strengthens the previous hypothesis of a single ancestor of cyst nematodes.

**Key words:** cone, cuticle, cyst, *Heterodera*, Heteroderinae, phylogeny, transmission electron microscopy (TEM).

The body wall (BW) cuticle of females of Heteroderinae sensu Luc et al., 1988 consists of layers which vary in number and composition among genera and are important to interpreting phylogeny of the group (3,6,12,15).

Shepherd et al. (12) found that in enlarged females, additional BW cuticle layers are added to the basic A and B layers present in vermiform second-stage juveniles (J2) and males. In species with lemon-shaped females, only one additional layer, C, was observed, whereas in species with round females, two additional layers, C and D, were reported. These observations strengthened the distinction between lemon-shaped and round-shaped cyst nematodes and support Skarbilovich's (13) 1959 placement of them, respectively, in separate subgenera, *Heterodera* and *Globodera*. Differences in cuticle layering also contributed to the eventual elevation of these subgenera to genera (4).

Investigations of BW cuticle layering of females were expanded to additional genera of Heteroderinae, and the phyloge-

netic significance was discussed (1,6). It was suggested that the absence of layer D in *Heterodera* females is due to a secondary loss or, alternatively, that parallel evolution of the cyst occurred in *Heterodera* and *Globodera*.

Subsequently, Wouts (15) proposed a complex phylogenetic scheme that favored evolution of Heteroderinae with a D layer (e.g., *Globodera*), independent from those reported to lack a D layer (e.g., *Heterodera*). Conversely, Baldwin and Schouest (3) proposed a computer generated (PAUP) parsimonious phylogenetic scheme that predicted secondary loss of the D layer in *Heterodera* as the most plausible hypothesis.

The hypothesized secondary loss of the D layer in *Heterodera* may be testable by additional fine-structural investigations. Shepherd et al. (12) described cuticle layers only at the midbody of young (unfertilized?) females of *H. schachtii* obtained from the greenhouse or field. The D layer was not detected in any of six species of *Heterodera*. This has been the basis of considerable controversy concerning the phylogeny of Heteroderinae. Baldwin (2) suggested the need to consider layering throughout the body of the specimens since in some Heteroderinae the D layer is more evident at the terminal region than at midbody. Ontogeny may also provide new insight on the body wall cuticle of Heteroderinae (9) and, specifically, the possible loss of the D layer in *Heterodera*. Shepherd

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et al. (12) noted that appearance and thickness of some layers depend on age.

In a study of the cone development in *Heterodera*, Cordero (7) presented evidence for the morphological variability of BW cuticle of the cone depending on the age of the specimens. It was found that as females mature, layers in addition to A, B, and C layers are added to the BW cuticle in the cone of *H. schachtii*. We propose further investigations to determine whether a thin D layer occurs in regions other than the cone and to examine possible changes in the layers of the midbody as *H. schachtii* matures.

#### MATERIALS AND METHODS

*Heterodera schachtii* was monoxenically cultured on *Beta vulgaris* L. cv. USH 11 Holly hybrid large, and females and cysts of known age were prepared for transmission electron microscopy (TEM). Sugar beet seeds were surface sterilized in 10% commercial bleach (0.525% NaOCl solution) for 10 minutes and germinated on 1% water agar. Germinating seeds were transferred to petri dishes containing Gamborg's B-5 plus White's organic nutrients at pH 6.5 (10,14). Gelrite (0.25%) was used as a gelling agent. Second-stage juveniles of *H. schachtii* were surface sterilized by four rinses with sterile water followed by 10-minute incubation in saturated aqueous Rifampicin solution. Prior to inoculation the J2 were again rinsed with sterile water. Cultures were incubated at 25 C with 16 hours illumination per day (3,000 lux, GroLux).

Infection was monitored using a Nikon inverted microscope equipped with Hoffman interference optics and a Garr time-lapse high resolution video recorder. Establishment of feeding site was recorded as time zero in the nematode's life cycle. Development of a given specimen was recorded and studied or stopped at a particular point for morphological studies. Thus, the time required by the nematode to achieve a certain developmental point un-

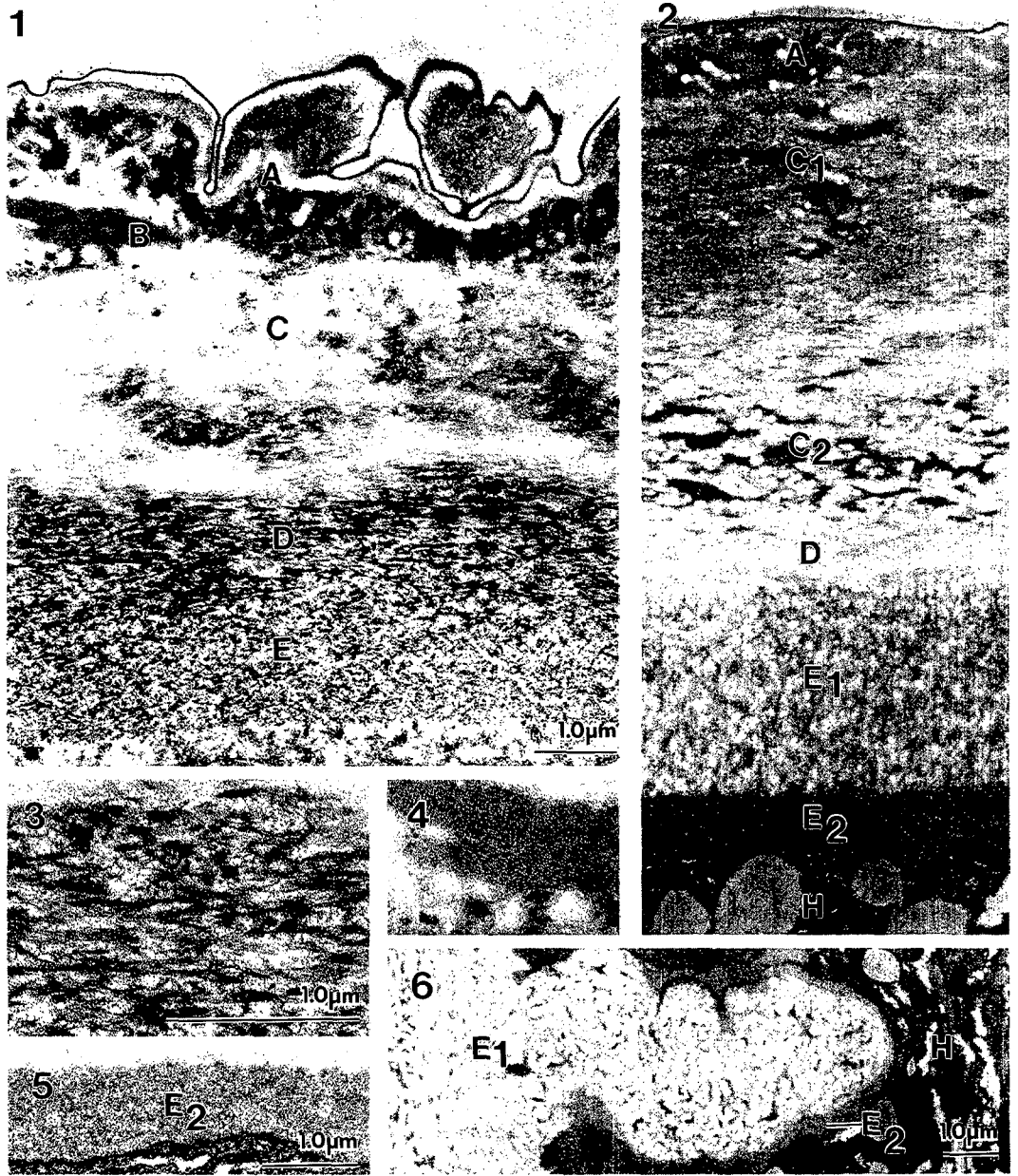
der our experimental conditions was determined.

Specimen preparation for TEM was as follows: Females were fixed 2 minutes at 60 C in a modified Karnovsky's fixative (11). The aqueous fixative solution included 2% paraformaldehyde, 1.5% glutaraldehyde, 0.2 M sucrose, and 0.1 M sodium cacodylate buffer. After the fixative had reached room temperature, the anterior end of each specimen was excised and removed. Fixation of the rest of the body continued overnight at room temperature. Specimens were rinsed 15 minutes in 0.2 M sodium cacodylate buffer; then fixation proceeded overnight at room temperature in an aqueous aldehyde-peroxide solution (5) which was modified to include 0.2 M sucrose and 0.2 M sodium cacodylate. All solutions were buffered at pH 7.2.

Postfixation was with osmium tetroxide fumes. Females were rinsed continuously for 30 minutes with 0.2 M sodium cacodylate buffer. They were left in a thin film of buffer and exposed to fumes from osmium tetroxide crystals in a storage dish sealed with Parafilm for a minimum of 4 hours at room temperature.

Dehydration was in a graduated ethanol series at 30-minute intervals, followed by slow infiltration with the firm formulation of Spurr's epoxy for 3 days. Sections were mounted on formvar carbon-coated grids. Staining was as reported by Baldwin (1), and specimens were examined using a Hitachi 600 TEM at 75 kV.

Terminology for BW cuticular layers is as previously established in the literature with A, the *outer* layer, being at the surface of the nematode, and layers B, C, D, and E progressively *internal* toward the hypodermis (1,6,12). Similarly, zone 1 of a given layer is closest to the outer surface of the nematode, relative to zones 2 and 3. Thickness of the BW cuticle is based on distance from the outer surface to the hypodermis as viewed in cross section. Thickness of cuticular layers or zones is based on the distance from the outer to the inner boundary of the layer or zone as viewed in cross section.



FIGS. 1–6. Transmission electron micrographs of cross sections of *Heterodera schachtii* females. 1) Body wall cuticle of a mature (ca. 4 weeks past final molt) female just anterior to the cone. Letters A, B, C, D, and E correspond to cuticular layers. 2) Midbody region of the body wall cuticle of an old (ca. 8 weeks past the final molt) *Heterodera schachtii* female just prior to cyst formation. Letters and scale same as Figure 1. Subscripts indicate zones within layers. H = hypodermis. 3) D layer showing fibers at midbody in a mature female. 4) Enlargement of a B layer from Figure 1. 5) Fine fibers of E<sub>2</sub> in the cone. 6) Bullae composed of zones E<sub>1</sub> and E<sub>2</sub> in the cone of an old *Heterodera schachtii* female. H = hypodermis.

RESULTS

The BW cuticle of *H. schachtii* is more complex than indicated by previous studies (Figs. 1, 2). In addition to the typical mid-

body A, B, and C layers, discussed below, a D layer and a two-zone E layer are also present in mature females. These additional layers, D and E, have not been pre-

viously reported. They were first observed at 4 weeks after the final molt (Figs. 1, 2) and were absent in younger specimens. Layer D is typically thin (ca. 0.8  $\mu\text{m}$  thick) and consists of helicoidally arranged fibers which are 20–30 nm thick (Fig. 3). Only two or three repeats of the helical pattern occur within a given region of the thin D layer. Internal to layer D, zone E<sub>1</sub> averages 3  $\mu\text{m}$  thick and consists of swirled fibers. The innermost zone E<sub>2</sub> has a flat edge contiguous with zone E<sub>1</sub> and an irregular margin adjacent to the hypodermis (Figs. 2, 5). Zone E<sub>2</sub> is about 1  $\mu\text{m}$  thick and consists of randomly oriented fine fibers (Fig. 5). Layer E, with zone E<sub>1</sub> and finally zone E<sub>2</sub>, appears later in development than layer D. The overall thickness of the cuticle is about 11  $\mu\text{m}$ .

Layers D and E are contiguous throughout the body up to the base of the vulva cone where the body contour changes. In this transitional region, layer D is greatly reduced and is absent in the cone. In the posteriormost part of the cone of mature females, zones E<sub>1</sub> and E<sub>2</sub> project into the body giving rise to bullae (Fig. 6). More posteriorly, zone E<sub>2</sub> becomes thinner and finally disappears. Conversely, zone E<sub>1</sub> widens as bullae enlarge.

Although previously reported layers A, B, and C are typical of other Heteroderinae, morphological variations exist within these layers. At midbody, layer A is relatively smooth without deep ridges (Fig. 2), whereas posteriorly distinct ridges are present (Fig. 1). The ridges are particularly conspicuous in young females. At midbody, layer A is thinner and its dark inclusions are more compact and less consistently observed than posteriorly in the same specimen. Layer B is difficult to discern at midbody, but it is clearly defined posteriorly (Figs. 1, 4). Layer C is resolved into two or three zones. The fine fibers in layer C<sub>1</sub> are oriented longitudinal to the body axis at midbody, but these fibers are irregularly oriented at the posterior end of the cone. Zones C<sub>1</sub> and C<sub>2</sub> are clearly defined at midbody; however, C<sub>1</sub> predominates at the posterior end of the cone.

## DISCUSSION

Layers of the female cuticle are a reliable character for testing hypotheses of phylogeny of Heteroderinae. Existence of a thin D layer, limited to portions of mature *Heterodera*, strengthens the proposed monophyly of cyst-forming Heteroderinae (1,3,6,8,9). Phylogeny of Heteroderinae has been controversial because of perceived inconsistencies in distribution of the D layer among cyst-forming species. However, the presence of a thin D layer in *Heterodera*, as well as parsimony arguments (3), supports the hypothesis of evolution of the cyst from a unique common ancestor and weakens the hypothesis of parallel development of the cyst. In addition, layer D also occurs in non-cyst-forming genera in Heteroderinae, and computer generated parsimonious phylogenetic analysis (PAUP) suggests monophyly of the genera sharing a D layer (3). Presence of a D layer, together with additional characters, supports ataloderines, which lack cysts, as a sister group of heteroderines (3).

*Afenestrata africana* Baldwin and Bell, 1985, a cyst-forming Heteroderinae, is considered a sister group of *Heterodera*, but a D layer has not been observed in *A. africana* (6). However, only field specimens of unknown age were available for this study. These were previously fixed in lactophenol, and it is not known which of the structural features may have been obscured. Developmental studies of the *A. africana* female cuticle may indicate a D layer in at least portions of the body. A plausible alternative to the hypothesis of a limited D layer in *A. africana* is that a transformation series ranging from a reduced D layer to none at all occurs within *Heterodera* and sister groups *Afenestrata* and *Cactodera betulae* (6). It should be noted, however, that *C. betulae* is distinct from other *Cactodera* by the apparent absence of layer D and by a number of additional characters (3,6). Shepherd et al. (12) indicated that the D layer was not only absent in *H. schachtii*, but that it was absent in five additional species of *Heterodera* examined; however,

a TEM micrograph is published only of *H. avenae*. Further investigation of development of the BW cuticle in a range of species of *Heterodera* could suggest complete loss of layer D in *H. avenae*, as well as some other species and species groups. Use of specific labels such as monoclonal antibodies to layer D would be particularly useful in screening for the layer and in establishing homologies of layers.

Presently, the D layer in Heteroderinae is interpreted as only two states, presence or absence of the layer (3,15). Detection of a greatly reduced D layer in older specimens of *Heterodera* could be interpreted as a third character state. We have noted that a transformation series might occur with additional character states, including further reduction of the D layer. In addition, the distribution and assembly of fibers of the D layer may be considered as additional character states. Layer D is absent from the cone of *Heterodera*, but it is present in the cone of *Cactodera cacti* (7). The D layer of these two genera is similar in the way the fibers are arranged. However, the arrangement of fibers of layer D in *Heterodera* differs from that of *Atalodera* spp. Wouts and Sher, 1971, *Sarisodera hydrophila* Wouts, 1971, *Thecavermiculatus gracililancea* Robbins, 1978, and *Bellodera* Wouts, 1985 (1,6, Baldwin, unpubl. obs.). In these non-cyst-forming species, the D layer is thick (5–7  $\mu\text{m}$ ) and is most pronounced in the cone. The arrangement of fibers in layer D of *Globodera* is distinct from that of *H. schachtii* because the D layer is broader (6–8  $\mu\text{m}$ ) and the periodicity of the pattern is repeated less frequently in *Globodera* (12). Such detailed differences in patterns of layer D may be useful for phylogenetic analysis of Heteroderinae and require further consideration.

Although an E layer was previously described for the non-cyst-forming heteroderine, *Atalodera ucrici* (6), this is the first report of zones E<sub>1</sub> and E<sub>2</sub>. Zone E<sub>2</sub> of *H. schachtii* is thin and may be homologous with the single zone E layer described for *Atalodera ucrici*. In both genera, the zones consist of randomly oriented fine fibers ad-

acent to the hypodermis. A similar layer also occurs in *Bellodera* (Baldwin, unpubl. obs.). Contrary to the single zone E layer in *A. ucrici*, zone E<sub>1</sub> in *H. schachtii* consists of swirled fibers, and homology of these layers is unlikely. Although zone E<sub>1</sub> has been described only in *H. schachtii* and associated with bullae, zone E<sub>1</sub> may be widely distributed among heteroderines. Evidence of E<sub>1</sub> should be investigated in the cone region of older specimens in additional species that form bullae.

In the present study, we detected differences in thickness within a given layer and zone at various body levels in the same specimen. These differences in thickness are particularly evident in zone A<sub>3</sub> between the midbody and the posterior region. In addition, Cordero (7) noted that in the cone of *H. schachtii*, cuticle layering changes with age and that the BW cuticle is completely developed only in cones of older specimens. These findings indicate that thickness of BW cuticle layers should be used as a character with caution, unless a standard body region and age of the specimen can be identified for these measurements. Deep invaginations of the cuticle of *H. schachtii* as illustrated by Shepherd et al. (12 [Fig. 9a]) are characteristic of young specimens as well as of the cone region. We suggest that the D layer was not previously observed (1,12) in *H. schachtii* because the females examined were too young; even in mature specimens it is thin and obscure. We have noted that in other heteroderines (e.g., *Globodera*) the D layer is thick and, unlike *H. schachtii*, apparently occurs in freshly molted adult females. Although the D layer is absent in the female cone of *H. schachtii*, in other genera (e.g., *Atalodera*) the D layer is thickest and most pronounced in the posterior region. We are convinced that in still other genera, including *Verutus*, *Meloidodera*, and *Cryphodera*, the D layer is absent regardless of age and body region (1). Future studies of female BW cuticle in Heteroderinae should investigate changes in the cuticle layers during postembryogenesis and throughout the different body regions. Such studies

should lead to a more sophisticated usage of cuticular layers as increasingly reliable for testing hypotheses of phylogeny in Heteroderinae.

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