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Morphogenesis of Larval Cuticle in the Polychaete *Phragmatopoma lapidosa*

A Correlated Scanning and Transmission Electron Microscopic Study from Egg Envelope Formation to Larval Metamorphosis

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Summary. The development of the egg envelope and its incorporation into the larval cuticle of the polychaete *Phragmatopoma lapidosa,* was studied by correlative scanning and transmission electron microscopy. The mature egg possesses an envelope composed of five zones including an outer granular zone formed by the tips of the egg microvilli. The formation of the granules is described and their functions are discussed. The entire egg envelope is retained as the larval cuticle up to the 16 h trochophore stage. From this stage to about the 60 h larval stage, the envelope is gradually lost and replaced by a cuticle consisting of branching microvilli. The cuticle of the 20 day larva is composed of highly branching microvilli penetrating a homogeneous electron opaque cuticle. The possible functions of the cuticle among the Annelida are discussed.

Key words: Egg envelope – Polychaeta, Sabellariidae – Larva – Cuticle – Fine structure.

Introduction

The fate of the egg envelope in the developing embryo of polychaetes varies widely according to the literature. Those reported to lose their egg envelope and form a new cuticle include *Perinereis marionii, P. cultrifera,* and *Nereis irrorata* (Herpin, 1926), *Capitella capitata* (Eisig, 1898), and *Spirorbis morchi* (Potswald, 1965), and those reported to incorporate the egg envelope into the larval cuticle include

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Sabellaria alveolata (Wilson, 1929), *S. vulgaris* (Novikoff, 1938), *Nephthys hombergi* and *Pectinaria koreni* (Wilson, 1936), *Capitellides giardi* (Day, 1937), *Diopatra cuprea* and *Autolytus faciatus* (Allen, 1959; 1964) and *Pomatoceros triqueter* (Segrove, 1941). *Arenicola cristata* is a case of conflicting reports as Wilson (1883) noted that the egg envelope was retained as the larval cuticle while Okada (1941) said it was lost. Similarly, Dales (1950) stated that the egg envelope of *Nereis diversicolor* is transformed into the larval cuticle but Smith (1964) said it is not retained.

As far as we know, no studies to date have investigated the ontogeny of the egg envelope and its subsequent fate during larval development in any polychaete. The purpose of this paper is to describe the development and fate of the egg envelope in the reef-building polychaete, *Phragmatopoma lapidosa* Kinberg 1887, using correlative transmission and scanning electron microscopy. Larval development in *P. lapidosa* has been reported previously (Eckelbarger, 1976; Eckelbarger and Chia, 1976).

Materials and Methods

Sexually mature females of *Phragmatopoma lapidosa* were collected during the summer of 1975 at Seminole Shores, Hutchinson Island, Martin County, Florida and maintained in the Johnson Science Laboratory at the Harbor Branch Foundation. Larval stages were reared in the laboratory from artificially-fertilized eggs following the procedures of Eckelbarger (1975). Procedures for specimen preparation for scanning electron microscopy (SEM) were previously reported in Eckelbarger and Chia (1976). For transmission electron microscopy (TEM), we encountered some fixation difficulties and hence the procedures were modified for fixing larval stages. Sexually mature females and juveniles were fixed for $1\,^1/_2$ h using the procedure for SEM tissue preparation. Late larval stages were also prepared with this procedure except for being fixed for one hour in a 5% glutaraldehyde solution rather than a 2.5% solution. Trochophores were fixed for one hour in a mixture of 6% glutaraldehyde and 1% paraformaldehyde buffered in 3% sucrose and 0.2 M Millonig's phosphate buffer (Millonig, 1961) at4° C. Specimens were then washed for 1 h at 4° C in three changes of 0.1 M Millonig's phosphate buffer containing 6% sucrose followed by post-fixation for one hour in 1% OsO_4 in 0.1 M Millonig's phosphate buffer at 21–23° C. Following fixation, tissue was dehydrated in ethanol, exchanged in propylene oxide and embedded in Epon after Luft (1961). Thin sections were cut on a Porter-Blum MT2-B ultramicrotome with a diamond knife, stained first with saturated uranyl acetate (Watson, 1958) and secondly with lead citrate (Reynolds, 1963), and examined with a Zeiss EM9-S2 electron microscope.

Results

Intraovarian Oocytes

The ovaries of *Phragmatopoma lapidosa* are paired structures located on the blood vessels of the intersegmental septa of the abdominal segments. (Fig. 1). The developing oocytes remain attached to the germinal epithelium throughout oogenesis whereupon they detach and float freely in the coelomic fluid.

In small, previtellogenic, intraovarian oocytes approximately 30–40 μm in diameter, the oolemma is elaborated into simple, non-branching microvilli with slightly swollen tips (Fig. 2). The microvilli are about 0.5 μm in length and their tips possess glycocalyx and an electron dense region along the inside of the plasma

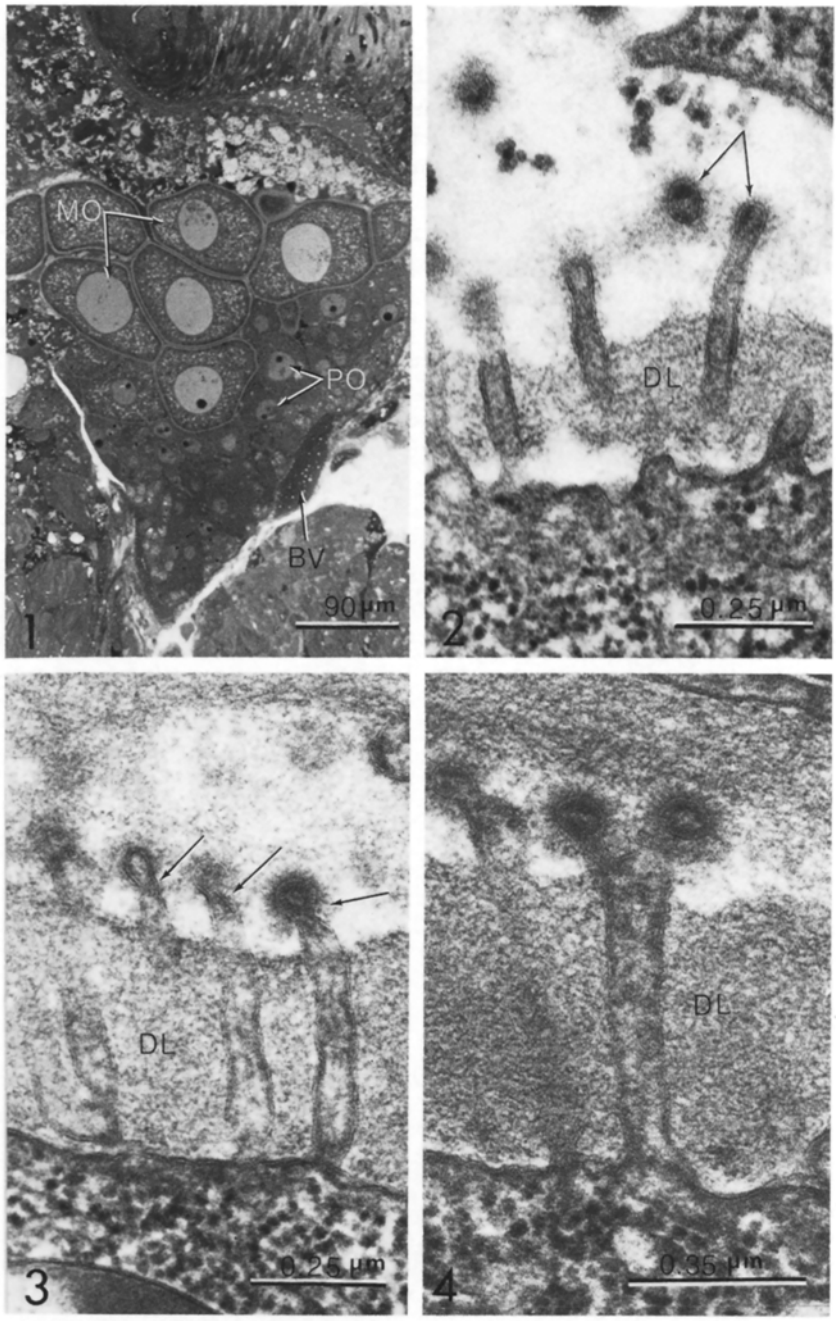

Fig. 1. Ovary attached to blood vessels (*BV*) of intersegmental septa with previtellogenic oocytes (*PO*) and mature oocytes (*MO*) visible

Fig. 2. Microvilli of previtellogenic oocyte showing electron dense material at tips (arrows) and developing dense layer (*DL*) of egg envelope

Fig. 3. Constriction of tips of microvilli (arrows). Note that the trilaminar membrane of each microvillus is still recognizable. *DL* dense layer

Fig. 4. Bifurcation of microvillus forming two surface granules. *DL* dense layer

membrane. An ill-defined, moderately electron dense, flocculent layer approximately 0.25–0.3 μm in thickness extends from the base of each microvillus to about half way to the tip. As vitellogenesis begins, the tips of the microvilli become slightly constricted (Fig. 3). The flocculent layer is now more electron dense, more uniform in thickness and extends to just below the tips of the microvilli. In the late stages of vitellogenesis, each microvillus bifurcates, and forms two spherical granules (Fig. 4). At this stage, the granule still possesses a trilaminar membrane surrounding an electron dense core.

In the mature egg (Figs. 5 and 6), the egg envelope has a more ordered appearance and consists of 5 definable zones totalling about 3.0 μm in thickness. Zone I is about 0.8 μm thick, lies just outside the oolemma, and consists of a loose network of fine, irregular fibrils. Zone II is about 1.5 μm thick and is composed of a moderately electron-dense layer of what appear to be closely packed fibrils. A tangential section through this zone, however, reveals it consists of the fine filaments of glycocalyx surrounding regularly-spaced microvilli (Fig. 7). Zone III is a thin electron-dense layer about 360 Å in thickness. Zone IV is composed of a monolayer of spherical granules about 0.145 μm in diameter which are spaced apart 160–240 Å and are resting on Zone III (Figs. 6 and 8). Extending from each granule are a number of long, irregular mucus strands which form a thin, PAS-positive jelly coat about 0.4 μm in thickness which has been designated Zone V (Fig. 8). A tangential section through Zone IV shows that each granule is composed of at least three concentric layers: an inner, dense core; a less dense intermediate layer and a lightly staining outer layer of radiating fibrils (Fig. 9). The trilaminar membrane which previously surrounded each granule (Figs. 2–4) is no longer recognizable.

Freshly spawned eggs are irregular in shape (Fig. 10) but soon become round in seawater. Examination of the egg envelope with SEM reveals the regularity of the surface granules (Fig. 11). It is possible to examine the underlying layer (Zone III) with SEM by mechanically abrading the egg surface and removing the granules. This layer is covered by a regular pattern of pits or depressions left by the granules. (Figs. 12 and 13). A comparison of the number of granules and microvilli in a given area of the egg surface results in a ratio of about 10 granules to every microvillus. Based on the known surface area of the mature egg and the diameter of each granule, it is estimated that approximately 5000 granules and 500 microvilli cover the egg surface.

Fertilized Egg

At fertilization the microvilli withdraw from the outer surface of the egg envelope to Zone I (Fig. 14), leaving behind faintly visible channels in Zone II which are easily observed in tangential section (Figs. 15 and 16), and isolating the surface granules from the oolemma. The irregular fibrils observed in Zone I of the unfertilized egg (Fig. 6) are still present, but a large number of small, randomly distributed, spherical particles approximately 180 Å in diameter have now appeared (Fig. 17). Zone II of the fertilized egg differs from that of the unfertilized egg (Figs. 6–8) by appearing to be more electron dense and composed of a fine granular material (Fig. 16).

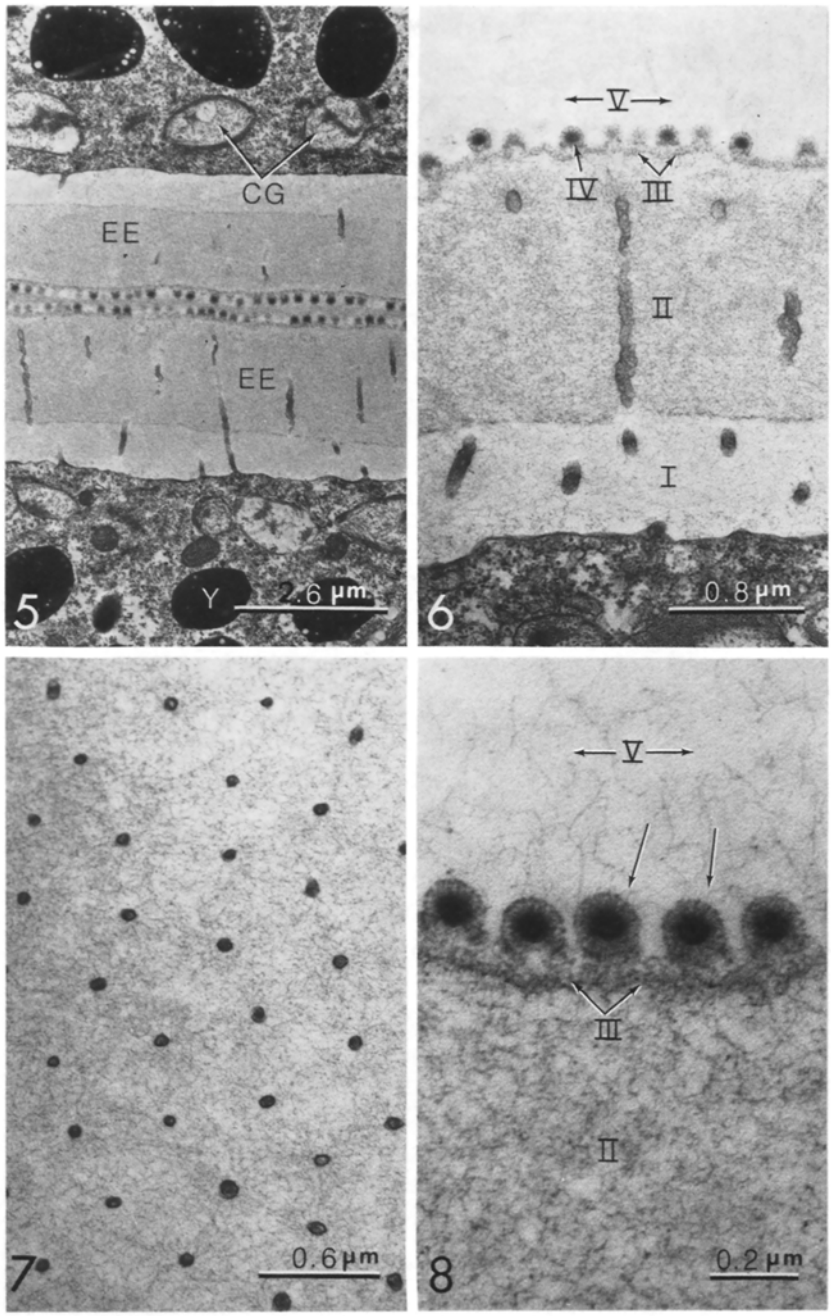

Fig. 5. Egg envelopes of two mature eggs within the ovary showing cortical granules (*CG*) adjacent to the oolemma. *Y* yolk

Fig. 6. Egg envelope of mature egg showing 5 Zones

Fig. 7. Tangential section through the microvilli of a mature oocyte showing their even spacing and glycocalyx coatings

Fig. 8. View of the outer region of the egg envelope showing mucus strands which comprise the outer jelly layer (Zone V) extending from the surface granules of Zone IV (arrows)

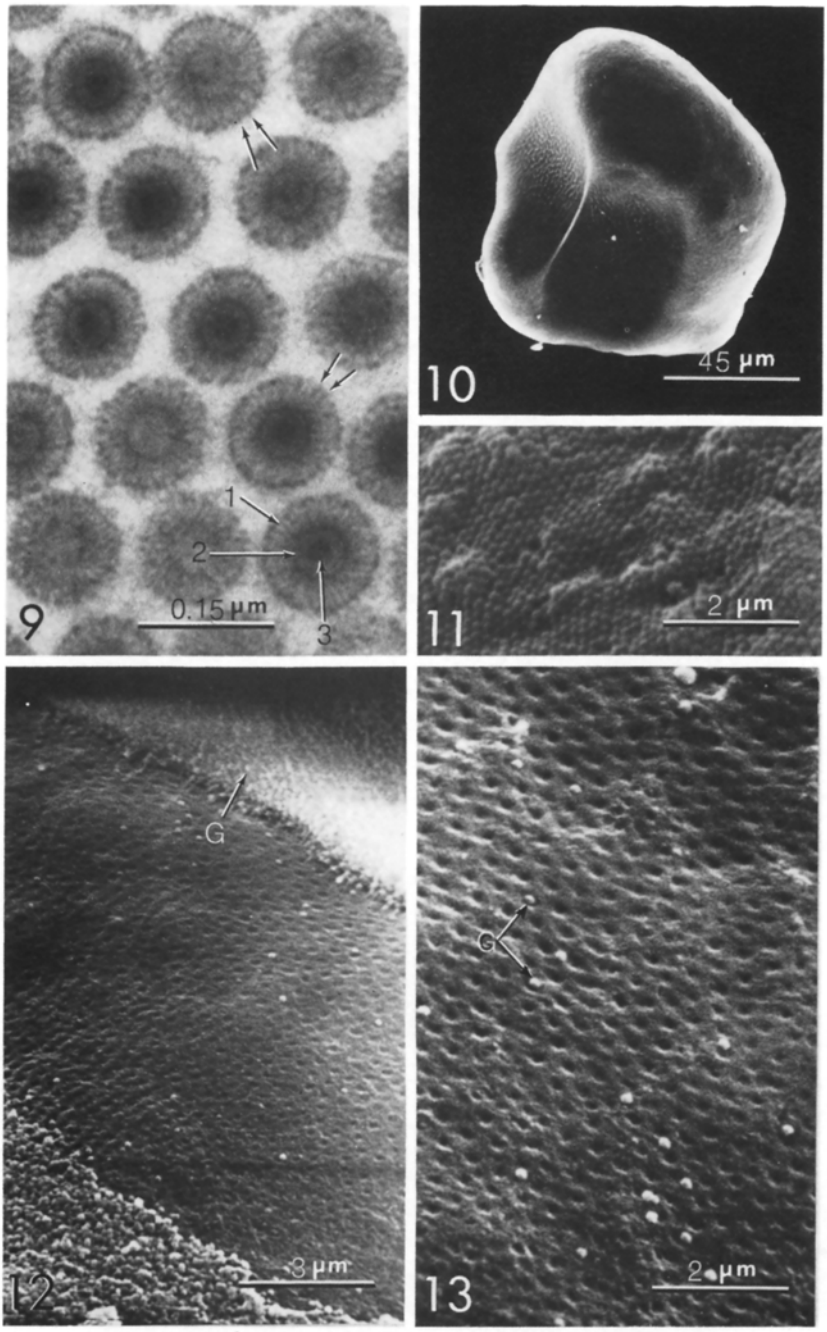

Fig. 9. Tangential section through the surface granules of Zone IV. Note the 3 concentric layers comprising each granule and the presence of fibrils radiating from their core (arrows)

Fig. 10. Scanning electron micrograph of freshly spawned, unfertilized egg with characteristic irregular shape

Fig. 11. Closeup of egg surface shown in Figure 10

Fig. 12. Scanning electron micrograph of surface of unfertilized egg with granular Zone IV removed to reveal pitted surface of Zone III. *G* surface granules

Fig. 13. Closeup of Zone III shown in Figure 12. Note that a few granules (*G*) still remain attached to Zone III

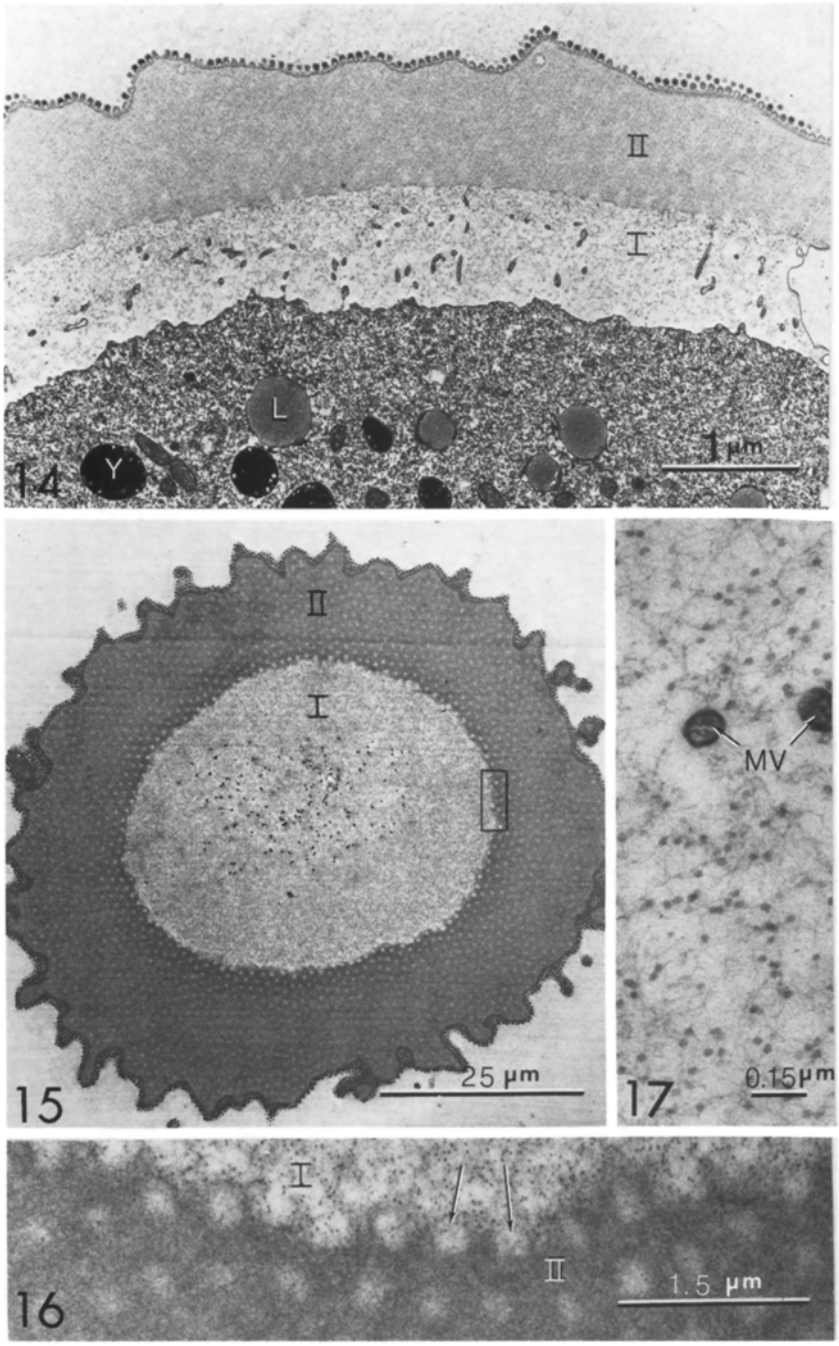

Fig. 14. Fertilized egg after withdrawal of microvilli from egg surface to Zone I. *L* lipid; *Y* yolk

Fig. 15. Tangential section through egg envelope showing regularly-spaced channels resulting from withdrawal of microvilli

Fig. 16. Closeup of region between Zones I and II of the egg envelope outlined by the rectangle in Figure 15. Arrows indicate the light staining areas in Zone II resulting from withdrawal of microvilli

Fig. 17. Closeup of Zone I of fertilized egg showing scattered particles and fine fibrils. *MV* microvilli

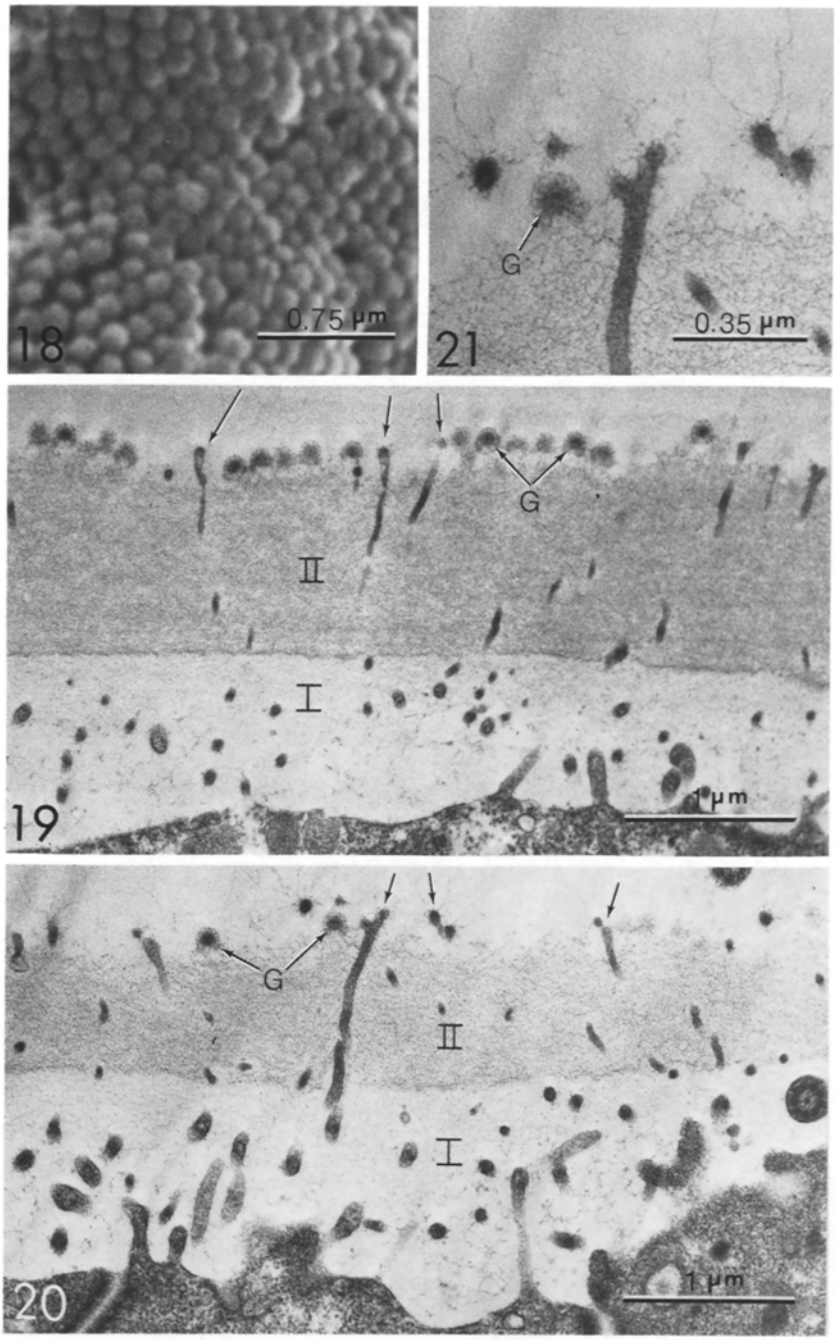

Fig. 18. Scanning electron micrograph of granular cuticle of 16 h trochophore larva

Fig. 19. Cross section through cuticle of 20 h trochophore. Note the loss of some surface granules, the disappearance of Zone III underlying the granules and the penetration of unbranched epidermal microvilli through the cuticle. *G* surface granules

Fig. 20. Cuticle of 40 h larva. Zone II is less defined than previously, most of the surface granules (*G*) have been lost and numerous microvilli with branching tips have appeared (arrows)

Fig. 21. Closeup of branching tips of microvilli viewed in Figure 19. Note strands of glycocalyx. *G* surface granule

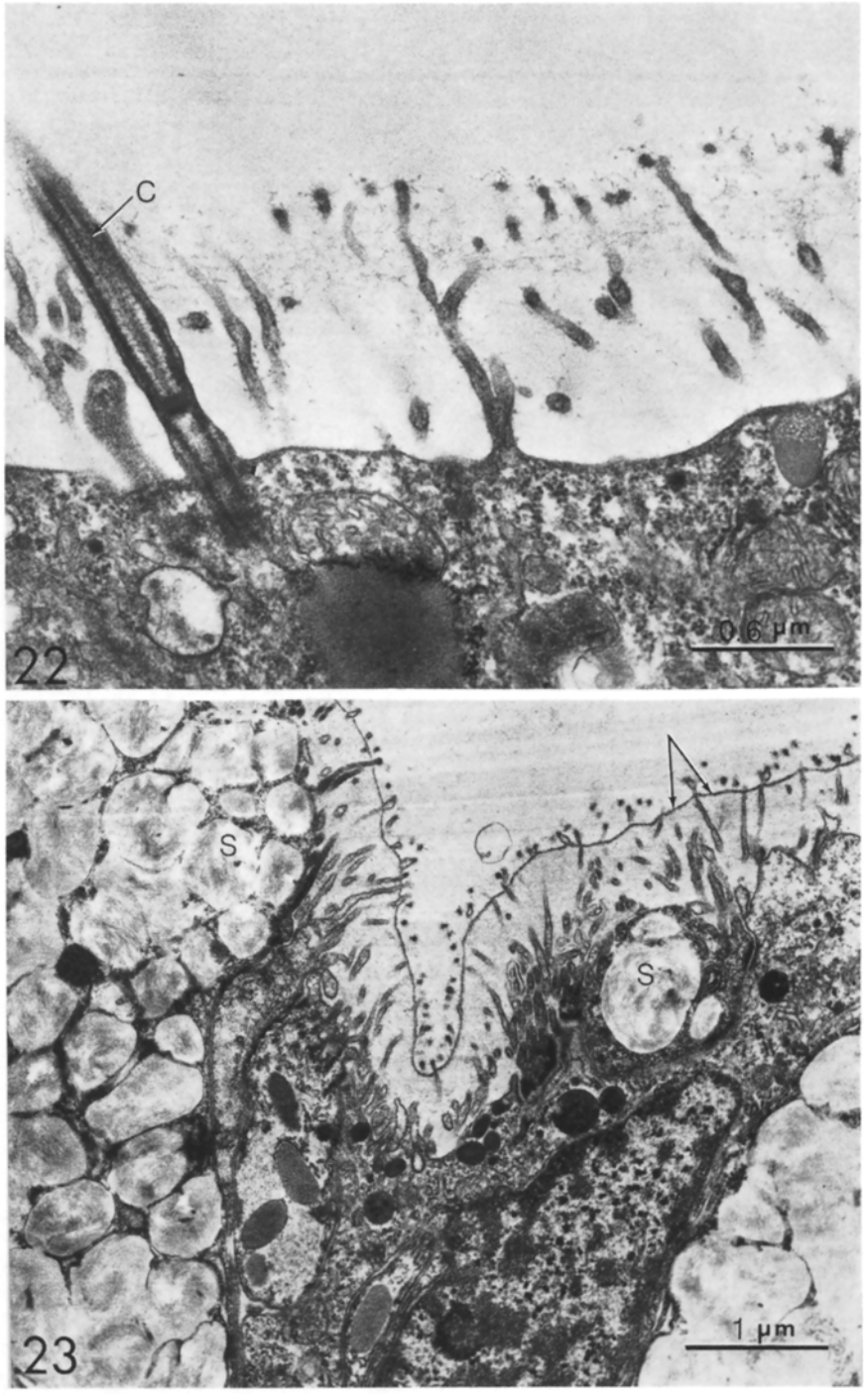

Fig. 22. Cuticle of 60 h larva with branching microvilli. Note complete absence of original zones of egg envelope. *C* cilium

Fig. 23. Cuticle of 20 day larva just prior to metamorphosis. Arrows indicate electron dense outer region of cuticle through which microvilli penetrate. *S* secretion droplet

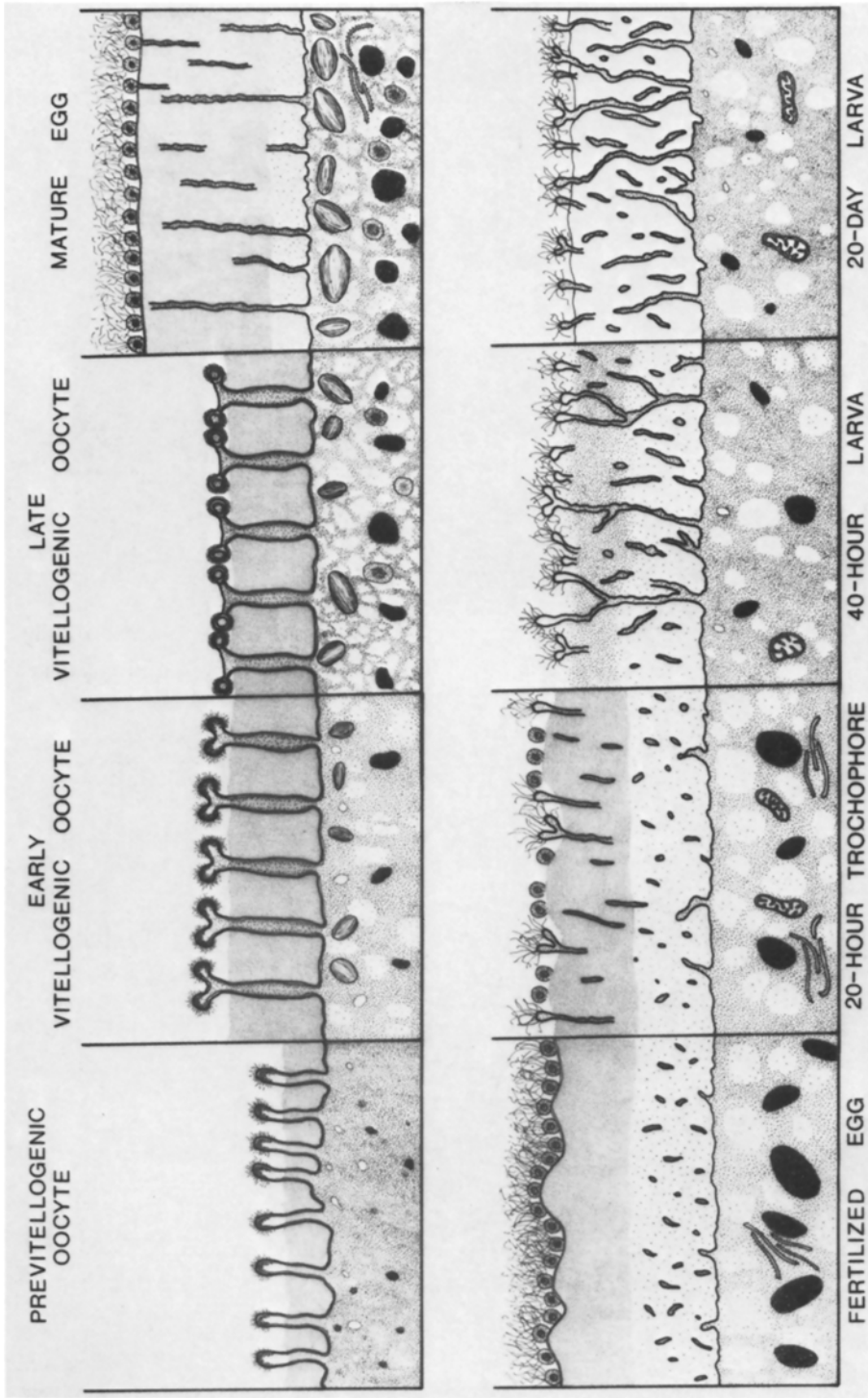

Fig. 24. Diagrammatic summary of the development of the egg envelope and its replacement by the larval cuticle of *Phragmatopoma lapidosa*

Trochophore

In the 16 h trochophore larva, the surface of the cuticle as viewed with SEM is basically indistinguishable from that of the egg envelope (Fig. 18). A cross-section through the cuticle of the 20 h trochophore shows that the jelly coat (Zone V) and Zone III have disappeared and some of the surface granules have been replaced by nonbranching microvilli which have arisen from the underlying epidermal cells (Fig. 19). The tips of the microvilli possess distinct strands of glycocalyx. The cuticle at this stage ranges in thickness from 1.2 to 2.7 μm with the thinnest portion corresponding to the region penetrated by the cilia of the prototroch.

Later Larval Stages

Forty hours after fertilization, most of the surface granules (Zone IV) have been lost and numerous microvilli with bifurcated tips have appeared (Figs. 20 and 21). Zone I of the egg envelope is still present but Zone II is now only a weakly staining network of scattered fibrils.

Sixty hours after fertilization, Zones I and II of the original egg envelope have disappeared although the strands of glycocalyx covering the distal half of each microvillus form a weakly staining, loosely defined layer (Fig. 22).

The cuticle of the 20 day larva, just prior to metamorphosis, ranges from 0.4–1.0 μm in thickness. The microvilli are highly branching and penetrate a weakly staining, homogeneous, non-fibrillar layer. The branching tips extend through the outer border of the cuticle which is thin and stains intensely (Fig. 23).

Figure 24 diagrammatically summarizes the development of the egg envelope and formation of the larval cuticle of *Phragmatopoma lapidosa.*

Discussion

In transmission (TEM) and scanning (SEM) electron microscopic studies of the egg envelopes of the sabellariid polychaetes, *Sabellaria alveolata* (Pasteels, 1965a, b) and *Phragmatopoma lapidosa* (Eckelbarger and Chia, 1976), respectively, a monolayer of surface granules was described. Franklin (1966) in a TEM study of the eggs of *Sabellaria vulgaris,* also reported the presence of surface granules and presented evidence that they are formed by constriction of the tips of the oocyte microvilli. Gwynn and Jones (1971) reported superficially similar "rounded bodies" on the surface of the eggs of the serpulid *Pomatoceros triqueter,* and found support for Franklin's proposition that the granules originated from the oocyte microvilli. The present study confirms Franklin's findings. Colwin and Colwin (1961, Figs. 3 and 18) also reported an "outer border layer" of granules in the eggs of the serpulid *Hydroides hexagonus,* which appear very similar to those described in sabellariid eggs although the mechanism of their formation was not investigated.

It is apparent that in the eggs of *Phragmatopoma lapidosa,* the number of oocyte microvilli is too small to account for the number of surface granules if it is assumed that two granules are produced by each microvillus, as evidenced by cross sections.

In tissue sections, no more than two granules have ever been observed in association with a single microvillus. However, this is still difficult to account for the 10 to 1 ratio of granules to microvilli estimated from SEM and TEM observations. There appear to be two possible explanations. First, some microvilli may have withdrawn after having formed surface granules or second, each microvillus may have repeatedly produced successive groups of granules, resulting in either case in a high ratio of granules to microvilli. The first possibility, however, is not likely as we have no evidence to indicate the withdrawal of microvilli. The second possibility seems most likely in *Phragmatopoma lapidosa.* A situation parallel to the second alternative is found in the adult lumbricids reported by Richards (1974) who described regularly arranged epicuticular projections of microvillar origin and suggested that due to the large number of projections relative to the number of microvilli, each microvillus may be the source of a group of epicuticular projections. In *Phragmatopoma lapidosa* eggs, new microvilli must also have arisen continuously throughout the growth phase of oogenesis to account for the fact that the intermicrovillar distance does not significantly change during oogenesis. We suggest that during most of the growth phase of oogenesis in *P. lapidosa,* new microvilli and their associated granules are formed to keep pace with the increase in surface area of the egg. Near the end of the growth phase, new microvilli are no longer formed and existing microvilli produce several successive groups of granules. This theory is supported by the fact that only near the end of oogenesis are numerous surface granules observed that are not attached to microvilli.

The function of the surface granules is unknown although Tyler (1965) suggested that the granular "outer border layer" in *Hydroides* and *Sabellaria,* possibly bear fertilizin receptors. In both Franklin's study (1966) of *Sabellaria alveolata* eggs and Gwynn and Jones' (1971) study of the eggs of *Pomatoceros triqueter,* it was suggested that the surface granules were involved in the acrosomal reaction. Richards (1974) suggested that the epicuticular projections in oligochaetes may serve as a means of mechanically stabilizing the acid mucopolysaccharide coat which forms a fine fibrous covering around the worms. The surface granules of sabellariid eggs may function in a similar way to stabilize the jelly coat surrounding the egg.

Epicuticular surface granules or particles have been observed in the adult polychaetes *Harmothoë imbricata* (Lawry, 1967; Holborow et al., 1969), *Scoloplos armiger* and *Panthalis oerstedi* (Storch and Welsch, 1970), *Glycera dibranchiata* (Chien et al., 1972) and *Lanice conchilega* (Schulte and Riehl, 1976) but the nature and origin of these granules were not studied.

Coggeshall (1966) in an ultrastructural study of the epidermis of the earthworm, *Lumbricus terrestris* L., concluded that the surface particles or "ellipsoidal bodies" may consist of pieces of cytoplasm separated from the underlying epithelial cells but found it difficult to believe that these isolated fragments could maintain their integrity and viability when separated from their parent cell. Ultrastructural studies on the cuticles of the oligochaetes, *Enchytraesus fragmentosus* (Hess and Menzel, 1967) and *Dero obtusa* (Krall, 1968), the eunicid polychaete *Diopatra neapolitano* (Misuraca and Zs.-Nagy, 1970) and the leech *Batrocobdella picta* (Desser and Weller, 1977) concluded that epicuticular surface particles originated from the tips of microvilli. Humphreys and Porter (1976) described epicuticular corpuscles in

Eisenia foetida which they felt strikingly resembled *Mycoplasma gallisepticum*, a pleuropneumonia-like organism (PPLO) but concluded that they were probably formed by epidermal microvilli. Potswald (1971), in a study of cuticular regeneration in the oligochaete, *Aeolosoma bengalense*, demonstrated that the epicuticular surface particles found in this and other oligochaetes probably have a microvillar origin. Removal of the cuticle in *A. bengalense* stimulates the formation of new microvilli and the apparent pinching off of their tips to produce another layer of surface particles. Once the tip of the microvillus pinches off, the remainder of the microvillus is withdrawn into the supportive cell. Rieger and Rieger (1976) in their study of the cuticle of the archiannelids *Trilobodrilus* and *Diurodrilus* found support for the view that various kinds of surface vesicles or bodies known from many annelids are derivatives of the tips of microvilli.

Potswald (1971) suggested that due to the great similarity between the formation of the primary envelope in the oocyte of *Sabellaria* and the cuticle of *Aeolosoma*, the primary envelope of *Sabellaria* might become the cuticle of the larva. Wilson (1929) and Novikoff (1938) had earlier reported that the "fertilization membrane" appeared to persist as the larval cuticle. From the present study, it is apparent that the egg envelope initially remains fully intact, serving as the larval cuticle through the trochophore stage and is then gradually lost and replaced by a new larval cuticle.

It should be noted that in a series of detailed studies on sipunculan development, Rice (1967; 1970; 1973; 1976) has found that in the majority of sipunculans, the egg envelope is transformed into the larval cuticle. In fact, Rice (1973) has called attention to the phylogenetic affinities between sipunculans and annelids based on, among other things, the transformation of their respective egg envelopes into the larval cuticles.

Little can be said at present with regard to the functional significance of the microvillar cuticle. Since the microvilli are in direct contact with the cuticle or mucus, it has been suggested that they play a role in secretion and maintenance of these extracellular materials (Lane, 1963; Coggeshall, 1966; Potswald, 1971). In view of the well-documented uptake of small organic compounds by free-living, soft-bodied marine invertebrates (Stephens, 1968; Ahern and Gomme, 1975), it has been proposed that the epidermal microvilli of marine invertebrates may be an adaptive feature in absorbing organic molecules from the environment (Chia, 1972). More recently, Rieger and Rieger (1976) suggested that the elaboration of the basic surface coating of the microvilli within the primitive Turbellaria represents the primitive condition within the Spiralia and that these primitive "cuticles" functioned not in a supportive role but by acting as a molecular filter by collecting and concentrating organic compounds within reach of the microvilli. The well developed microvilli seen in most annelids could conceivably play a significant role in the interaction of the organism with its environment.

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