# Ultrastructure of the eggshell of *Heterodera schachtii* and *H. glycines* (Nematoda : Tylenchida)

Roland N. PERRY and Marcus W. TRETT

*Department of Nematology, Rothamsted Experimental Station, Harpenden,*
*Ultrastructure de la coque de l'œuf de* Heterodera schachtii *et* H. glycines *(Nematoda : Tylenchida)*

SUMMARY

Ultrastructural studies demonstrate that the eggshells of *Heterodera schachtii* and *H. glycines* consist of a chitinous layer and a lipid layer. The chitinous layer comprises an osmiophilic outer portion and a more substantial, less-densely staining inner portion. The lipid layer is composed of a thin, amorphous outer layer external to a predominantly tetra- or pentalaminate inner layer. An outer vitelline membrane was not identified in either species. During embryonation, a secondary vitelline membrane, initially indistinguishable from the inner lipid layer, separates to form the epicuticle as the first-stage juvenile cuticle is secreted. The inner lipid layer was not detected in eggs of *H. schachtii* from cysts with extensive fungal contamination and this is discussed in context of possible fungal enzyme action and mechanical abrasion by the enclosed juvenile.

RÉSUMÉ

*Ultrastructure de la coque de l'œuf de* Heterodera schachtii *et* H. glycines *(Nematoda : Tylenchida)*

L'étude ultrastructurale de la coque de l'œuf chez *Heterodera schachtii* et *H. glycines* a montré que celle-ci comprend une couche chitineuse et une couche interne lipidique. La couche chitineuse est constituée d'une partie externe osmiophile et d'une partie interne, plus importante, moins densément colorée. La couche lipidique est composée d'une fine couche basale et d'une membrane plus importante comportant elle-même quatre ou cinq couches. Lors de la formation de l'embryon, une membrane vitelline secondaire est formée, qui au début ne se distingue pas de la membrane de l'œuf et qui se sépare ensuite pour former l'épicuticule lorsque la cuticule du juvénile de premier stade est secrétée. Ces membranes de la coque de l'œuf n'ont pu être distinguées sur les œufs de *H. schachtii* provenant de kystes très contaminés par des champignons; ce point est discuté dans le contexte d'une éventuelle action enzymatique du champignon et d'une abrasion mécanique par le juvénile.

The precursor of enhanced juvenile metabolism and subsequent activity leading to hatch of *Globodera rostochiensis* appears to be a change in eggshell permeability which is likely to be governed primarily by the lipid layer (Perry, 1986). Following the discovery of this layer in the eggshell of *G. rostochiensis* (Perry, Wharton & Clarke, 1982), the nature of the permeability change was examined by Clarke and Perry (1985). They considered that hatching agents, by binding to or replacing membrane bound $Ca^{2+}$, may change lipoprotein membrane structure. In several respects, the changes in $Ca^{2+}$ content of *Heterodera schachtii* eggshells in response to various treatments parallel the pattern for *G. rostochiensis* indicating the existence of lipoprotein membranes in eggshells of *H. schachtii* (Clarke & Perry, 1986).

The hatching mechanism of *H. glycines* appears to involve zinc rather than $Ca^{2+}$ and eclosion may be mediated by a zinc-dependent enzyme (Tefft & Bone, 1984). The possible involvement of enzymes in altering eggshell permeability was reviewed by Perry and Clarke (1981) and recently Tefft and Bone (1985) reported the occurrence of leucine aminopeptidase activity in the egg supernatent of *H. glycines*.

Eggshell permeability changes may, therefore, be central to the hatching of *H. schachtii* and *H. glycines* but the presence of lipoprotein membranes remains to be confirmed. We have now examined the eggshell ultrastructure of these two species using a novel cryofracture-fixation technique (Trett, in preparation).

## Materials and methods

Cysts of *H. glycines* were obtained from pot-grown soybean plants. Cysts of *H. schachtii* originated from two sources : either from a field population after sugar beet stored in moist soil for 12 months at 5° or from the same population increased on cabbage plants (cv. Hispi) grown in pots containing steam-sterilised loam. The latter cysts had virtually no fungal contamination. In both cases, cysts were extracted from moist soil (Shepherd, 1970). Difficulties in preparation of nematode eggs for

transmission electron microscopy (Bird, 1971) have led to the development of a cryofracture-fixation technique that has given better results with *H. schachtii* and *H. glycines* than earlier methods (Perry, Wharton & Clarke, 1982). In brief, cysts, held between aluminium foil sheets and frozen in liquid nitrogen (— 170°), were fractured and permitted to thaw at 4° in 2.5 % glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.2; 280 mOs $kg^{-1}$) containing 11 mM calcium chloride and 56 mM magnesium chloride (Trett, in preparation). Subsequent processing followed the methods of Trett (1984) and Trett and Perry (1986). Ultrathin sections were viewed in a Philips 201 electron microscope at 100 kV accelerating voltage.

## Results

The eggshells of *H. schachtii* and *H. glycines* appeared identical in structure. In each the chitinous layer (CL) comprises a densely-staining outer layer (OCL), 0.02-0.05 µm thick and a more substantial inner layer (ICL), 0.25-0.30 µm thick (Figs 1a, b, d and 2). Throughout, the chitinous layer is fine textured and granular. High magnification of this layer indicated a fibrous sub-structure. In both species the chitinous layer lies immediately external to the lipid layer (LL) which is composed of a thin, amorphous outer layer (OLL) and a multi-laminate, membrane-like structure, termed here the inner lipid layer (ILL; Figs 1b and 2). Unlike the chitinous layer, the outer lipid layer has a less compacted structure that probably results from leaching of saturated lipids during processing; different dehydration regimes significantly affected the appearance of this layer.

Although the inner lipid layer of some specimens was found to comprise six laminae, tetra- and pentalaminate conditions predominate (Fig. 1c). In both species the inner lipid layer measures between 0.02 and 0.03 µm thick and exhibits a lamina separation of approximately 30 nm (Fig. 1c). This layer was frequently seen to be detached from the outer lipid layer (Figs 1b and d) but, with the exception of small remnants, could not be located in eggshells of *H. schachtii* taken from the field cyst population.

Examination of embryonating eggs showed that, during early stages of development, the secondary vitelline membrane (SVM) is closely apposed against the inner lipid layer, from which it is indistinguishable. However, where embryonating cells (EJ) have separated from the eggshell, discrete membranes can be identified (Fig. 1a). Later in development, the secondary vitelline membrane becomes more permanently separated as the neotenic, first-stage juvenile cuticle is secreted beneath it and it becomes the epicuticle (EP; Fig. 2). A further feature observed in several eggshells of both *H. schachtii* and *H. glycines* was the presence of densely-staining, flocculant material adhering to the outer surfaces (UM; Fig. 1d). This frequently formed a continuous outer layer and closely resembled degenerating uterine cells.

## Discussion

Despite some confusion in the terminology used, the fine structure of the eggshells of *H. schachtii* and *H. glycines* appears to conform to that described for other tylenchids (Bird, 1968; Bird & McClure, 1976; Wharton, 1980; Perry, Wharton & Clarke, 1982). However, the outer vitelline membrane, present in the eggs of *Meloidogyne javanica*, *Rotylenchus reniformis*, *Tylenchulus semipenetrans* and *Pratylenchus minyus* (Bird & McClure, 1976), was not identified in eggs of either species examined in the present study. The significance of this is uncertain. It is possible that the outer vitelline membrane degenerates within the uterus after eggshell formation. This may not be true of all members of the Heteroderidae as an outer " vitelline layer " has been reported in the eggshell of *G. rostochiensis* (Perry, Wharton & Clarke, 1982).

The sub-division of the chitinous layer into distinctive outer and inner components (Fig. 2) is justified on the grounds of their differing chemical composition. Whilst both have the same fine, granular sub-structure, which would appear to reflect the presence of fibrous sub-units, the outer chitinous layer is markedly more osmiophilic than the inner layer. This is probably not an artefact resulting from slow penetration of osmium fixative into the eggshells as prolonged osmification did not increase the observed thickness of the outer layer. It is possible that the outermost layer represents a zone into which unsaturated lipids, or substances with similar chemical properties, have diffused from the uterus. Steric hindrance or cross-reaction with the chitin and/or proteins of this region, during eggshell formation, may determine the thickness of this layer. The specific hydrophilic/lipophilic balance of this boundary layer will be of considerable importance in determining the relative mobility of diffusable substances, such as root diffusates, within the eggshell.

The presence of the lipid layer may also place constraints on the movement of substances through the eggshell in either direction. Eggshell permeability changes after initial embryonation have been observed in hookworms of the genus *Ancylostoma* (Matthews, 1985) and it is possible that similar changes occur in *H. schachtii* and *H. glycines* during embryonation but prior to permeability changes induced by hatching agents (Clarke & Perry, 1986). If such changes do occur during egg development, they may correlate with the separation of the secondary vitelline membrane from the inner lipid layer observed in the present study.

In fully embryonated eggs of *G. rostochiensis*, structural changes in the lipoprotein, membrane-like

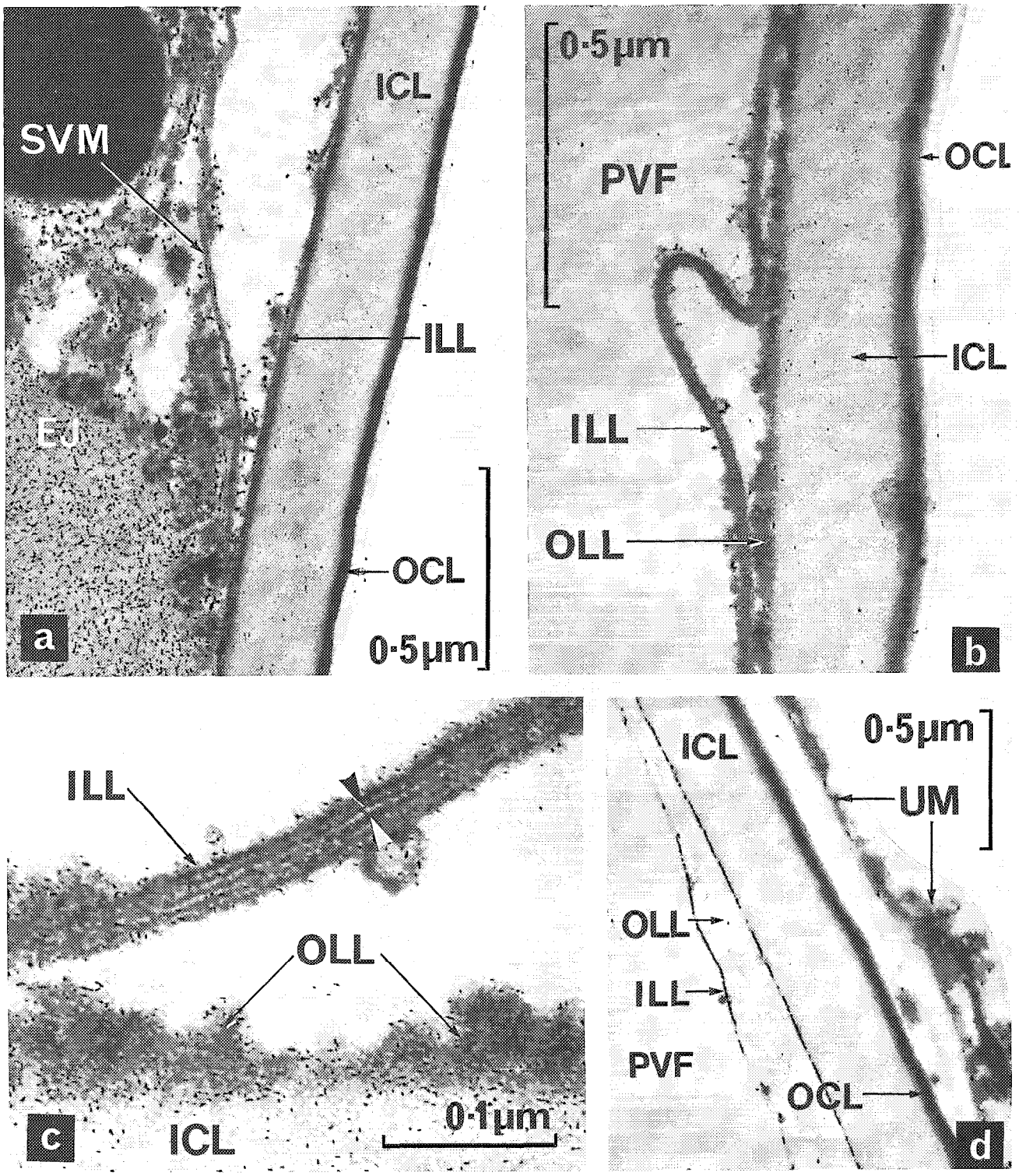
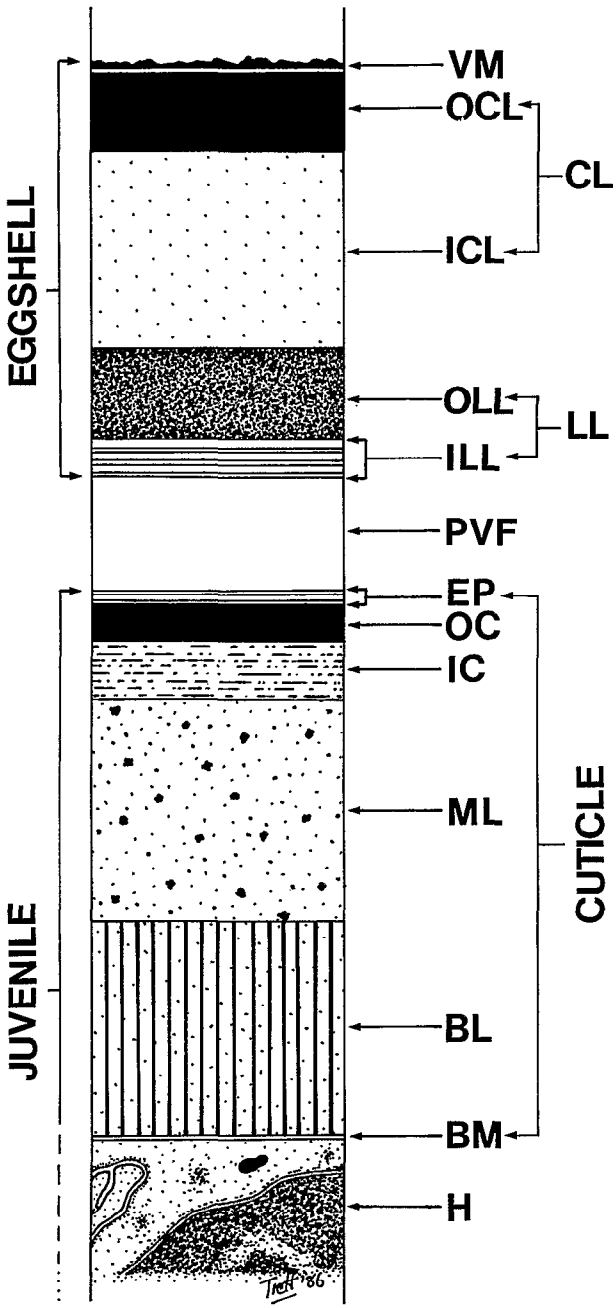



Fig. 1. Sections through the eggshells of *H. schachtii* and *H. glycines*. a : Section through the shell of an embryonating egg of *H. schachtii* showing separate secondary vitelline membrane (SVM) and inner lipid layer (ILL); b : Eggshell of embryonated *H. schachtii* showing inner lipid layer (ILL) detached from amorphus outer lipid layer (OLL); c : Detail of same inner lipid layer (ILL) shown in 1b. Note multilaminate condition with a mean lamina separation of 30 nm (arrowheads); d : Sections through the shell of an embryonated egg of *H. glycines* showing detached coating of densely-staining material (UM) that resembles degenerating remnants of uterus.

Key to abbreviations used in figures — BL : basal layer; BM : basal membrane; CL : chitinous layer; EJ : tissues of embryonating juvenile; EP : epicuticle; H : hypodermis; IC : inner cortical layer; ICL : inner chitinous layer; ILL : inner lipid layer; LL : lipid layer; ML : median layer; OC : outer cortical layer; OCL : outer chitinous layer; OLL : outer lipid layer; PVF : perivitelline fluid/space; SVM : secondary vitelline membrane; UM : uterine material; VM : vitelline membrane/vitelline layer.




structures of the lipid layer may result from hatching agents binding to or replacing membrane-bound $Ca^{2+}$ (Clarke & Perry, 1985); this may account for the observed changes in eggshell permeability that lead to hatching of the juvenile. Similar changes in the $Ca^{2+}$ content of eggshells in response to treatment with hatching agents were found in *H. schachtii* (Clark & Perry, 1986). As the unit membranes of both the inner lipid layer and the juvenile epicuticle are derived directly from the secondary vitelline membrane of the embryo, both probably contain the same protein moieties. Consequently, host root diffusates may exert a direct effect on the juveniles as well as the eggshell reflecting the bimodal action of diffusates on cyst nematodes such as *G. rostochiensis* (Perry, 1986).

The presence of an inner lipid layer in eggs from cysts of *H. schachtii* from cabbage plants and its absence in eggs from the field may be highly significant. Hatching tests (Perry & Beane, unpubl.) indicate that the presence of such membrane-like structures in encysted eggs of *H. schachtii* correlates with negligible hatch in water; a large water hatch may be obtained only when this layer has become disrupted.

It is unlikely that the cysts of *H. schachtii* from cabbage or sugar beet will have experienced differences in exposure to active host diffusates during development, as cabbage root diffusates enhance total hatch and rate of hatch in the same way as sugar beet root diffusates (Shepherd, 1962). The main difference in the two batches of cysts is in the degree of fungal contamination. Cysts from cabbage contain virtually no fungal growth whereas those from sugar beet were almost all internally contaminated with fungus. Although this did not affect hatch in response to host root diffusates (Perry & Beane, unpubl.) it may provide an explanation for the disappearance of the inner lipid layer; fungal enzymes, such as lipases, could penetrate the eggshell and disrupt both inner and outer lipid layers. In addition, the inner lipid layer may also be broken down by mechanical action of the juvenile while cysts were in the field as envisaged by Wilson (1958) in the hatching process of *Trichostrongylus retortaeformis*. The osmotic pressure of perivitelline fluid in eggs of *H. schachtii* is sufficiently low to permit movement of the unhatched juveniles (Perry, Clarke & Hennessey, 1980). These and other factors involved in disruption of the eggshell membrane are currently under investigation.

Fig. 2. Diagrammatic reconstruction of a section through the eggshell and juvenile cuticle of a generalised embryonated tylenchid egg. Terminology used to define eggshell layers attempts to unify and rationalise that employed by earlier workers. The vitelline membrane (VM) has not been observed in eggs of *H. schachtii* and *H. glycines*. The outer chitinous layer (OCL) and outer lipid layer (OLL) may vary in thickness between species and, in the case of the outer lipid layer, may be extremely reduced. The epicuticle (EP), which may also be multilaminate, is derived from the secondary vitelline membrane and is closely apposed against, and indistinguishable from, the inner lipid layer (ILL) during early stages of development.

Abbreviations : see legend of Figure 1.

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